Effect of amoxicillin clavulanate on the intestinal microbiota and metabolomes of mice administered yogurt and *Bifidobacterium animalis* subsp. *lactis* BB-12

By

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ABSTRACT

One of the most common indications for probiotic treatment is the prevention of antibiotic-associated diarrhea (AAD). *Bifidobacterium animalis subsp. lactis* BB-12 is a safe and well-tolerated strain that has a long history of use as a probiotic in dietary supplements and fermented milk products. Our aim was to understand the capacity of the antibiotic amoxicillin-clavulanate (AMC) to alter the intestinal microbial composition and metabolome of mice when administered concurrently with either BB-12 or yogurt or both combined using 16S rRNA DNA sequencing, qPCR, and NMR metabolomics. To study the effects of BB-12, yogurt and AMC, three cohorts of mice were used, and treatments were given through oral route in drinking water.

AMC resulted in significant reductions ($p<0.05$) in male mouse body weight throughout whole study and reduction in water intake for first five days when consumed in the drinking water over a ten-day regimen. AMC administration also resulted in significant ($p<0.05$) loss in bacterial cell numbers in the cecum and feces overtime. The fecal microbiota changed within one day of the start of AMC administration. These changes included significant reductions in bacterial alpha-diversity and increases in proportions of Pseudomonadota (formerly called Proteobacteria), most notably *Erwiniaceae* and *Enterobacteriaceae*. Although there were differences in taxonomic enrichments between mouse study cohorts, the cecal contents of all mice given AMC contained lower concentrations of the short-chain fatty acids (SCFA) acetate, butyrate and propionate and higher quantities of arginine, glutamine, glycine, proline, serine and threonine.

Overall, even after recommended dose of AMC was administered, antibiotics impacted severely on 16S rRNA cell counts, BB-12 cells in yogurt, gut microbial community and on cecal metabolites specifically SCFA and amino acids.

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CHAPTER 1

LITERATURE REVIEW

Amoxicillin-Clavulanate and BB-12 effects on Antibiotic associated diarrhea

1. Introduction

Antibiotics have been widely used to inhibit the growth of bacterial pathogens and consequently to treat bacterial infections for decades [1]. While antibiotics are saving many lives, they also inhibit and inactivate commensal bacteria which are crucial for human and animal health [1]. These negative effects of antibiotics are most common in gastrointestinal tract (GI) microorganisms which is termed as 'gut microbiome' [1, 2]. The inactivation of commensal bacteria results in gut dysbiosis (disruption to the microbiota homeostasis), which cause many diseases in human and animals [1]. The human gut contains around 400 species of bacteria and maintaining a healthy balance of these microorganisms is crucial for efficient gastrointestinal function [2]. The GI tract/gut is colonized by diverse bacterial phyla such as Bacteroidota, Bacillota, Actinomycetota, Pseudomoadota and Verrucomicrobia. The GI tract's microbial density and diversity were found to be greatest in the cecum and colon [2]. The colon contains the majority of microorganisms, reaching 3-fold more than the microorganisms inhabiting in the small intestine and with diverse bacteria genera mainly in ileum and colon, *Enterococcus, Bacteroides, Bifidobacteria, Peptococcus, Peptosteptococc, Ruminococcus, Clostridia,* and *Lactobacilli* [3]. These bacterial genera are severely affected by the use of antibiotics, which may cause a variety of changes in the gut microbiome (i.e., gut dysbiosis).

Overuse of antibiotic promotes colonization of *Clostridium difficile,* an opportunistic pathogen causing antibiotic-associated diarrhea (AAD) [4]. AAD has been associated with altered gastrointestinal tract microbiota, decreased short chain fatty acid (SCFA) metabolism, luminal carbohydrate accumulation, subsequent pH changes, decreased water absorption, and, finally, diarrhea [4]. AAD is more than an unwanted adverse effect of antibiotic therapy; it is associated with prescription noncompliance and overuse of second-line antibiotics [4].

Almost all antibiotic types such as broad and narrow spectrum have been linked to AAD, but those with broad-spectrum coverage in particular ampicillin, cephalosporins, fluoroquinolones, amoxicillin, extended-coverage penicillin, and clindamycin are known to be the most common ones [5]. Several studies have shown that amoxicillin-clavulanate (AMC) is the most common antibiotic that caused high incidence of AAD [5, 6]. AMC proportionally reduced bacteria such as Bifidobacteria, Lactobacilli and Clostridia and ultimately cause the diarrhea (AAD) especially in the infants [4, 5, 6].

The treatment of AAD which is caused by *C. difficile* requires antibiotic therapy with glycopeptides (vancomycin) or metronidazole in more severe instances [5]. Another strategy for treating or preventing AAD is to use of non-pathogenic living microorganisms capable of reestablishing the intestinal ecosystem's homeostasis [5]. Numerous organisms, including selected strains of *Lactobacillus acidophilus, Lactobacillus bulgaricus, Bifidobacterium longum*, *Bifidobacterium animalis subsp. lactis* BB-12, *Enterococcus faecium*, and yeast such as *Saccharomyces boulardii* have been utilized in the treatment or prevention of AAD [1, 5].

Additionally, AAD not only affects the gut microbiome but also reduces the proportion of important metabolites such as SCFA [2, 5]. Therefore, restoring metabolic function, including SCFA generation by the microbiota, may be one strategy for mitigating AAD. Few studies have examined the effect of probiotics on the structure and function of the gut microbiota community, particularly in the setting of antibiotic-induced abnormalities [2]. As a result, well-designed experiments assessing these endpoints with well-characterized probiotics are critical for furthering mechanistic understanding of probiotic action in ameliorating AAD symptoms [2].

Overall, AAD can cause community-wide microbiota perturbations due to the broad spectrum of antibiotics. Specifically, AMC causes repression of protective microbiota by promoting the growth of harmful indigenous bacteria, which may manifest clinical symptoms of AAD in susceptible individuals [4, 5].

2. Antibiotic mediated effects:

2.1.Gut Microbiome

Antibiotics can have a direct or indirect effect on the gut microbiome. Antibiotics are administered purposely to eradicate pathogenic bacteria; nevertheless, due to their broad-spectrum activity, subsets of commensal species are also killed or suppressed indiscriminately [7]. Notably, different antibiotics or their combinations have distinct antimicrobial spectra and hence affect the microbiome differently. For example, vancomycin reduces fecal microbial diversity and the absolute proportion of gram-positive bacteria, most notably those belonging to the Firmicutes phylum, whereas amoxicillin has no discernible effect on total bacterial numbers or microbial diversity [7]. Not only did a combination of antibiotics containing ampicillin, gentamicin, metronidazole, neomycin, and vancomycin significantly reduce the total number of bacteria in the gut, but it also significantly altered the composition of the gut microbiota. Thus, when we utilize antibiotics to investigate the impact of microbiota on host health, we must first choose appropriate medicines and understand how the antibiotics chosen will alter the gut microbiome [7].

Antibiotic therapy reduces the alpha diversity of the gut microbiome [1,7]. A seminal study in three healthy persons showed that five days of a normal dose (500 mg/BID) ciprofloxacin had a substantial effect on approximately one-third of the bacterial taxa detected in the study [1]. Additionally, antibiotic exposure in newborns and early children may have a profound effect

on the microbiota at important developmental stages. For example, at 4 weeks of age, the microbiomes of infants exposed to ampicillin and gentamicin perinatally demonstrated a decrease in *Actinobacteria* species (*Bifidobacterium and Lactobacillus*) and an increase in *Proteobacteria* and continued to show a decrease in the alpha diversity of these species at 8 weeks of age [1]. Bokulich *et al.*, 2016 showed that early antibiotic treatment in children for first two year of life resulted in a decrease in the intestinal microbiome's alpha diversity and specific reductions in the *Clostridium* and *Ruminococcus* species [8] Additionally, they demonstrated that early antibiotic exposure impairs the stability and maturation of the gut microbiome [8]. Similarly, another continuous cohort of infants from birth to age 3 showed a decrease in the alpha diversity of their microbiota after exposure to AMC [1]. Additionally, they demonstrated that the species identified in the microbiota of infants exposed to antibiotics were dominated by a single strain rather than by several strains of the same species. These results were similar to the previous study, those individuals had a deficiency of *Clostridium* species [1].

All in all, most of the broad-spectrum antibiotics can cause AAD, but AMC is one of the important antibiotics with highest rate (>75% cases of diarrhea with *C. difficile*). AMC is made of amoxicillin and clavulanic acid [9]. Amoxicillin is one of the most commonly used antibiotics in the primary care setting. It is an amino penicillin, created by adding an extra amino group to penicillin to battle antibiotic resistance. Amoxicillin is in the class of beta-lactam antimicrobials. Beta-lactams act by binding to penicillin-binding proteins that inhibit a process called transpeptidation (the cross-linking process in cell wall synthesis), leading to activation of autolytic enzymes in the bacterial cell wall. This process leads to lysis of the cell wall, thus destroying the bacterial cell [9]. This mechanism is applicable to kill the microorganism which cause disease in animals and humans.

Amoxicillin is active against both gram-positive and gram-negative bacteria, including *Enterococcus* species, *Listeria monocytogenes, Streptococcus spp., Haemophilus influenzae, Moraxella catarrhalis, Corynebacterium diphtheria, Escherichia coli, Klebsiella pneumoniae, Salmonella* spp., *Shigella* spp., and *Borrelia* species. Additionally, the inclusion of clavulanic acid broadens the spectrum to methicillin-sensitive *Staphylococcus aureus* (MSSA), *Neisseria species, Proteus species, Pasteurella multocida*, and *Capnocytophaga canimorsus,* etc. [10]. This combination of amoxicillin clavulanate primarily increases in Enterobacteria with varying effects bacteria spp. such as *Bifidobacterium, Lactobacillus,* and *Bacteroides.,* as well as a general decrease in diversity [10]. Many studies showed the high impacts of AMC or amoxicillin (alone) on gut microbiome. For instance, Kabbani. T.A *et. al.,* 2017 reported AMC usage was correlated with a decrease in the prevalence of the species *Roseburia* and an increase in the prevalence of *Escherichia, Parabacteroides*, and *Enterobacter*. Microbiota changes returned to baseline levels after two weeks following therapy but were not totally restored [11].

Mangin *et al.,* 2020 [4] investigated AMC effects on acute bronchitis in 18-month-old children. Both overall bacteria and Bifidobacteria counts remained constant throughout antibiotic therapy. However, alterations at the species level in Bifidobacterium indicated a decline in diversity. Amoxicillin treatment eliminated the *Bifidobacterium adolescentis* species and significantly reduced the occurrence rate of *Bifidobacterium bifidum* but had no effect on *Bifidobacterium longum* or *Bifidobacterium pseudocatenulatum*, *Bifidobacterium catenulatum.* However, they did not examine the long-term impacts [12]. Similarly, Lode. H. 2001, *et. al.* reported that AMC administration to healthy individuals increased the frequency of enterococci and *Escherichia coli* strains in the aerobic microflora, but decreased *Bifidobacteria, Lactobacilli,* and *Clostridia*

strains. There were no significant changes in the numbers of anaerobic cocci and Bacteroides [12].

2.2 Bacterial metabolites

Metabolomic analysis is capable of detecting and identifying a large number of small molecules present in biological samples, allowing for the evaluation of both microbial and host-derived metabolites [14]. Feces may provide a more accurate representation of the direct microbial metabolic products produced in the gut than serum metabolites, which represent metabolites that eventually enter the systemic circulation and may have a bigger effect on the host [14]. Antibiotics also affect host immunity by modifying bacterial metabolites and signals transferred from the gut microbiota to the host, most notably those detected by intestinal epithelial and immune cells [7]. Antibiotics have been shown to have a dramatic effect on lipids, bile acids, amino acids, and amino acid-related compound concentration, according to metabonomic study [7]. Short chain fatty acids (SCFAs) are generated in the large intestine by bacteria during the fermentation of fiber. Secondary bile acids effectively suppress *Clostridium difficile,* a spore-forming gram-positive anaerobic bacteria that is the main cause of antibiotic-associated diarrhea [7]. Cefoperazone, clindamycin, and vancomycin combination is associated to the loss of the *Lachnospiraceae*, and *Ruminococcaceae* families and a decreased ability of conversion of primary bile acids to secondary bile acids in the large intestine, hence raising the risk of *C. difficile* infection [7]. Otherwise, antibiotic-induced changes in 6 to 8 weeks old mice in amino acids, particularly proline, have been implicated with *C. difficile* colonization [7].

Antibiotic AMC showed more effect on fecal metabolomics compared to other antibiotics. In 2-day-old newborns, a combination of ampicillin and gentamycin resulted in decreased

fecal amounts of GABA, tryptophan, and ornithine [15]. Another study in 1-week-old newborns found that a combination of several beta-lactam antibiotics such as AMC resulted in decreased fecal concentrations of metabolomics generated by bacteria [7, 15]. AMC therapy has been projected to change between 4.4% and 87% of known fecal end metabolic products in humans [15].

3. Effects of Antibiotic associated diarrhea (AAD):

3.1 Strategies, evidence and mechanism of action

Antibiotic-associated diarrhea (AAD) occurs in association with the administration of antibiotics and is one of the most common adverse effects of antibiotics administration in human [12]. Numerous enteric pathogens have been linked to AAD. Overgrowth of *Clostridium difficile (C. difficile)* is the bacterial agent most frequently associated with AAD [12]. *C. difficile* diarrhea most frequently affects elderly, immunocompromised, hospitalized adults, although it can also affect youngsters [13]. AAD can occur in up to one-third of individuals using a given antibiotic, but the prevalence and severity of AAD varies per drug. Cephradine + gentamycin sulfate caused more severe diarrhea in mice than lincomycin hydrochloride + ampicillin sodium or ceftriaxone sodium + erythromycin lactobionate. Numerous investigations concluded that AAD was mostly caused by antibiotic-induced alterations or dysbiosis in microbial composition and activity [13].

AAD has detrimental effects on gastrointestinal tract homeostasis, as indicated by the following: (a) a decrease in the total population and biodiversity of the gut microbiota; and (b) a decrease in short-chain fatty acid (SCFA) synthesis [13]. Another possible outcome of AAD is that beneficial metabolic activities of gut microorganisms are inhibited. Global alterations in the

composition and quantity of the gut microbiota (even in the absence of pathogenic microbe overgrowth) might cause global colonic metabolism to be perturbed, resulting in AAD [16].

Antibiotic-associated diarrhea can also be caused by other enteric infections, the direct actions of antimicrobial drugs on the intestinal mucosa, and the metabolic repercussions of lower concentrations of fecal microbiome. *Salmonella, C. perfringens type A, Staphylococcus aureus*, and perhaps *Candida albicans* are additional enteric infections that can cause diarrhea. Recently, a distinct genotype has been linked to antibiotic-associated diarrhea [17]. Infection with either subtype results in diarrhea that typically disappears within 24 hours. There is no specific treatment, and few laboratories offer the essential diagnostic tests to diagnose this infection.

Antibiotics may significantly reduce the concentration of typically prevalent fecal anaerobes. As a result, the metabolism of carbohydrates may decrease, resulting in osmotic diarrhea, and the rate of breakdown of primary bile acids, which are powerful colonic secretion agents, may decrease. These both mechanisms are clearly established as a cause of AAD according to many studies [18].

3.2 AAD caused by AMC

AMC is a commonly recommended antibiotic, particularly for infants and teenagers [4]. These two compounds are frequently used in combination to treat a variety of pediatric infectious disorders, including respiratory disease, sinusitis, urinary tract infections, and skin and soft tissue infections [19]. The combination of a β-lactam class of antimicrobial, amoxicillin, combined with a β-lactamase inhibitor, clavulanic acid, has been characterized to have bactericidal effects against bacteria that secrete β-lactamase [19,20]. The use of broad-spectrum antibiotics and anti-

microbials, such as AMC, has been widely established to disrupt the community-wide microbiota, suppressing beneficial bacteria while promoting the growth of harmful indigenous bacteria, which may manifest clinical symptoms of AAD in susceptible individuals [20].

Young and Schmidt examined the short-term effects of amoxicillin/clavulanic acid on a male patient with acute sinusitis who had antibiotic-associated diarrhea. It was demonstrated that, with the exception of *Bifidobacterium*, the major bacterial groups were partially restored 14 days following antibiotic therapy [16]. No sequences belonging to butyrate-producing Clostridium cluster XIV a were identified during antibiotic therapy, but this cluster reappeared two weeks after drugs were stopped. The decrease in this cluster in this case study may be a result of antibiotic-associated diarrhea [16].

4. AAD treatments

4.1 Prevention and treatment options for AAD

In mild to severe cases of AAD, rehydration and cessation of the triggering drug or substitute are critical first steps. If necessary, antibiotics with a low risk of causing diarrhea such as quinolones, metronidazole, co-trimoxazole, parenteral aminoglycosides, or tetracyclines can be used [19]. However, any antibiotic therapy may alter the normal equilibrium of the intestinal microorganism with a potential of pathogenic organisms emerging and an abnormal growth of *C. difficile.* The theory of live microbes may have a role in the treatment or prevention of AAD has been demonstrated by the introduction of many 'physiologic' non-pathogenic organisms [19]

They are referred to as 'probiotics' or 'biotherapeutic agents' and are described as "live bacteria that confer a health benefit on the host when administered in sufficient doses". Many

randomized controlled clinical trials have been conducted to examine the beneficial effect of probiotics on gut health in a variety of illnesses (antibiotic-associated and viral diarrhea, irritable bowel syndrome, necrotizing enterocolitis, and others) [22]. While there are robust clinical trials documenting the efficacy of some probiotics in preventing AAD, the mechanism(s) of how probiotics prevent AAD are unclear [22]. Enhancing metabolic function, including SCFA generation by the microbiota, may be one approach for mitigating AAD.

Bacteria spp., notably *Lactobacillus rhamnosus* or *Saccharomyces boulardii,* and fecal microbiota transplantation have been shown to be useful in preventing or treating AAD. Advances in the understanding of microbial features may pave the way for the development of treatments that specifically target the gut microbiota in the prevention and treatment of AAD [16].

4.2 Probiotics for AAD

Probiotics are frequently advised during antibiotic use regarded to potentially reduce the risk of AAD. Numerous bacterial species, including members of the *Bacillus, Bifidobacterium*, *Clostridium, Lactobacillus, Lactococcus, Leuconostoc*, and *Streptococcus* genera, have been examined in clinical investigations for their ability to mitigate AAD [22]. Additionally, the yeast *Saccharomyces boulardii* has also investigated in clinical studies for AAD prevention [20, 22]. A Cochrane review published in 2019 identified 33 randomized clinical trials with 6,352 participants fulfilling the inclusion criteria [22]. Probiotics were found to have a considerable beneficial effect on AAD prevention (number needed to treat for an additional beneficial outcome (NNTB), 95 % confidence interval (CI) 7 to 13). Consistent with a previous Cochrane review (2015), when 5 billion colony forming units (CFUs)/day were consumed, the risk ratio of getting AAD was dramatically lowered. 5–40 billion CFU/day of *L. rhamnosus* or *S. boulardii*, the two most

often used species, were suggested to be the most effective for avoiding AAD in children receiving antibiotics. Nonetheless, the Cochrane analysis assigned a moderate level of assurance to the evidence due to minor concerns about bias and inconsistency amongst probiotic strains utilized [22].

Another study demonstrated that supplemented probiotics during *Helicobacter (H.) pylori* treatment demonstrated a reduction in antibiotic-induced alterations, with larger shifts in the microbiota in the antibiotic-only group. The same research team also demonstrated that the shift in functional gene families was greater in the antibiotics group than in the probiotics group. This protection of the microbiota has been examined in a few BB-12 studies [23].

4.3 *Bifidobacterium animalis* subsp. *lactis* (BB-12) persistence in the GI tract and AAD prevention

Probiotics are a common dietary strategy for modulating gut microbiota, as they are widely considered safe for human consumption. Members of the genus Bifidobacterium are widely utilized as probiotics due to their capacity to prevent and treat a wide variety of gastrointestinal problems in animals and humans, including colonic transit disorders and intestinal infections [24]. *Bifidobacterium animalis* subsp. lactis BB-12 (BB-12) is the most well-documented probiotic species in the genus *Bifidobacterium* because it exhibits superior gastric acid and bile tolerance, contains bile salt hydrolases, and has strong mucus adhesion properties all of which are desirable probiotic characteristics [24]. BB-12 has been used successfully in a variety of clinical trials on infants, children, adults, and the elderly, and numerous beneficial effects have been reported, including the management of infantile colic, the improvement of the immune system, the reduction of the risk of infections in early childhood, and the rebalancing of the disturbed gut

microbiota caused by severe acute malnutrition [22]. Additionally, BB-12 has been incorporated into dietary supplements, fermented milk products, and newborn formulas throughout the world [24].

Bifidobacterium animalis subsp. *lactis* BB-12 showed promising results to reduce AAD. In 343 patients undergoing a seven-day antibiotic therapy, a randomized, double-blind, placebo-controlled research examined the efficacy of BB-12 and LA-5 in preventing AAD [23]. Fourteen days of intervention were assessed using a symptom diary card for AAD. After 14 days of treatment, the incidence of AAD was considerably reduced in the probiotic group to 10.8%, compared to 15.56% in the placebo group. The probiotic group experienced considerably less diarrhea (2.32 days) than the placebo group (4.58 days). Severe diarrhea was substantially more common in the placebo group than in the probiotic group. These findings indicate that BB-12 and LA-5 have the potential to significantly lessen the duration and severity of AAD [24].

In one study, patients being treated for *H. pylori* were randomized to receive placebo or probiotic supplemented yogurt with BB-12 and *Lactobacillus acidophilus* LA-5. After antibiotic treatment both groups observed depletion of bifidobacteria in stools, but the probiotic group showed restored levels of bifidobacteria in four weeks compared to the placebo group [22, 23]. One way in which BB-12 may minimize antibiotic-induced disruption of the microbiota is by protecting an intact mucus layer and epithelial cell lining in the gastrointestinal tract. In vitro studies have demonstrated that fermentation products from BB-12 increase tight junction strength compared to controls and other probiotic strains.

Bifidobacterium lactis strain Bb12® is a probiotic bacterium that is commonly found in probiotic yogurt. Probiotic yogurt containing this microbe has been shown to have anti-diabetic effects, including decreased blood LDL-cholesterol in type 2 diabetes patients, increased HDL

cholesterol in adult women, and enhanced glucose tolerance during pregnancy [23]. In addition, Bb12® treatment has been demonstrated to increase faecal secretory IgA excretion in premature newborns. Probiotic health claims have been proved with varied levels of proof, with only a few being supported by double blind randomized controlled trials [22, 23].

4.4 Mechanistic basis for AAD prevention

One of the most common indications for probiotic treatment is the prevention of antibiotic-associated diarrhea (AAD). Data from several studies are consistent with the notion that antibiotic-induced disruption of commensal bacteria in the colon results in a significant reduction of short chain fatty acid (SCFA) production and a concomitant reduction in Na-dependent fluid absorption resulting in AAD. Probiotics were shown to prevent AAD in numerous clinical trials. According to Marco. M *et al.,* 2020, probiotics reduce the risk for AAD via modulating the gut microbiota, altering nutrient and bile acid metabolism, inducing epithelial solute transporter activity, supporting intestinal barrier function, and influencing the immune system [22].

Numerous mechanisms by which probiotics can influence the makeup of the gut microbiome have been explored previously. Specific molecular processes include the suppression of intestinal pathogens by producing antimicrobial chemicals, competitive exclusion via the use of limited nutrient resources or adhesion to the epithelium, and promotion of indigenous microbial activity. Probiotic metabolic byproducts may be absorbed by members of the gut microbiota through cross-feeding interactions [22, 25]. For example, in a model of the human intestinal microbial ecology, a propionogenic bacterial consortia was recently found to recover fecal propionate levels and modify bacterial composition following antibiotic treatment (M-SHIME) [22].

SCFA is one the important metabolites which is reduced in AAD cases. SCFA are rapidly absorbed by the colon and stimulate Na- dependent fluid absorption via a cyclic AMP-independent process with Na-H, SCFA-HCO3, and Cl-SCFA exchanges. Therefore, restoration of the SCFA is very important in the treatment and prevention of AAD. There are several lines of evidence that illustrated that probiotics help to restore SCFA by the production of organic acids such as lactate and acetate or by providing a more hospitable environment for SCFA producing bacteria [20].

Antibiotics interfere with intestinal mechanism and increase colonic primary bile acids, which are chemicals that block epithelial ion transport proteins [24]. Reduced levels of secondary bile acids changed by bacteria also enhance susceptibility to *C. difficile* infection. Probiotics have been found to modify the composition of bile acids in healthy volunteers fed *S. boulardii* CNCM I-745. Individuals receiving amoxicillin-clavulanate had increased amounts of cholic acid, a major bile acid, and lower levels of secondary bile acids in their feces. In individuals treated with *S. boulardii* CNCM I-745, these alterations were reversed [24, 20]

All in all, probiotics strains are very important in the prevention of the AAD.

5. Ongoing clinical study

The current mice study was based on a clinical study of Daniel Merenstein *et. al.,* 2020, where they completed two Phase I trials with a probiotic yogurt fortified with *Bifidobacterium animalis* subsp. lactis BB-12 (BB-12). They are currently conducting a Phase II, double-blind, randomized-controlled trial of this yogurt product on 300 children ages 3-12 taking prescription antibiotics for a respiratory tract infection. The present study advanced this Phase II clinical trial, because it addressed whether consuming BB-12 in yogurt was more effective at reducing risk for

AAD than in a dietary supplement format (powder). This study also aimed to elucidate the underlying mechanisms of BB-12 benefits on gut health.

6. Objective of the present study

AAD is defined as clinically unexplained diarrhea that occurs in connection with antibiotic administration. To ameliorate and prevent AAD, probiotics supplementation is considered to be an attractive therapeutic strategy. *Bifidobacterium animalis subspecies lactis* BB-12 is a safe and well-tolerated strain that has a long history of use as a probiotic and is currently under investigation for the capacity to prevent AAD in children taking antibiotics. However, it is not yet clear exactly how consumption of BB-12 or other probiotics can prevent AAD. It is also not known whether the carrier matrix in which probiotics are consumed influences its efficacy. To better understand how *B. lactis* BB-12 alters intestinal function to prevent AAD, this study investigated the effects of feeding the BB-12 strain to mice administered the antibiotic amoxicillin-clavulanate (AMC). To investigate the effects of different delivery formats, BB-12 was provided to the mice in either yogurt or a supplement (freeze-dried powder) format.

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CHAPTER 2

Effect of amoxicillin clavulanate on the intestinal microbiota and metabolomes of mice administered yogurt and *Bifidobacterium animalis* subsp. lactis BB-12

1. Introduction

Antibiotic-associated diarrhea (AAD) is a common and challenging side effect of antibiotic use [1]. AAD is defined as clinically unexplained diarrhea that occurs in connection with antibiotic administration [2]. Although AAD is usually a benign, self-limiting disorder, it may lead to persistent infections with *Clostridium difficile* and other bacterial species, such as *Clostridium perfringens*, *Klebsiella oxytoca*, and *Staphylococcus aureus* [3].

To ameliorate and prevent AAD, probiotics supplementation is considered as an attractive therapeutic strategy. Probiotics are live microorganisms that, when administered in adequate amounts, confer a health benefit on the host [4]. Clinical studies have shown a role for probiotics in preventing AAD [5]. A recent comprehensive Cochrane review of probiotics for the prevention of pediatric AAD showed that probiotics confer a moderate beneficial effect for AAD prevention (number needed to treat for an additional beneficial outcome (NNTB) 9, 95% CI 7-13) [6]. In a recent American Gastroenterological Association (AGA) clinical practice guidelines on the role of probiotics in the management of gastrointestinal disorders, the use of certain probiotic strains of lactobacilli, bifidobacteria, and *Saccharomyces* was also recommended for prevention of *C. difficile* infection for adults and children on antibiotic treatment [7].

Thus far, only few studies investigated the mechanistic basis for probiotic prevention of AAD [8]. These studies have shown that probiotics can reduce antibiotic-induced changes in gut microbiome as well as directly affect intestinal epithelial and immune cell function to prevent AAD [8]. However, the precise mechanistic details for probiotic effects remain to be determined. Moreover, because probiotics are consumed in different carrier matrices (e.g., fermented foods, dietary supplements), investigations of probiotic effects on AAD may also need to take carrier matrix into account [9].

Bifidobacterium animalis subsp. lactis BB-12 is a safe and well-tolerated strain that has a long history of use as a probiotic in infant formula, dietary supplements, and fermented milk products [10]. BB-12 survives passage through the human digestive tract [11]. The colonization, although temporary, has been established through extended recovery of BB-12 from feces for up to two weeks post-consumption, supporting that BB-12 can persist in the intestine [12,13]. Human studies have indicated that BB-12 can induce immunoregulatory gene expression [14,15] and alter the composition of the gut microbiota [16]. Studies performed *in vitro* also reported that BB-12 can adhere to intestinal cells and inhibit intestinal pathogen growth [17], and BB-12 fermentation products may increase intestinal tight junctions' expression compared to other probiotic strains [18].

The capacity of BB-12 to impact host physiological responses was shown in numerous human studies [10]. Although published reports showing AAD prevention are limited to two human studies on multi-strain preparations, both studies reported a significant reduction in AAD incidence [18]. To better understand how BB-12 alters intestinal function to prevent AAD, this study investigated the effects of BB-12 administration on mice given the antibiotic amoxicillin-clavulanate (AMC) in accordance with an ongoing clinical study investigating the ability of BB-12 to impact antibiotic-induced reductions in SCFA (NCT04414722) [19]. AMC was used because it is frequently prescribed for children with upper respiratory infections [8]. To investigate the effects of different delivery formats, BB-12 was provided to mice in either yogurt or saline from a freeze-dried powder in comparison with mice fed yogurt or saline alone.

2. Materials and Methods

2.1 Preparation and enumeration of *B. lactis* BB-12 freeze-dried powder

Freeze-dried *B. lactis* BB-12 (BB-12) cells were provided by Chr. Hansen (Chr. Hansen A/S, Hoersholm, Denmark) and stored at -20°C. Viable, freeze-dried BB-12 cells were enumerated according to a protocol recommended by Chr. Hansen (*personal communication* Dr. Mirjana Curic-Bawden). Briefly, 500 mg of freeze-dried BB-12 powder was thawed for two hours at 23°C before being suspended in 2.5 mL physiologic saline (0.9 % NaCl). The suspension was then transferred into a Whirl-Pak bag (Nasco, Fort Atkinson, WI) and mixed twice in a Smasher (AES BioMerieux, Durham, NC) at the fast speed setting for 2 min each time. The samples were then incubated for 20 min at 23°C prior to repeating the mixing for 2 min twice at the fast speed setting. Serial dilutions were plated on De Man, Rogosa and modified Sharpe (MRS) (Fisher Scientific, BD) agar with cysteine, and viable cell numbers were enumerated after 48 h incubation at 37°C under anaerobic conditions in a GasPak jar (Becton, Dickinson and Company, NJ). For daily preparation of freeze-dried BB-12 cells for mouse consumption, cell suspensions from freeze-dried powder were adjusted to a total of 1 x 10^7 BB-12 cells in 50 µL in physiologic saline (0.9 % NaCl). Cell numbers administered to the mice were verified by colony forming unit (CFU) enumeration on MRS after 48 h incubation at 37°C under anaerobic conditions.

2.2 Yogurt preparation

Yogurt prepared on three separate dates, i.e., 01/17/2019, 02/06/2019 and 02/28/2019 was received from Pennsylvania State University and stored at 4°C for up to two weeks before use. The yogurt was made using the starter cultures YF-L702, a mixture of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. bulgaricus, as previously described [15]. BB-12 was added at the end of the yogurt fermentation and was confirmed to have at least 1×10^{10} colony forming units per 100 mL serving of BB-12 at the end of the 30-day shelf life [11, 15] The BB-12 and

control yogurts were diluted in saline (0.9 % NaCl) to obtain a total of 1×10^7 cells in 50 µL of yogurt for mouse consumption. Cell numbers administered to the mice were verified by colony forming unit (CFU) enumeration on modified MRS with cysteine culture after 48 h incubation at 37°C under anaerobic conditions.

2.3 Mouse study

The study was approved by the University of California, Davis Institutional Animal Care and Use Committee (IACUC) Protocol Number 20474. Six-week-old male and female C57BL6/J mice (Jackson Laboratory, Sacramento, California, USA) were received in three cohorts (10 male and 10 female mice per cohort, total 60 mice). The mice were housed in pairs of the same sex and were maintained on 12 h light- dark cycle with ad hoc access to water and chow (Lab Diet # 5001, Newco Specialty, Hayward, CA) for the duration of the study.

After acclimation for seven days, mice were randomly assigned to one of five groups (n = 6 male and 6 female mice per treatment group). Amoxicillin-clavulanate (1 mg/mL; Neta Scientific Inc, Hainesport, NJ) (AMC) was introduced daily to new drinking water in four groups of mice over 10 days. The AMC dose was consistent used in prior human studies [11, 14]. Starting on the first day of antibiotic administration, each mouse was administered either physiologic saline (0.9% NaCl) (Sal Ab), 10^7 BB-12 cells in saline (BB-12 Ab), yogurt (Yog Ab), or yogurt containing 10^7 BB-12 cells (Yog BB-12 Ab) orally from the tip of a gavage bulb [20]. A fifth group of mice was administered saline but not antibiotics (Controls, Con).

Mouse weight and water intake were measured every 24 h. Freshly expelled fecal samples were collected immediately prior to the initiation of antibiotic administration (t = 0), and then after 1, 5, and 10 days of the study. Fecal samples were frozen immediately in liquid nitrogen and stored

at -80 °C until further processing. At the time of sacrifice, the mice were anesthetized under CO_2 prior to blood collection via cardiac puncture and termination was then ensured death by cervical dislocation. Ileal and cecal contents, colonic tissues, and liver were collected, and frozen immediately in liquid nitrogen, and stored at -80 °C until further processing.

2.4 Sample preparation for genomic DNA and metabolomic analysis

Cecal genomic DNA and metabolite extractions were conducted on the same day to reduce the impact of freeze-thaw, as reported previously [22]. In brief, approximately 200 mg cecal contents were suspended and homogenized in 1.5 mL ice-cold Dulbecco's phosphate buffered saline (DPBS, 1X, pH 7.4) followed by vigorous vortexing, incubation on ice for 5 min, and centrifugation at 14,000 ×g for 5 min at 4°C. The resulting pellet and supernatant were used for DNA and metabolite extraction, respectively.

2.5 Genomic DNA extraction

Genomic DNA was extracted from pelleted cecal contents using 200 mg mouse feces using mechanical lysis and phenol-chloroform extraction as described [23]. For extraction of DNA from *B. lactis* BB-12 and *Lactobacillus plantarum* NCIMB8826, the strains were incubated in MRS for 24 h at 37°C prior to DNA extraction from BB-12 as described above. DNA was purified with the Wizard SV gel and PCR Clean-Up system (Promega, Madison, WI) according to the manufacturer's protocol. The DNA concentration was measured with the Qubit 3.0 fluorometer using the Qubit double-stranded DNA (dsDNA) HS assay kit (Life Technologies, Eugene, OR).

2.6 Quantitative PCR for BB-12 and total bacterial cells

The *B. lactis* BB-12 gene encoding elongation factor Tu (*tuf*) was used for BB-12 enumeration using previously described primers, 5'ACAAGCAGATGGATGAGTG3' and 5'AGAAGAACGGCGAGTGAC3' [21]. Estimates of gene copy numbers were made using primers targeting 5'GTGSTGAYGGYYGTCGTCA3' and 5'ACGTCRTCCMCNCCTTCCTC3' of the 16S rRNA gene [21]. Reactions were performed on an Applied Biosystems 7500 Real-time thermocycler using 0.2 μM of each primer and PowerUp™ SYBR™ Green Master Mix (Thermo fisher) under the following conditions: 2 min at 50 °C, 2 min at 95 °C, and 40 cycles of 15 sec at 95 °C, 15 sec at 60 °C and 1 min at 72 °C. Standard curves of BB-12 and *L. plantarum* genomic DNA were used to enumerate quantities of *Bifidobacterium* and total 16S rRNA gene copy numbers, respectively, as previously described [22].

2.7 16S rRNA gene amplicon DNA sequencing

PCR amplification of the V4 region of 16S rRNA genes contained in cecal and fecal contents with 30 PCR cycles at 94°C for 45 sec, 54°C for 60 sec, and 72°C for 30 sec was performed as described previously [24]. Briefly, ex-Taq DNA polymerase (TaKaRa, Otsu, Japan) and primers F515 forward (5′-GTGCCAGCMGCCGCGGTAA-3′) and R806 reverse (5′-GGACTACHVGGGTWTCTAAT-3′) primers with a random 8-bp barcode on the 5′ end of F515 were used [25]. Negative controls were included for each barcoded primer to confirm primer purity. PCR products were pooled and then gel purified with the Wizard SV gel and PCR clean-up system (Promega, Madison, WI). Ion Torrent S5 libraries were prepared from a pooled amplicons ligated with non-barcoded ion A and Ion P1 adapters. They were prepared by using Ion 510™, 520™,

530™ Ion Chef Kit (Life Technologies Corporation, A34461) and 530 Chip Kit (Life Technologies Corporation, A27764) for 200 base-read libraries. The prepared libraries were then sequenced on an Ion S5 XL Sequencer (Life Technologies Corporation).

The sequence output BAM file was converted to FASTQ format using BED Tools [26] and reads shorter than 200 bp were removed. Data analyses was performed on the remaining sequences. Specifically, raw FASTQ files were demultiplexed and quality filtered using the Quality Insights into Microbial Ecology 2 (QIIME2) software (version 2019.10) [27]. Demultiplexing was performed using the "demux emp-single" command after barcodes were removed using the "extract_barcodes.py" command from QIIME software (version 2018.4). Quality filtering was performed using the "deblur denoise-16S" command. After denoising, both sets of samples were rarified for alpha and beta diversity and taxonomic analysis.

The cecal samples were rarefied to a depth of 5,547 and fecal samples were rarefied to a depth 2,871 reads per sample prior to analysis. In total, insufficient read numbers were obtained from five mice for further analysis (cecum: saline (one mouse), yogurt (two mice); feces: BB-12 (two mice)). For taxonomic classification, the QIIME2 naïve Bayes classifier was trained on the SILVA 16S rRNA gene database version 13–8 [28] clustered at 99%. SILVA was used because it provided more accurate assessments of bacterial taxonomy with a mock community compared with the 16S rRNA Database Project [29].

2.8 Metabolome analysis

For metabolite analysis, the supernatant was filtered using a 0.22 m pore size syringe filter (Millex-GP syringe filter, Millipore, Billerica, MA) and then with a filter with a 3 kDa molecular weight cut-off (Amicon ultra centrifugal filter, Millipore, Billerica, MA). An internal standard

(DSS (Disodium-2,2-dimethyl-2-silapentane-5-sulfonate)-d6 in 5 mM, with 0.2% sodium azide in 99.8% D2O) was added to the filtrate and the pH of each sample was adjusted to pH 6.8 ± 0.1 with 1 M and 0.1 M of NaOH and HCl to minimize pH-based peak movement. 180 µL aliquots were subsequently transferred to 3 mm Bruker NMR tubes (Bruker, Brillerica, MA), stored at 4 °C until spectral acquisition. ^{1}H NMR spectra were acquired at 298K using the NOESY ^{1}H presaturation experiment ('noesypr1d') on Bruker Avance 600 MHz NMR spectrometer (Bruker BioSpin, Germany). Spectral acquisition, processing parameters, and the estimation of metabolites concentration performed as previously described [22].

2.9 Statistical analysis

Statistical analyses were performed with Prism 7.0 GraphPad Software version 8.0 for Mac (GraphPad Software, San Diego, CA, USA). Data are presented as mean ± SEM unless stated otherwise. Analysis of differences between the different groups were analyzed with the Kruskal-Wallis multiple comparison test followed by Dunn's post hoc analysis, with p values ≤ 0.05 considered to be significant. A linear mixed model regression analysis was used to assess the effect of sex on colon length and percentage of body weight change controlling for the potential random variable confounding effect of cohort by using SAS 9.4 (SAS Institute, Cary, NC, USA) as described previously [30]. Pearson correlation was used to correlate metadata to the Shannon diversity index. p-values <0.01 were considered statistically significant.

Significant differences in Bray-Curtis and weighted UniFrac distances between the fecal and cecal microbiota were determined with PERMANOVA with pairwise comparisons using the QIIME2 beta-group-significance script. Sample richness/evenness was estimated using the Shannon index, Observed OTU & Faith PD using QIIME2. Unless specified otherwise, microbiome

data statistical analyses and figure construction were performed in R version 3.6.2 [31]. Spearman's Rank Correlation Coefficient was used for correlations between the cecal microbiota and metabolome with p adjusted < 0.05 considered significant. PCoA was made based on Euclidian distances using multivariate analysis for cecal metabolome data.

3. Results

3.1 Mouse body weights and water intake were affected by amoxicillin-clavulanate (AMC) administration

The body weights of mice given AMC changed over time in a sex-dependent manner irrespective of BB-12 or yog (yogurt) intake. The body weights of male mice given AMC were lower compared to control mice throughout whole study (10 days) (Linear mixed model regression analysis, $p<0.05$) (**Fig 1A**). This result was not observed in females receiving AMC. Female mice that received yog + Ab (with or without BB-12) gained more body weight compared to mice given only antibiotics (saline + Ab mice) (Two-way ANOVA, $p<0.05$) **(Fig. 1B).** These changes in body weight were consistent between the three cohorts of mice tested for each treatment group (Two-way ANOVA, $p > 0.05$) **(Fig 1).**

For the first three days all male and female mice administered AMC (with or without yog and BB-12 fed) on average consumed 2.3-fold less water per day compared to control mice (**Fig. 1C and Fig. 1D**). After day three of the study, their water intake increased and was equivalent to the saline controls for both sexes (**Fig. 1C and Fig. 1D**). With regards to water intake, there were no significant differences between male and female mice and between three cohorts tested (data not shown).

3.2 AAD and yogurt altered the mouse digestive tract

Although there were no differences in stool consistency with AMC administration (data not shown) and colon lengths were not affected in the AMC controls, mice given AMC had enlarged ceca, irrespective of treatment (**Suppl Fig. 1**). Amongst mice given yogurt (with or without BB-12), the colon lengths were significantly longer compared to controls to which no antibiotics were administered (Two-way ANNOVA, $p < 0.05$) (**Suppl Fig. 2**). Sex differences and cohort effects were not found between colon lengths of mice (Two-way ANNOVA, $P = 0.59$).

3.3 *Bifidobacterium tuf* copy numbers were at or below the detect limit in most mice

qPCR targeting the *tuf* gene encoding elongation factor Tu in *Bifidobacterium* was used for quantification of bifidobacteria and BB-12 in the mouse cecal and fecal contents. Bifidobacteria were detected in very low and variable numbers (approximately 100 *tuf* gene copy numbers) per gram (**Fig 2**). Notably, bifidobacteria were detected in the saline controls, a finding indicative that the *tuf* primers are not 100 % strain specific [32] (**Fig. 2)**. Numbers of *Bifidobacterium* were noticeably significantly higher in the cecal contents of mice given BB-12 in saline (one- way ANNOVA, $p<0.05$), and were detected at levels 3-fold higher than fecal contents **(Fig. 2D).** There were no significant differences between mouse cohort and sex (one-way ANNOVA, $p >0.05$).

3.4 AMC caused significant losses in bacterial numbers in the mouse intestine

AMC treatment resulted in at least 3-fold reductions in bacterial cell numbers within one day of administration ($p < 0.0005$, Two-way ANNOVA) **(Fig. 3)**. Cell numbers, estimated as 16S rRNA gene copy numbers, reduced from 10^8 cells/g to 10^3 cells/g, irrespective of yogurt or BB-12 administration. Notably, the numbers of bacterial cells were different between three cohorts of mice such that consistent reductions in bacterial amounts occurred only for cohorts two and three

from 10^8 cells/g to 10^3 cells/g, but not for cohort one which is 10^8 cells/g to 10^5 cells/g **(Fig. 3).** These reductions were sustained on day 5 and 10, and in a cohort consistent manner. Cell numbers in cecal contents were higher than in the stools (**Fig. 3)**. The numbers of bacteria in cecal contents decreased by 1.5-fold, from an average +/- 7.5 std dev to average +/- 5.2 std dev among mice given AMC **(Fig 3)**.

3.5 AMC resulted in significant changes to the intestinal microbiota composition

The bacterial composition in the mouse fecal contents was significantly affected by AMC administration, regardless of BB-12 or yogurt consumption (Bray-Curtis diversity; $p<0.05$, Kruskal-Wallis test) (**Fig. 4A)**. This change occurred within one day of AMC administration **(Fig. 4A).** Bacterial beta-diversity in the cecum was also affected by AMC, and on 5th and 10th day significant differences was observed between the BB-12 and yogurt BB-12 treatment groups. Noticeably, on 10^{th} day beta diversity of control was significantly different from all AMC treated mice except for yogurt BB-12 fed mice **(Fig. 4B, Suppl. Fig. 3)**. Additionally, there was a cohort effect on the cecal and fecal microbiota, where cohort one clustered separately from cohorts two and three, even among saline controls (Kruskal-Wallis, $p = 0.0012$) **(Fig. 4A, Fig. 4B)**.

Consistent with the observed differences in bacterial-beta diversity, AMC also impacted bacterial alpha diversity in fecal and cecal contents (Shannon-Index, Faith PD and Observed OTU) **(Suppl. Fig. 4 and Suppl. Fig. 5**). Alpha diversity declined significantly in the fecal contents within one day after the start of AMC administration and remained low for the remainder of the study (Kruskal- Wallis, $p>0.05$) (**Suppl Fig. 4)**.

Prior to antibiotic administration, all mice contained high relative abundance of *Muribaculaceae, Lactobacillaceae, Lachnospiraceae, Staphylococcaceae, Ruminoccocaceae, Oscillospiraceae,* and *Rikenellaceae,* comprising, on average, 85% of the fecal microbiota **(Suppl Fig. 6)**. The feces of the saline control mice maintained high relative proportion of these taxa over the course of the ten-day study. The cecal contents of those mice was also mainly comprised of *Staphylococcaceae, Lactobacillaceae, Muribaculaceae, Lachnospiraceae,* and *Ruminoccocaceae* (**Suppl. Fig. 9)**.

After one day on AMC, the proportions of these bacteria declined in all AMC-treated mice, including those receiving BB-12 in either saline or yogurt format ($p<0.005$, Kruskal-Wallis test) **(Suppl Fig. 6)**. For mice given AMC, the relative proportions of *Acholeplasmataceae, Sutterellaceae,* and *Staphylococcaceae* were higher after the first day (**Fig. 5**). Proportions of *Streptococcaceae* and *Leuconostocaceae* were significantly higher ($p > 0.05$, Kruskal Wallis) on day 5 (**Fig. 5**). After ten days of AMC intake, proportions of *Enterobacteriaceae* and *Bacteroidaceae* were higher in the stool compared to the saline (sham) controls **(Fig. 5)**. Similarly, the cecal contents were enriched by *Staphylococcus, Lactobacillus, Muribaculaceae, Lachnospiraceae, Ruminoccocaceae* in control mice, compared with AMC treated mice. However, *Akkermansiaceae, Paenibacillaceae, Streptococcaceae* and *Clostridiaceae* were significantly higher ($p > 0.05$, Kruskal Wallis) in AMC treated and BB-12 fed mice with or without yog, compared with control mice **(Fig. 5)**.

Differences were observed among three cohorts of mice, with regard to the impacts of AMC on fecal microbiota composition. Cohort one contained significantly higher proportions of Bacteroidota and Actinomycetota, compared with cohorts two and three. These differences were observed in control mice and after one day of AMC administration. Cohort one was enriched by

Bacteroidota and cohorts two and three had significantly higher proportion of Bacillota (Kruskal Wallis, $p < 0.05$) **(Suppl. Fig. 7)**. No significant cohort effect was observed in Pseudomonadota (Kruskal Wallis, $p > 0.05$). Consistent with the elevated numbers of *tuf* gene copy numbers in the cecal contents of mice given BB-12 in saline, proportions of *Bifidobacteriaceae* were significantly higher at day 5 feces and day 10 cecal contents of those mice (Kruskal Wallis; $p > 0.005$) **(Fig. 5)**. Proportions of *Muribaculaceae* was not as reduced when BB-12 was consumed in yogurt and saline in fecal content on day 1 and 5 compared with only AMC treated mice with and without yogurt (Kruskal Wallis; $p = 0.0045$) **(Suppl fig. 6)**. In the cecal contents, *Bifidobacteriaceae* was more abundant in BB-12 in powder form (saline) than yog + BB-12 **(Suppl Fig. 9)**. In addition, cohorts' effect was observed where cohort one was significantly different than cohort two and three (Data not shown) similar to fecal microbial community.

3.6 AMC reduced SCFA and altered amino acid concentrations in the mouse cecum.

Multivariate analysis of over 35 metabolites detected in the mice cecal contents showed that AMC resulted in significant changes to the cecal metabolome (One-way ANNOVA, $p < 0.05$) **(Fig.6A**). Despite significant differences between one of the cohorts compared to the other two **(Suppl. Fig. 11)**, mice given AMC had significant reductions (4.5-fold, on average) of the SCFA acetate, butyrate, and propionate compared to saline administered controls **(Fig. 6B)**. These reductions were also observed for mice given BB-12 in saline or yogurt **(Fig. 6B)**. Similarly, isobutyrate, valerate, isovalerate, inosine, methionine, hypoxanthine, uracil, nicotinate, and taurine were found in lower levels in AMC administered mice (One-way ANNOVA test; $p < 0.05$) **(Suppl Fig. 10A)**. Other metabolites were enriched with AMC intake, including lactate, proline, threonine, pyruvate, creatine, creatinine, arginine, and putrescine **(Suppl Fig. 10B)**.

As found for the intestinal microbiota, mice fed BB-12 also exhibited differences for certain cecal metabolites. All AMC administered groups except for those given BB-12 in yogurt contained higher concentrations of formate, glycine, and glutamine in the cecal contents (One-way ANNOVA test; $p<0.05$) **(Suppl. Fig. 10)**. Compared with mice given BB-12 in saline, mice provided with BB-12 in yogurt contained higher levels of thymine, dimethylamine, fumarate, methyl and lower levels of ornithine, tyramine, acetone, sarcosine, aminobutyrate and hydroxybutyrate **(Suppl. Fig. 10)**.

3.7 Cecal metabolites were correlated with specific microbial taxa

SCFA such as acetate, butyrate, propionate, valerate, isobutyrate (BCFA) and other metabolites such as aspartate, inosine, taurine, uridine were highly correlated with the proportions of *Ruminococcaceae*, *Muribaculaceae, Lactobacillaceae, Akkermansiaeae, Anaerovoraceae, Bacteroidaceae, Rikenellaceae* and *Oscillospiraceae* in the mouse cecum, a finding consistent with the significant reductions in those taxa with AMC intake. SCFA were also negatively correlated with *Erwiniceae* and *Enterobacteriaceae* proportions (**Fig. 7)**. Concentrations of the amino acids such as arginine, threonine, glutamine, glycine, proline, and serine were negative correlated with the proportions of *Muribaculaceae, Akkermansiaceae, Lachnospiraceae, Acholeplasmataceae* and *Oscillospiraceae,* (**Fig. 7)**.

4 Discussion

Antibiotics are very important drugs for the treatment of various diseases. Despite their importance, these drugs can also cause unwanted negative effects on the composition and function

of normal human and animal microbiome [40]. Probiotics may be able to prevent or reverse antibiotic-induced disruptions to the gut microbiome [40]. In this study we explored the capacity of *Bifidobacterium lactis* BB-12 in different delivery matrices to sustain the gut microbiota and metabolome during AMC intake in three different cohorts of mice. The mixture of amoxicillin and clavulanic acid (AMC) is one of the most frequently prescribed antibiotics in the western world, however it can cause antibiotic associated diarrhea (AAD), especially in infants and adults [3, 40].

In this study, AMC resulted in significant reductions in only male body weight compared to control mice at all time points. While oral antibiotic-treated male mice drink less than control mice, these body changes may not only due to less water intake alone but also depend on microbiome and metabolic changes. In addition, AMC induced inflammation on cecum which is consistent with many studies [41]. Cecal inflammation was observed may be by accumulation of mucus and undigested fibers caused by the absence of gut microbiota [42]. Interestingly, we observed yogurt effect was more dominant than AMC on the mouse colon length may be due to fermented bacteria in yogurt had anti-inflammatory effect on colon which was caused by AMC.

Our 16S rRNA results showed that AMC induced pro-found alterations of the intestinal microbiota consistent with existing data [43], reduced alpha-diversity and reduction in 16S rRNA cell counts, which was observed already within one day of AMC intake. These results are consistent with prior mice study where, gut microbiome changed within sixteen hours after the first dose of antibiotics in mice [47]. AMC is combination of amoxicillin and clavulanate where amoxicillin is the class of beta-lactam antimicrobials and beta-lactams act by binding to penicillin-binding proteins that inhibit a process called transpeptidation (the cross-linking process in cell wall synthesis), leading to activation of autolytic enzymes in the bacterial cell wall. This process leads to lysis of the cell wall, thus destroying the bacterial cell [44]. Therefore, as expected, AMC intake

inhibited Gram-positive bacteria such as Bacillota (formerly called Firmicutes) and Bacteroidota, including families *Lactobacillaceae*, *Muribaculaceae, Staphylococcaceae, Ruminoccocaceae, Oscillospiraceae,* and *Rikenellaceae* families. Conversely, Gram-negative bacteria in the Pseudomonadota (formerly called Proteobacteria) phylum were enriched by AMC intake, which is comprised by facultative and obligative anaerobic bacteria, comprising several known pathogens in humans [45]. Only two families, *Erwiniceae* and *Enterobacteriaceae* of phylum Pseudomonadota significantly enriched maybe they are resistant towards AMC in cecal and fecal contents [46]. This finding is also consistent with prior human and mice studies showing AMC mainly increases in Enterobacteria and anaerobic bacteria such as *Bifidobacterium* sp, *Lactobacillus* sp and clostridia decreased significantly ($p < 0.05$). Also, they found Bacteroides were not markedly altered during the administration period similar to our study findings [46].

Interestingly, we observed changes in the bacterial community was based on the notable cohort and time-dependent differences [46]. Consistent with AMC dominant effect our investigation shown that AMC may eventually result in the eradication of the anaerobes such as phylum Bacillota, Bacteroides and family such as *Bifidobacteriaceae* [48,49]. As the high susceptibility of the Bifidobacterium genus to amoxicillin-clavulanic acid was previously demonstrated in vitro [37, 38], it seems that the microbial community changes occurred during antibiotic administration was based on time points. Members of the normal Bifidobacterium cannot be completely eradicated by this antimicrobial treatment. The antimicrobial effect is probably dose-related, and removal of the antibiotic allows prompt regrowth of their population [50]. Therefore, *Bifidobacteriaceae* were not observed as a dominant family (<5%) even in the BB-12 fed mice. According to many research, yogurt fermentation may influence the survival of BB-12 [50], however, our qPCR quantification results shown that delivery matrix powder form had a greater survival rate than the

yogurt form for BB-12 in both cecum and feces which is contrary to many studies [51]. These contrary results observed due to AMC may had strong effects on the yogurt resulting in eradication of almost anaerobic bacteria including BB-12 compared to saline/ powder form.

Furthermore, host-microbiota metabolic interactions are dynamic, so antibiotic-induced changes in the gut microbiota can result in the alteration of host metabolism [52]. SCFAs, being essential bacterial metabolites would bear the brunt of the impact first [53]. Consistent with this, we found that AMC treatments significantly decreased levels of SCFAs, including acetate, butyrate, and propionate however, the level of formate, another SCFA, showed an opposite response to antibiotic exposures in cecum. Correlation network analysis revealed that the gut microbiota was more closely related to SCFAs in mice. We found that *Ruminococcaceae*, *Muribaculaceae, Lactobacillaceae, Akkermansiaeae, Bacteroidaceae, Rikenellaceae* and *Oscillospiraceae* had strong relationships with SCFA production, which are significantly reduced in cecal contents in AMC treated mice. These results were observed in prior study in only female mice [54].

The gut microbiota also has a key role in the supply of both BCAAs and AAAs to maintain host amino acid homeostasis [54, 55]. In this study, several associations were observed between the gut microbiota and amino acids in mice. Moreover, Sridharan *et al.,* 2014 [55], reported that amino acid and SCFA metabolism was largely affected by the gut microbiota, of which Pseudomonadota could be the most important moderator [55]. Interestingly, we identified that *Erwiniceae* and *Enterobacteriaceae* in the Pseudomonadota phylum had highly negative relationships with SCFA and amino acids such as glutamine, glycine and proline and these families were significantly increased in AMC treated mice compared to control mice. Yet another SCFA, formate oxidation contributes to the inflammation-associated bloom of phylum Pseudomonadota especially Enterobacteriaceae family [56]. In our study we found that, *Erwiniceae* and *Enterobacteriaceae* showed

positive correlation with formate. Therefore, may be cecum inflammation in AMC treated mice was a result of higher proportion of *Erwiniceae* and *Enterobacteriaceae.* Interestingly, we observed that formate was significantly increased for all AMC treated mice except for yogurt BB-12 mice. Therefore, yogurt and BB-12 combination may have positive effect in the restoration of formate level.

Finally, to compare the effect of BB-12 and AMC we used genetically homogenous C57Bl/6J mice grown in a similar condition to ensure that the experimental condition is identical for each mouse. Thorough statistical analyses showed that cohort 1 had different microbiome in GI tract compared to cohort 2 & 3 at each time point in AMC treated mice. No cohort effect was observed for body weight, water intake, or metabolome data. This indicates that cohort 1 mice may have behaved differently on a genotype-by-genotype basis than cohorts 2 and 3 which is consistent with previous C57Bl/6J mice results [42].

5 Conclusion

Our microbiota results suggest that high dose of AMC had very strong effects on microbial composition and cecal metabolites indicated by significant reduction in male body weight, cecal inflammation, reduction in 16S rRNA cell numbers, reduction in SCFA and amino acids and significant increase in the proportion of phylum Pseudomonadota which is a sign of gut dysbiosis. Overall, effects of BB-12 and yogurt were minimal in AMC treated mice. Additionally, we found that survival rate of BB-12 in powder form was higher compared to addition of yogurt.

Future research should extend these findings and include using low dose of amoxicillin clavulanate through metagenomic and metatranscriptomics analysis of the microbiota, while also expanding the clinical outcomes.

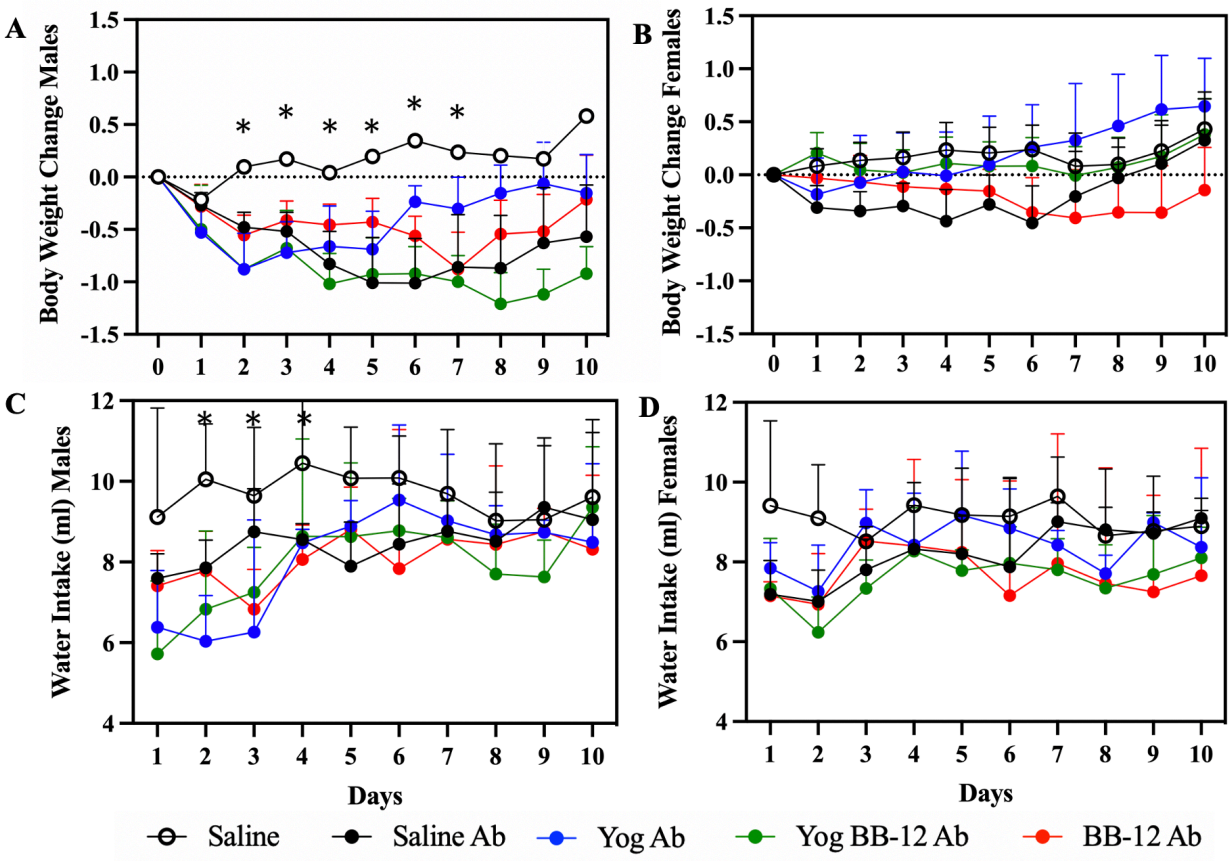


Figure 1. Weight change and water intake over the 10-day study. Percent change in mouse body weight for (**A**) males and (**B**) females and water intake (ml) for **(C)** males **(D)** females, relative to the day when AMC was first added to the drinking water. Average water intake (ml) quantified for two mice per cage, every 24 h starting on the first day after AMC and the study. Body weight data and water intake are presented as mean ± SEM (n = 12 for body weight, n = 6 cages with two mice per cage). Significant differences were determined using the Two-way ANOVA (* $P<0.05$). '*' represents significant difference between saline (control) and all antibiotic (Ab) treated mice for each day.

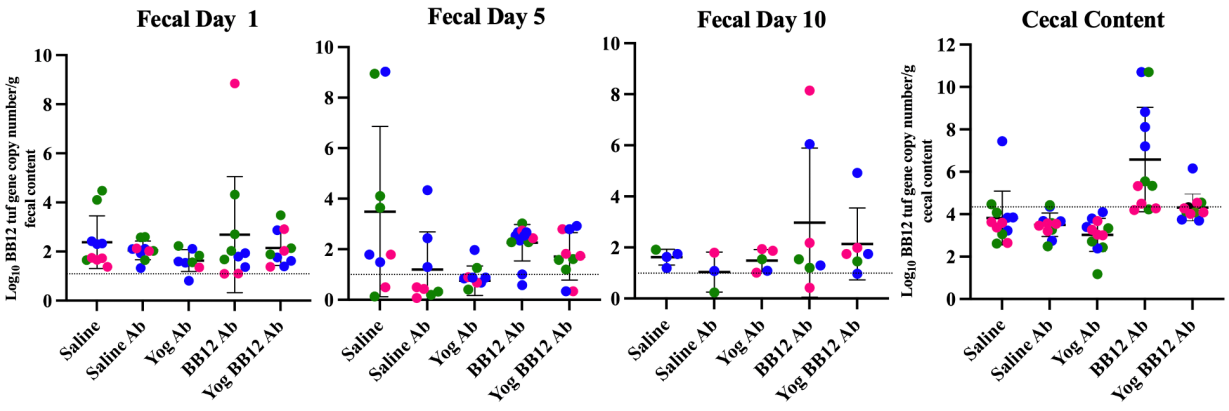


Figure 2. *Bifidobacterium tuf* copy numbers in mouse fecal and cecal contents. Each point represents one fecal or cecal or sample. Data are presented as mean ± SD (n=12 mice per treatment for fecal contents (day 1 and 5) and cecal contents, n=8 mice for fecal contents at day 10). The broken horizontal line indicates the lower detection limit of *Bifidobacterium tuf* gene copy numbers. Blue dots indicate mouse cohort 1, green dots cohort 2 and pink dots indicate cohort 3.

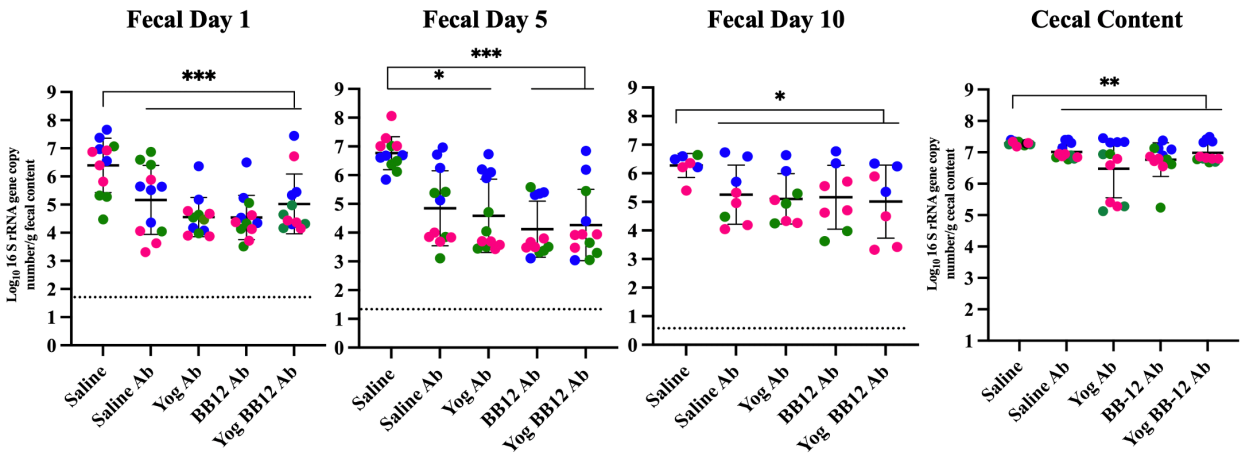


Figure 3. Bacterial cell number estimates in mouse cecal and fecal and cecal contents. The dashed horizontal line indicates the lower limit of detection limit of 16S rRNA gene copy number. Blue circles - cohort 1, green circles - cohort 2 and pink circles - cohort 3. Data are presented as mean ± SD (n=12 mice per treatment for fecal contents (day 1 and 5) cecal contents and n=8 mice for fecal contents at day 10). Significant differences were determined using the Two-way ANOVA test (* P<0.05, ** P < 0.01, *** P< 0.001, **** P<0.000

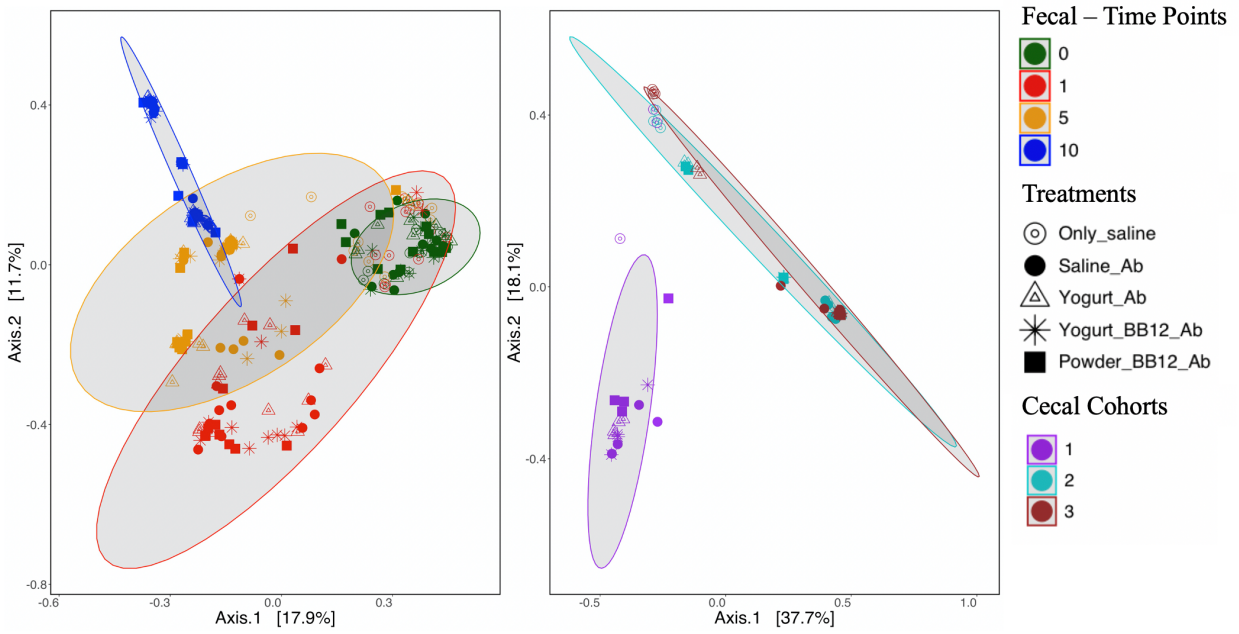


Figure 4. Bacterial beta diversity in mouse feces and cecal contents. PCoA plots on beta diversity based on Bray- Curtis distances for 16S rRNA bacterial communities in (A) fecal and (B) cecal contents (1 time point). Confidence ellipse plots for each group represented at a 95% level based on different time points for fecal contents and for three the cohorts in cecal contents. Cohort difference also observed in fecal content was similar to cecal content (data not shown).

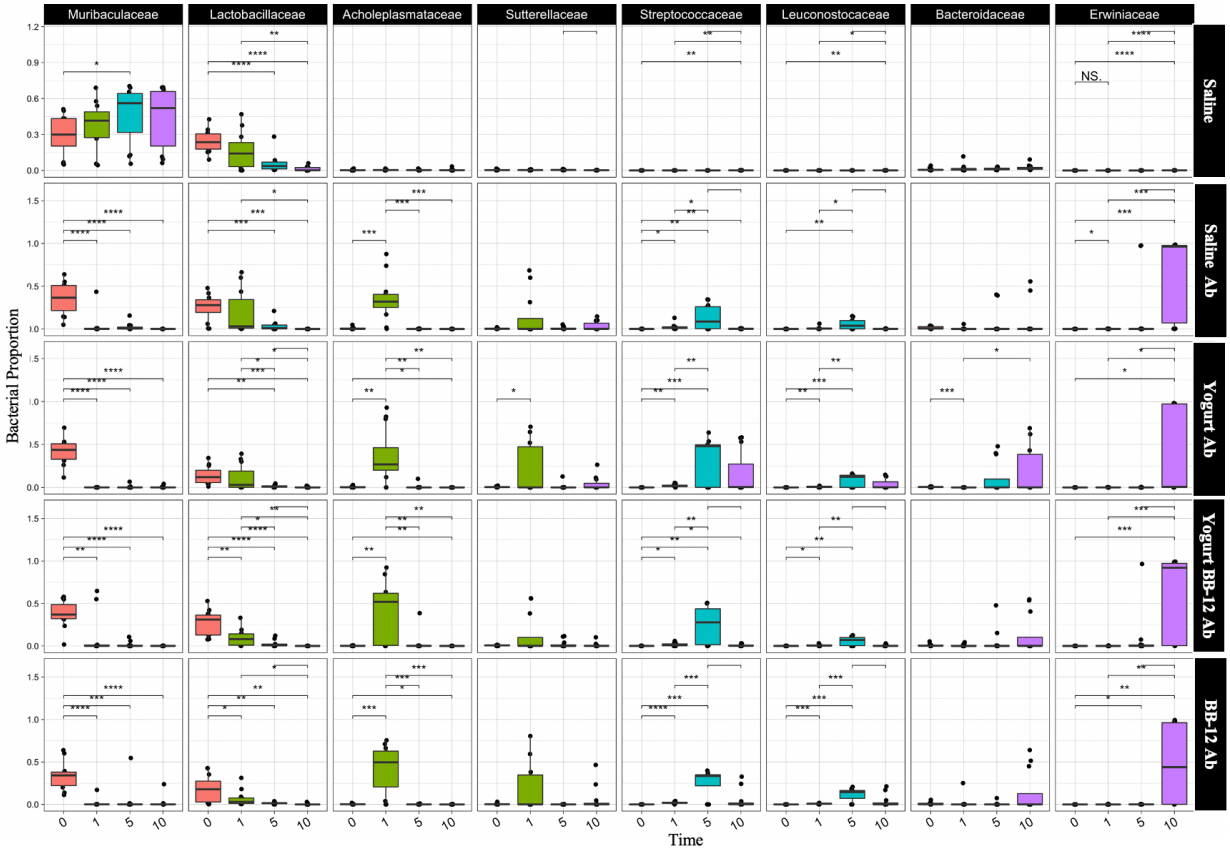


Figure 5. Bacterial taxa detected in significantly different proportions in fecal samples. Bacterial families found in significantly different proportions in fecal samples in at least one time point are shown. Significant differences were determined using the Kruskal- Wallis test (* P<0.05, ** P < 0.01, *** P< 0.001, **** P<0.0001).

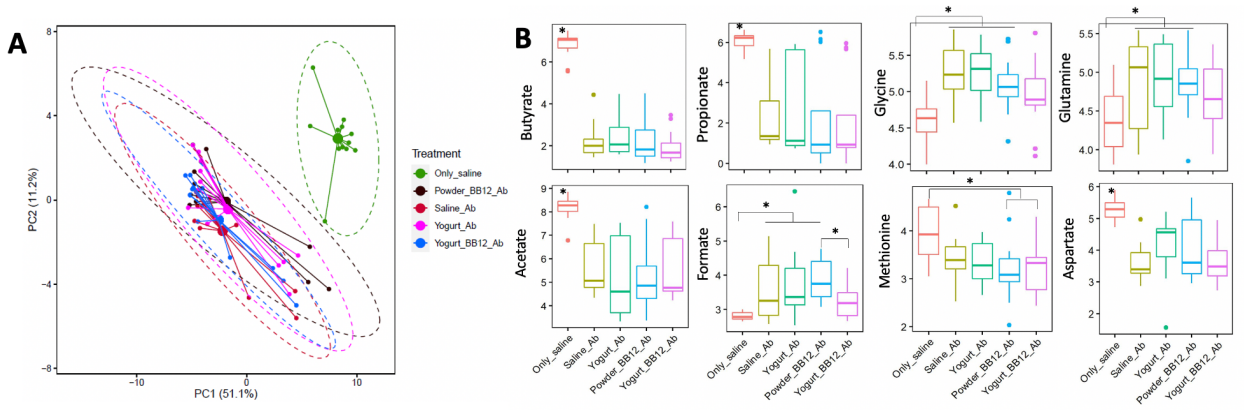


Figure 6. AMC resulted in reduced cecal SCFA and altered amino acid levels. (A) PCoA plot made based on Euclidian distances for cecal metabolome. **(B)** Metabolites found in different quantities in mouse cecal contents. Significant differences were determined for SCFA and amino acids using the ANNOVA test followed by post hoc analysis (* $P<0.05$).

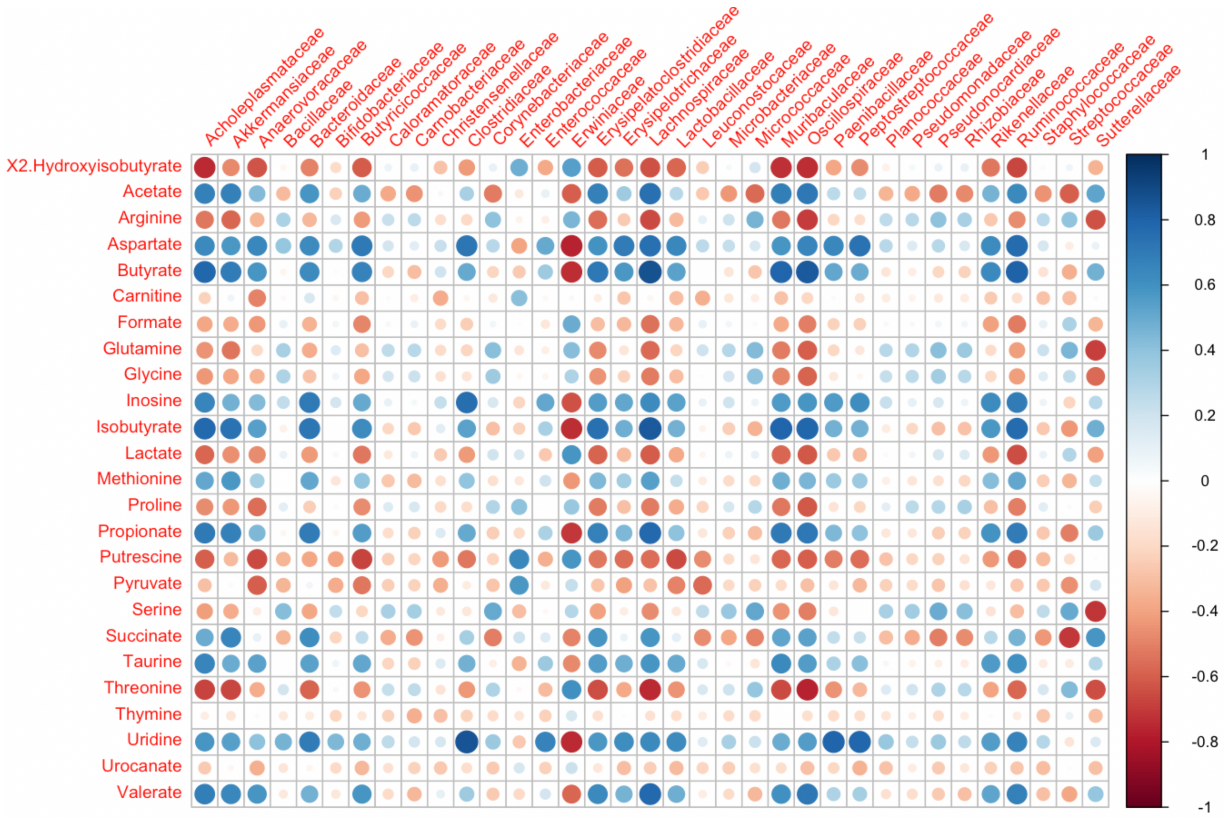
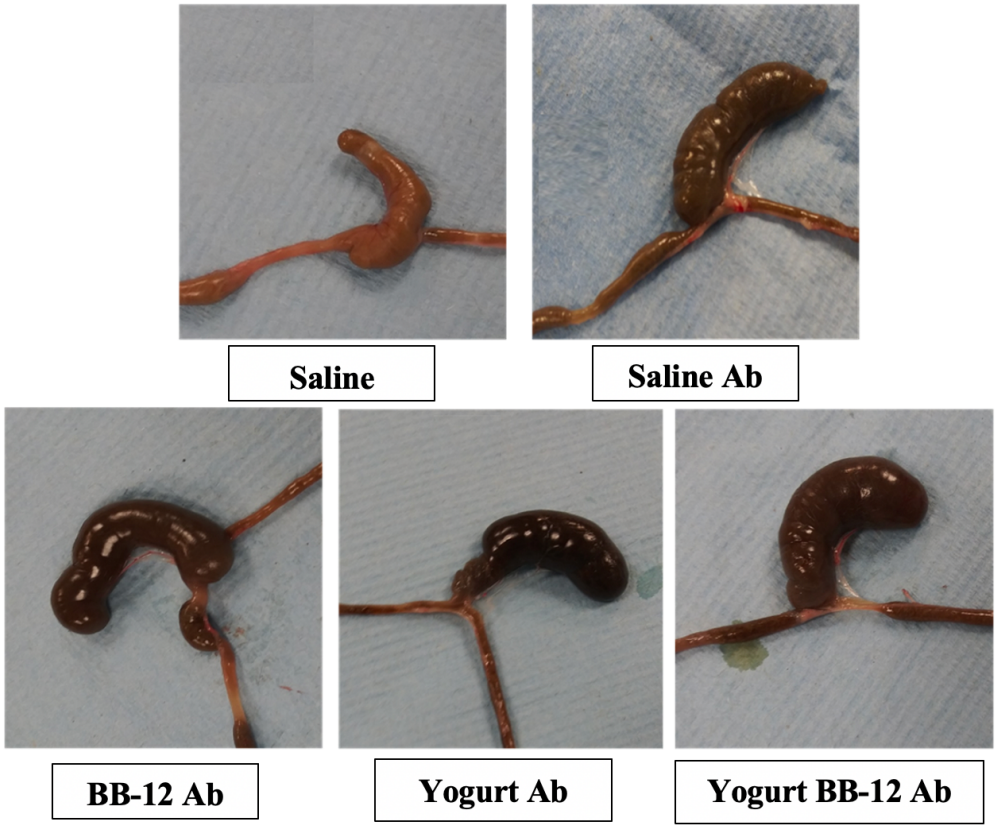
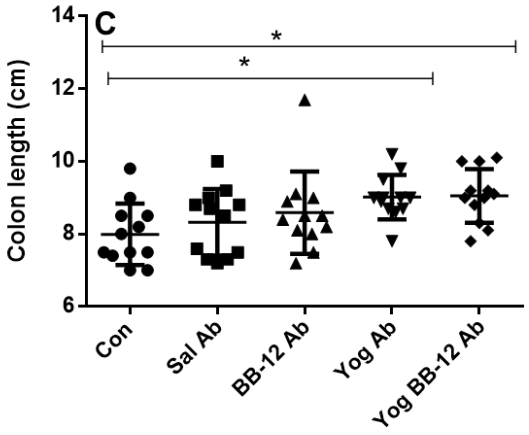


Figure 7. Correlation between cecal metabolome and microbiota. Pairwise correlations of metabolites and family level taxonomic abundances in the cecum. Positive correlations are shown in blue and negative correlations in brown. Blue represented significant positive correlation ($P < 0.05$, 1 indicate perfect positive correlation (dark blue), brown represented significantly negative correlation ($P < 0.05$, -1 represent perfect negative correlation (dark brown)), and white represented that the correlation was not significant ($P > 0.05$) according to Spearman's rank correlation coefficient.

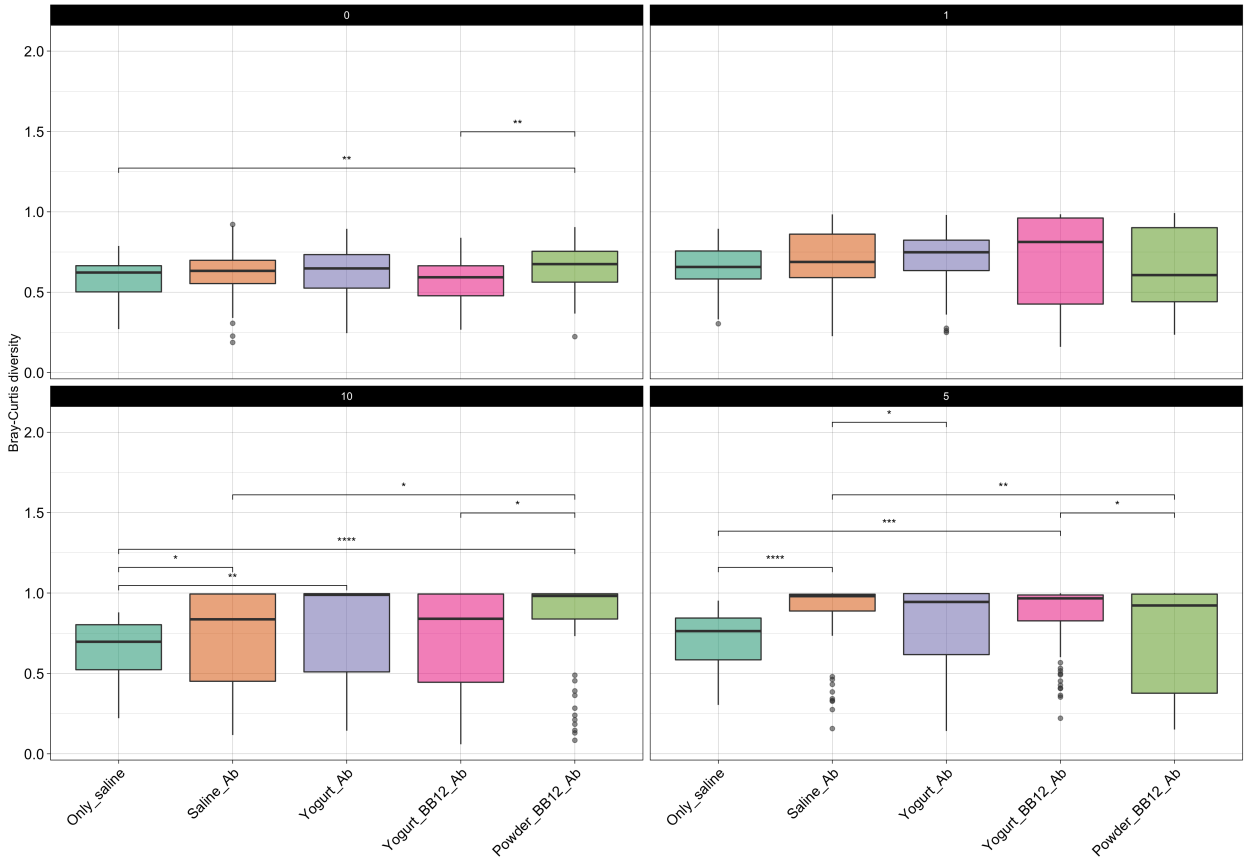
Supplemental Figures



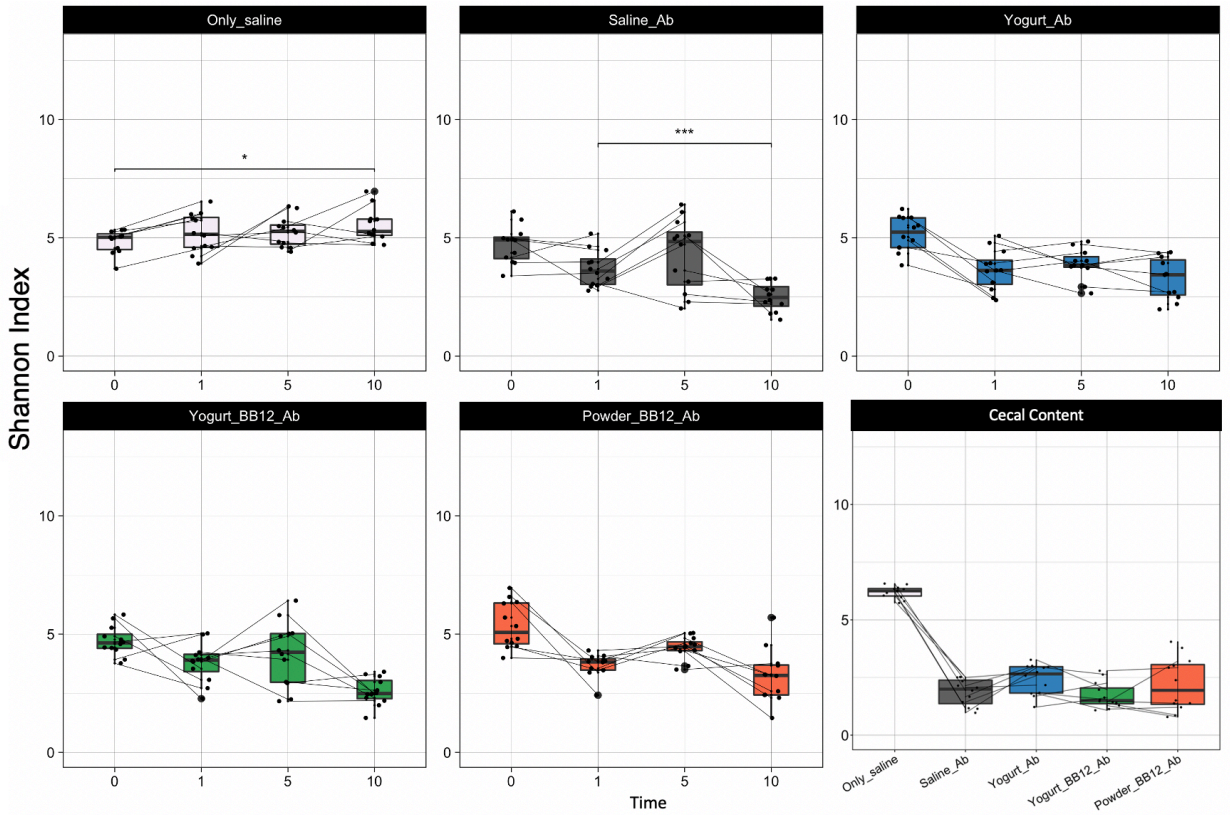
Suppl. Fig. 1. Antibiotics resulted in enlarged cecum sizes of mice on day 10. Representative images are shown for each treatment.



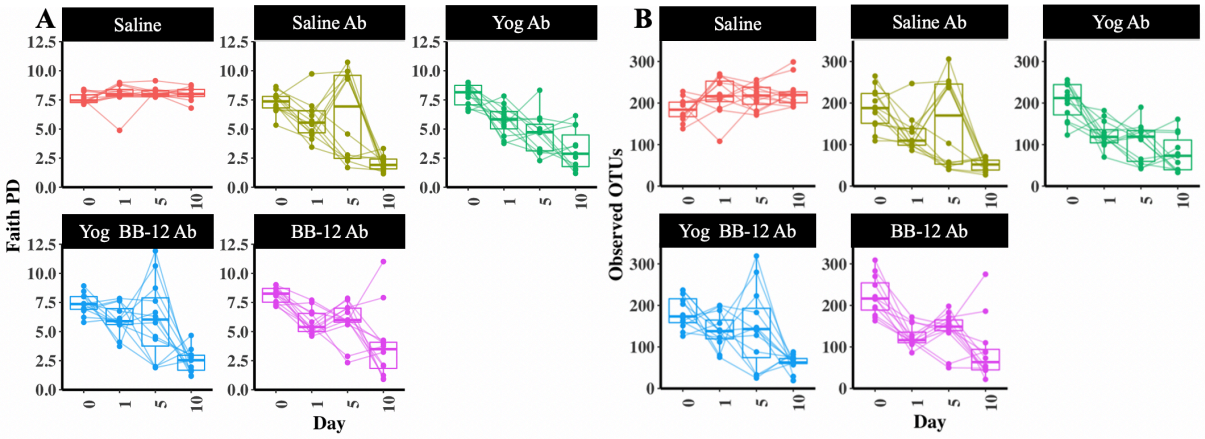
Suppl. Fig 2. Colon length of mice. Colon lengths were measured during necropsy from the proximal colon to the anus on day 10. Data are presented as mean ± SEM (n = 12 mice). *, $p < 0.05$ according to Two-way ANOVA test.



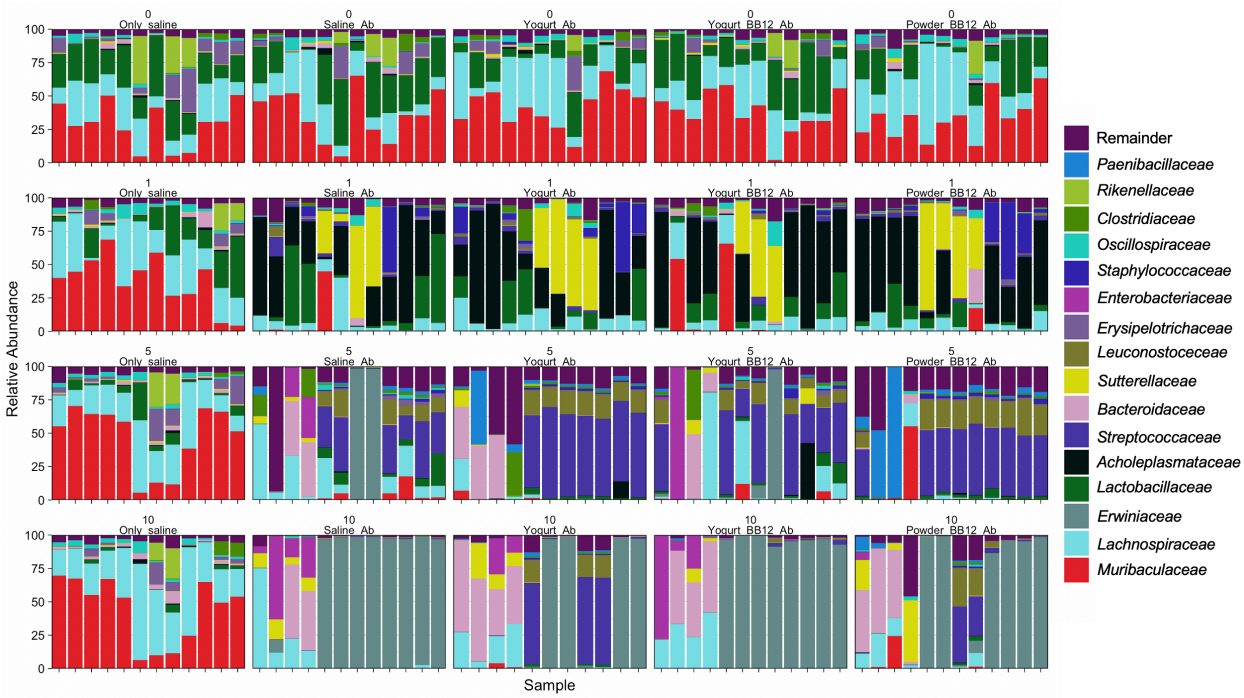
Suppl. Fig 3. Bacterial beta diversity in fecal contents. Bray curtis diversity at different sampling times of different treatments showed on x axis. Significant difference was determined using Kruskal- Wallis test (* P<0.05, ** P < 0.01, *** P< 0.001, **** P<0.0001)



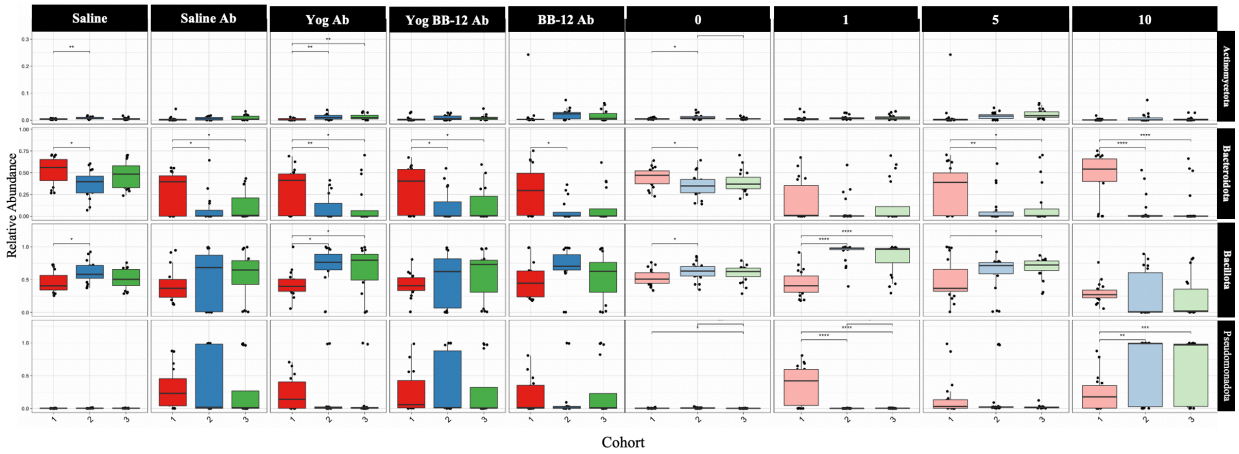
Suppl. Fig 4. Bacterial alpha diversity in fecal and cecal contents. Shannon diversity at different sampling times of different fecal content and cecal content. Significant difference was determined using Kruskal- Wallis test (* P<0.05, ** P < 0.01, *** P< 0.001).



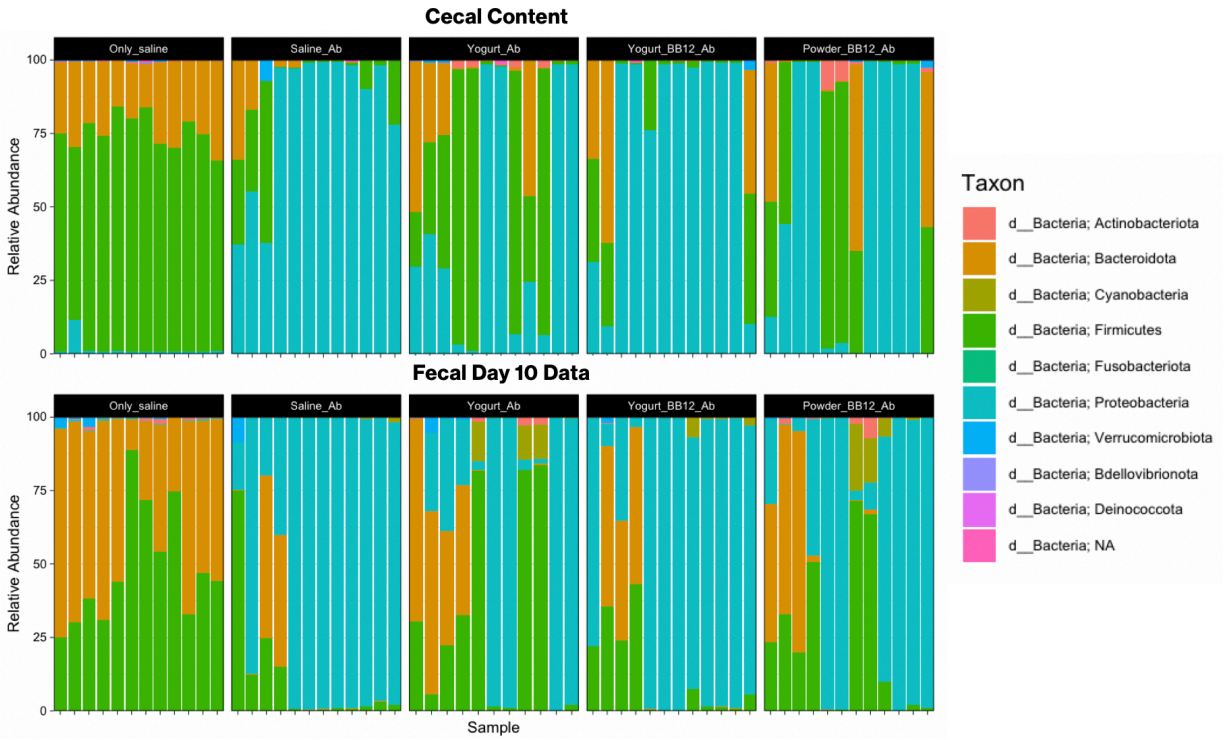
Suppl. Fig.5. Alpha diversity of fecal contents changes over time in different treatments. Box plots represent fecal alpha diversity according to **(A)** Faith PD and **(B)** Observed OTUs.



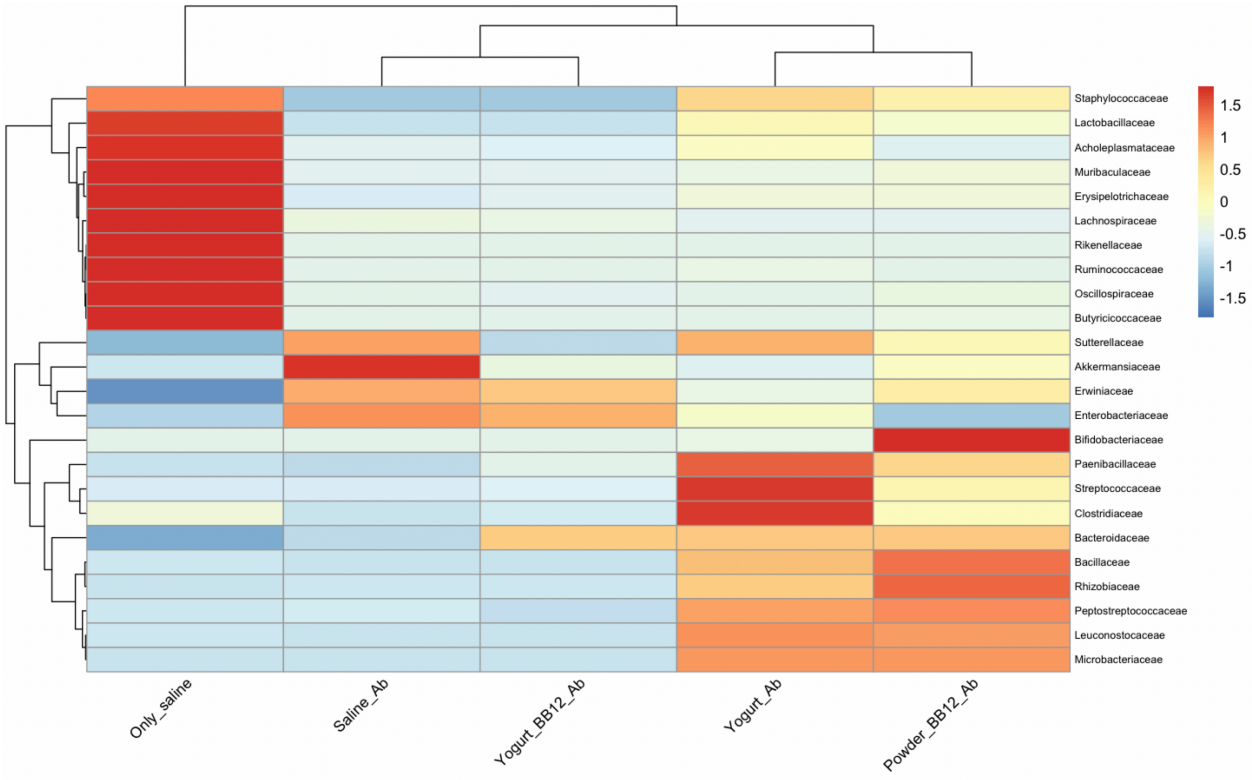
Suppl. Fig. 6. Relative abundance of bacteria using taxa bar plot. Top 15 families detected in individual mouse fecal contents over time in different treatments.



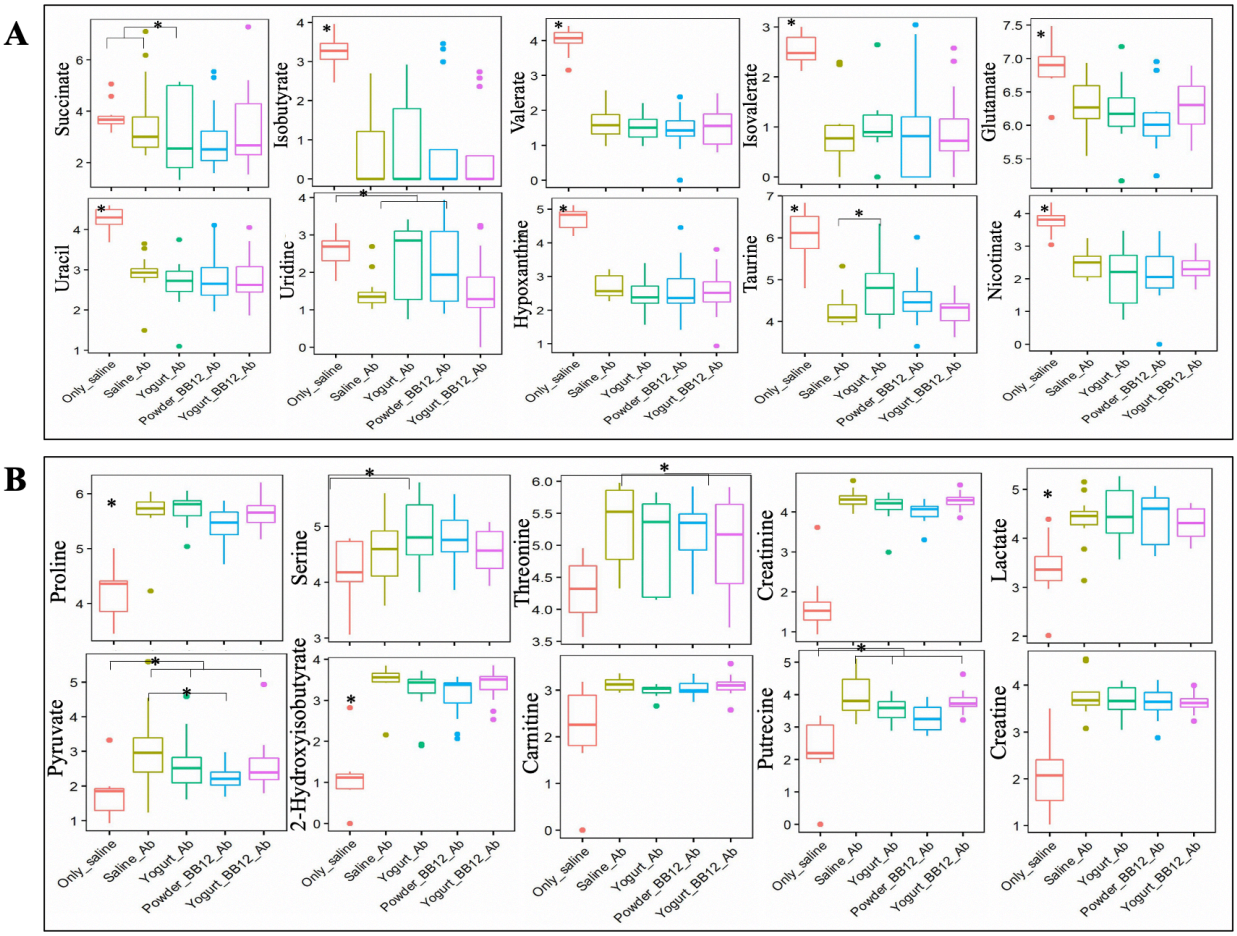
Suppl. Fig. 7. Bacterial phyla in the mouse fecal contents in three cohorts. Proportions of bacterial phyla detected in individual mouse fecal contents in three cohorts. X axis (bottom) represents three cohorts and Y represent relative proportion of bacterial phylum (right) in different treatments and time points represented on X axis (top). Significant differences were determined by Kruskal- Wallis test (* P<0.05, ** P < 0.01, *** P< 0.001, **** P<0.0001)



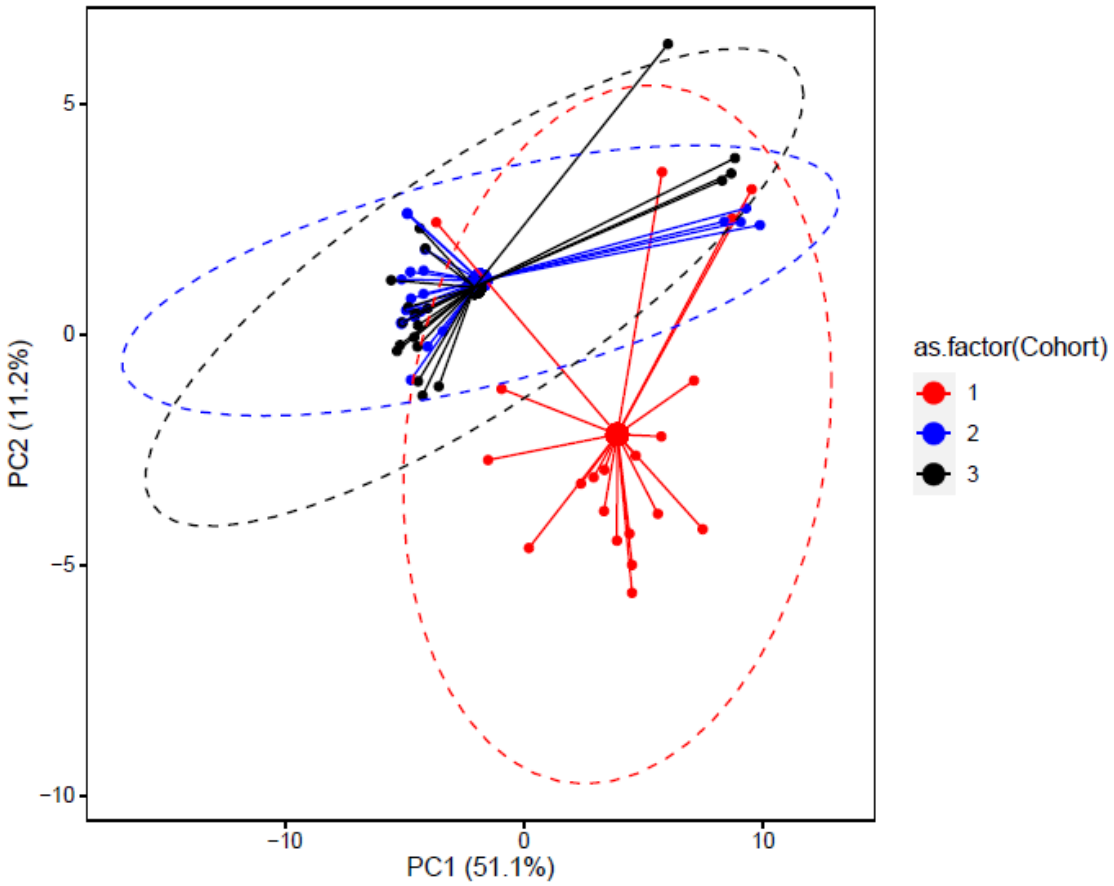
Suppl. Fig. 8: Proportions of bacteria in fecal and cecal contents on day 10. Taxa bar plot represented the relative abundance of bacteria on phylum level in cecal content and fecal samples on day 10.



Suppl. Fig. 9. Heat map of cecal bacteria based on bacterial proportion. Cecal microbiota represents family level in different treatments.



Suppl. Fig. 10. Significantly different cecal metabolites affected by AMC. (A) Metabolites reduced due to AMC effect **(B)** metabolites increased in AMC treated mice**.** Significant differences were determined for SCFA, amino acids and other metabolites using the ANNOVA test followed by post hoc analysis (* P<0.05).



Suppl. Fig. 11. Cohort effect on cecal metabolites. PCoA plot made based on Euclidian distances using multivariate analysis for cecal metabolome. Cohort one clusters away from cohort two and three.

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