Prevention of enteric bacterial infections and modulation of gut microbiota composition with conjugated linoleic acids producing *Lactobacillus* in mice

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Abstract

Probiotics are recognized to outcompete pathogenic bacteria by receptor-mediated colonizing and secreting functional metabolites which have direct antimicrobial activities towards pathogens and/or improving host's gut health and immunity. We have constructed a *Lactobacillus casei* (LC) probiotic strain, LC^{+mcra}, by inserting *mcra* (myosin cross-reactive antigen) gene, which stimulates the conversion of conjugated linoleic acids. In this study, we evaluated the protective roles of LC^{+mcra} against pathogenic *Salmonella enterica* serovar Typhimurium (ST) and enterohaemorrhagic *E. coli* (EHEC) infection in BALB/cJ mice. Through a series of *in vivo* investigation, we observed that LC^{+mcra} colonized efficiently in mice gut and competitively reduced the infection with ST and EHEC in various locations of small and large intestine, specifically cecum, jejunum, and ileum (p<0.05). The cecal microbiota in ST-challenged mice with LC^{+mcra} protection were positively modulated with higher relative abundances Firmicutes but lower Proteobacteria plus increased bacterial species diversity/richness based on 16S metagenomic sequencing. Based on cytokine gene expression analysis by qRT-PCR, mice pretreated with LC^{+mcra} were found with attenuated bacterial pathogen-induced gut inflammation. Furthermore, mice fed LC^{+mcra} daily for one week could protect themselves from the impairments caused by enteric infections with ST or EHEC. These impairments include weight loss, negative hematological changes, intestinal histological alterations, and potential death. This *in vivo* study suggests that daily consumption of novel conjugated linoleic acids over-producing probiotic might be efficient in improving gut intestinal microbiome composition and preventing/combating foodborne enteric bacterial infections with pathogenic *Salmonella* and diarrheagenic *E. coli*.

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48 ## Author summary

49 Numerous bacteria colonize throughout the gastrointestinal tract and form a complex
50 microbial ecosystem known as gut microbiota. A balanced microbial composition is crucial
51 for maintaining proper gut health and host defense against pathogenic microbes. However,
52 enteric bacterial infections could cause illness and even lead to death of host when
53 foodborne pathogens like *Salmonella* and enterohaemorrhagic *E. coli* (EHEC) invade gut
54 intestine and cause imbalance of gut microbiota. Beneficial microbes in gastrointestinal
55 tract such as *Lactobacillus* and their secreted bio-active metabolites, are potential bio-
56 agents to improve gut immunity and outcompete bacterial pathogens. In this study, to
57 evaluate roles of novel *Lactobacillus* strain LC^{+mcra} which produce higher amount of a
58 group of beneficial secondary metabolites called conjugated linoleic acids, we have shown
59 that daily oral administration of this LC^{+mcra} for one-week in mice lead to higher proportion
60 of beneficial bacterial colonization in different locations of intestine and a significant
61 reduction of pathogenic *Salmonella* and EHEC colonization. Furthermore, mice fed with
62 LC^{+mcra} restore and modulate *Salmonella* infection-induced negative impact on gut
63 microbiota composition and protect themselves from various levels of physiological
64 damage.

65

66 # Introduction

67 The majority of human gut epithelial surfaces are colonized and safeguarded by a
68 tremendous number of microorganisms including bacteria, viruses, fungi and protozoans
69 which are known as common gut microflora; each of them is crucial in forming and

70 balancing a complex ecosystem with microbial diversity [1]. These large number of
71 microorganisms build up a microbial genetic repertoire approximately 100 times greater
72 than that of the human host. Diversity of these microbes, specifically number of diverse
73 bacterial species, is essential for good health and immunity of host [2]. According to recent
74 reports, human distal gastrointestinal (GI) tract can house more than 1000 distinct bacterial
75 species, and the total number was estimated to be larger than 10^{14} CFU/gm of fecal material
76 [3]. Bacteroidetes, Firmicutes, Proteobacteria, and Actinobacteria are the prevalent
77 bacterial phyla in human gut microbiota and each of these phyla contains dozens of
78 bacterial genus and hundreds of species [4–6].

79 In a homeostasis gut ecosystem, most of the commensal bacteria colonize and
80 survive symbiotically, whereas conditions such as immunodeficiency, malnutrition, and
81 antibiotic-therapy cause dysbiosis and imbalance of commensal bacteria that induce
82 pathogenesis and cause diseases [7,8]. Furthermore, broad-spectrum antibiotic therapy or
83 any other detrimental conditions may disturb the gut ecosystem balance long-term or lead
84 to chronically irritated bowels, reducing the number of beneficial bacteria and increasing
85 the number of opportunistic pathogens and their toxic products that further weaken the host
86 defense and/or induce inflammation and damage [9]. As a consequence of imbalanced gut
87 microflora, opportunistic pathogens, their produced metabolites, proteins, and/or toxins
88 can take over the gut ecosystem and negatively impact host gut health.

89 *Salmonella* and diarrheagenic *Escherichia coli* generally infect human gut intestine
90 through consumption of contaminated foods and/or drinks [10–12]. Once these Gram-
91 negative enteric pathogenic bacteria arrive in host gut, their complex type III secretion
92 systems are activated, enabling them to introduce effector proteins directly into cell

93 cytoplasm. Series of these cascades induce systematic infections causing acute or chronic
94 inflammation and other serious disorders in the host [13]. However, such enteric illness is
95 usually facilitated by compromised gut immunity and dysbiotic gut microbiota which
96 provide those enteric bacterial pathogens with weakened colonization resistance [14]. On
97 the other hand, traditional antibiotic therapy has been found to lyse enterohemorrhagic *E.*
98 *coli* (EHEC) which further increases the risk for post infectious sequelae Hemolytic-
99 uremic syndrome (HUS) in the patients [15,16]. In such situations, procommensal
100 strategies by application of probiotics, prebiotics, and synbiotics can be considered as
101 priority in prevention and treatment of foodborne such bacterial pathogen-induced enteric
102 illness [11,17,18]. With a promising scheme, it allows an establishment or recovery of the
103 healthy enteric microbial ecosystem by introducing native, exogenous, or genetically
104 engineered beneficial probiotics without inducing deleterious effects (like antibiotics) on
105 human commensal gut bacteria [14,19].

106 Recently, we constructed and reported the role of a multi-functional *Lactobacillus*
107 *casei* probiotic strain overexpressing myosin cross-reactive antigen gene (*mcra*), named as
108 LC^{+mcra} [20]. Several groups of researchers have demonstrated the health-beneficial effects
109 of conjugated linoleic acids, such as anti-carcinogenesis, anti-oxidant, and anti-microbial
110 effects [14,21,22]. Similarly, we have also revealed the anti-pathogenic and anti-
111 inflammatory properties of linoleic acids over-producing *L. casei* (LC^{+mcra}) based on *in*
112 *vitro* examination. Here in this study, we aimed to evaluate the protective roles of LC^{+mcra}
113 on modulating/recovering gut intestinal microflora composition and combating/alleviating
114 foodborne enteric bacterial pathogenic infections *in vivo* based on mice model.
115

Results

Probiotics preventing ST infection induced physiological abnormalities in mice

The weight of each mice was monitored every day for the purpose of investigating if probiotics preventive administration could rescue mice from weight loss due to ST/EHEC infection (Fig 1). Within the entire 4-week rearing, a total of 12 mice in control group (no probiotic given), 7 mice in group given wild-type probiotic LC strain, and 1 mouse in group given linoleic acid over-expressed mutant LC^{+mcra} strain were sacrificed due to their health abnormality induced by ST infection. These sacrificed individuals included 8 mice from control and 5 mice from LC treatment found self-death due to ST challenge, but none from LC^{+mcra} treatment, which provided us the ST survival rates as 60% in control group, 75% in LC group, and 100% in LC^{+mcra} group. The death of the mice was generally accompanied with extreme (>20%) weight loss to approximately 8-10 g.

At the end of week 2, the average weight of mice in control group reached approximately 14-16g, whereas both groups of mice which were given either LC or LC^{+mcra} gained weight at range of 1-2g more compared to the control group of mice. Once mice were challenged with ST, the average weight gain trend of mice in control group which was not given probiotic was suspended and remained at 14.65 g during 1st week of post-challenge. Then the weight of those mice decreased to 14.36 g and 13.47 g at 2nd and 3rd post-infection weeks, respectively. However, the mice which were administrated LC^{+mcra} kept continuing to gain average weight. In spite of the negative effect induced by ST infection, mice which were given LC^{+mcra} gained weight at 16.88 g, 17.02 g, and 19.12 g at the 1st, 2nd, and 3rd week of post-infections, respectively. The wild-type probiotic, LC fed mice exhibited mild effects in maintaining the average body weight during the first two

139 weeks of ST infection and gained approximately 1.5 g weight at the end of 3rd post-
140 infection week. On the other hand, we failed to observe any negative effects including
141 average weight loss induced by EHEC infection. However, the oral administration of
142 LC^{+mcra} was more effective than LC in promoting the weight earning of mice by 1.1g and
143 1.4 g averagely at the 2nd and 3rd post-infection weeks compared with control group.
144

145 **Reduction on colonization of ST and EHEC in probiotics fed mice**

146 Either LC or LC^{+mcra} was orally administrated to mice in order to examine their
147 colonization ability in mice gut and evaluate their preventive role in altering enteric
148 pathogenic bacterial colonization and infection in gastrointestinal tract of mice using
149 BALB/cJ mice model. According to the colonization data collected from two individual
150 mice trials, both LC and LC^{+mcra} were able to colonize well in gut of BALB/cJ mice but
151 the genetically modified probiotic strain, LC^{+mcra} could colonize in the mice gut more
152 aggressively compare to the wild-type LC strain. Further, both LC and LC^{+mcra}
153 significantly reduced the colonization and infection of both enteric bacterial pathogens, ST
154 and EHEC in BALB/cJ mice. We found that mice fed with LC^{+mcra} could defend ST
155 infection remarkably and recover fully within a week of challenge. Specifically, mice
156 highly colonized with LC^{+mcra} strain were able to reduce significantly (approximately 1 log
157 CFU/g) cecal colonization with ST compare to the group of mice which were given wild-
158 type LC strain at all three time points (14, 21, and 28 d) (Fig 2A).

159 To compare the colonization of ST in jejunum, we observed that LC or LC^{+mcra} pre-
160 administrated mice were colonized with lower number of ST at rang of 1.0 to 2.3 log CFU
161 ST per gram jejunum fluids at 1st week post-infection, 0.9 and 2.5 log CFU/g at 2nd week

post-infection, and 1.3 and 3.7 log CFU/g at 3rd week post-infection (Fig 2B). Similarly, LC and LC^{+mcra} pre-administrated mice were colonized with ST in lower rate of 1.7 and 2.2 log CFU per gram ileum fluids at 1st week post-infection, 0.9 and 1.9 log CFU/g on 2nd week post-infection, and 1.2 and 3.4 log CFU/g on 3rd week post-infection to the control mice (Fig 2C).

The significant reduction on ST gut intestinal colonization was also observed in form of decreased ST fecal shedding. On the 8th day after mice were challenged with ST, both groups of mice administrated with either wild-type probiotic LC or genetically modified probiotic LC^{+mcra} strain were colonized with reduced number (0.8 to 1.1 log CFU/mL) ST in feces but the differences became unsubstantial at the 9th day. However, notably major effectiveness of LC^{+mcra} started to appear in mice after 1st week post-infection, at which 1.3 log CFU/mL less ST was recovered from mice feces. In the subsequent two weeks, LC^{+mcra} fed mice were observed with 1.1 and 2.1 log CFU/mL continuous ST reduction on fecal shedding.

On the other hand, mice which were pretreated with LC barely reduced the EHEC colonization in jejunum and ilium, whereas mice pretreated with LC^{+mcra} showed significant influence in EHEC colonization resistance (Fig 3). Specifically, LC^{+mcra} fed mice were capable of significantly reducing the colonization of EHEC at 2.3, 1.6, and 0.9 log CFU/g in cecum, 1.6, 1.8, and 2.7 log CFU/g in jejunum, and 2.8, 1.8 and 2.1 log CFU/g in ileum at the 1st, 2nd, and 3rd week post-challenge. Meanwhile, consequential decreased EHEC fecal shedding was detected in LC^{+mcra} fed mice as well. However, only insignificant reductions (0.1 to 0.5 CFU EHEC less per mL feces) were found during the first two days after EHEC challenge on EHEC-free mice (the 8th and 9th day). The LC^{+mcra}

185 administration substantially lowered 0.9, 1.9, and 2.2 CFU/mL EHEC fecal shedding at the
186 1st, 2nd, and 3rd post-infection weeks in comparison with control.
187
188 **Efficient colonization of LC^{+mcra} in mice gut**
189 In order to examine the correlation between probiotic colonization and reduction on
190 intestinal bacterial pathogens, we also compared the colonization level of both LC and
191 LC^{+mcra} in different portion of mice gut (Fig 4). The one-week daily oral administration led
192 to high and stable cecal colonization level of LC^{+mcra} above 10^6 CFU/g throughout 3 weeks
193 afterwards, which were significantly higher than wild-type LC. A similar trend was found
194 in mice jejunum, whereas, LC^{+mcra} only exhibited numerical higher ileum colonization than
195 wild-type LC.
196 The fecal shedding number of administered LC were observed to raise after 1st day
197 consumption (Fig 4D). Specifically, LC^{+mcra} fecal shedding colonies gradually increased
198 from 4.8 log CFU/mL, reached 5.8 log CFU/mL at the next day of final daily administration,
199 and slightly decreased around 5 log CFU/mL after 3 weeks. Whereas, fecal shedding
200 colonies of wild-type LC were observed significantly lower (by 0.4-1.5 log CFU/mL) than
201 LC^{+mcra}. They reached 5.1 log CFU/mL as peak at the next day of final daily administration
202 and ended up with lower than 3.5 log CFU/mL after 3 weeks.
203
204 **Mice hematology**
205 The hematological changes in mice with ST infection with or without pretreated with
206 probiotic strains at various time points were summarized in Table 2. When compared with
207 control group mice with placebo, ST challenge resulted in dramatic increase of red blood

208 cells (RBC) but decrease of white blood cells (WBC) and platelets (PLT). Both pre-
209 treatment of LC or LC^{+mcra} alleviated the increment of RBC and loss of WBC/PLT in mice
210 during Salmonellosis. LC^{+mcra} treatment on mice could further help the mice maintain their
211 normal levels of RBC, WBC, and PLT.

212 To further evaluate the WBC composition in blood collected from the mice
213 challenged with ST with or without pre-treated with probiotic strains, we investigated
214 neutrophils, lymphocytes, monocytes, eosinophils, and basophils counts in different time
215 points, which is summarized in Table 3. The numbers of neutrophils and lymphocytes in
216 blood collected from mice challenged with ST were found to be notably reduced, whereas
217 the monocytes, eosinophils, and basophils levels in mice with salmonellosis were detected
218 to be significantly higher. The pre-treatments with either probiotic strain, wild type LC or
219 mutant LC^{+mcra} was able to maintain the normal WBC composition under ST infection,
220 including all five cells studied, at the same levels statistically in comparison with control
221 group.

222

223 **Mice histopathology**

224 The histological examination of mouse cecal sections is shown in Fig 5. Tissue of cecum
225 collected from the control group mice and mice challenged with ST challenge (Fig 5A and
226 5D) with administration of LC^{+mcra} (Fig 5C and 5F) exhibited normal intestinal villi,
227 microvilli, and goblet cells. In comparison, salmonellosis induced variable levels of
228 histological alterations and abnormalities consisting of severe goblet cell depletion,
229 villi/microvilli elimination, and inflammatory infiltrations between circular folds were
230 found in cecum sections from ST infected mice which were not pretreated with probiotics

(Fig 5G, 5H, 5I, 5J, 5K, and 5L). However, the tissue of cecum collected from the mice administrated with LC showed symptoms of salmonellosis, but the induced histopathological changes were mild, such as slight goblet cell reduction and slight changes of villi/microvilli (Fig 5B and 5E).

Regulation on expression of intestinal inflammatory cytokine genes

The regulation of cecal inflammatory cytokine gene expressions during 3-week ST infection as well as 1-week probiotic pre-administration was displayed in Fig 6. Specifically, ST infection induced up-expression of 4 pro-inflammatory cytokines IL-1β, IL-6, INF-γ, TNF-α genes and 1 anti-inflammatory cytokine IL-10 gene in mice cecal tissue cells. The up-regulation levels ranged from 2.2 to 7.8 log folds with the highest values for INF-γ gene and the highest expression at two weeks after ST challenge (Day 21). Another anti-inflammatory cytokine TGF-β gene was found down-regulated in ST infected mice cecum by 1.5 to 2.9 log folds. The expression of intestinal inflammation-related cytokine genes in LC^{+mcra} pre-treated mice were manipulated at a positive manner. For example, all 4 pro-inflammatory cytokine genes provoked by ST were suppressed significantly by 1.3 to 5.3 log folds through three weeks after challenging compared to mice with no probiotic protection; expression of anti-inflammatory cytokines IL-10 and TGF-β genes were stimulated notably in comparison with either control or ST infection with no probiotic prevention.

Modulation on murine gut microbiota composition

To compare the gut microbiome composition in various groups of mice, we randomly

selected their cecal contents (5 mice from each group) for 16S metagenomic sequencing and taxonomic classification. According to the taxonomic profile at the phylum level (Fig 7A), Firmicutes were the dominant phylum (63.51%) in mice control group giving placebo (primary control), which was followed by Bacteroidetes (29.37%). The relative abundance of Proteobacteria was 0.92% with individual variation between 0.54% to 1.35%. Significant difference in gut microbial community phylum composition was observed in ST infected mice (Fig 7B), in which group, though the dominant phylum is still Firmicutes (61.26%), the relative abundance of Bacteroidetes was notably decreased to 17.81% and the relative abundance of Proteobacteria boosted to 14.86%. One-week daily administration of probiotic (LC or LC^{+mcra}) positively shaped the phylum level gut microbiota composition in mice with ST challenging (Fig 7C and 7D). To specify, in comparison with ST infected mice with no probiotic protection (secondary control), the dominance of Firmicutes were raised by 5.67 and 13.34% in LC and LC^{+mcra} pretreated groups, respectively. The relative abundances of cecal Proteobacteria were also reduced by 13.17 and 14.17% in LC and LC^{+mcra} pretreated mice groups, respectively.

At genus level (Fig 8), *Bacteroides* was identified being the highest abundant (18.50%) in primary control group of mice cecal contents, followed by *Ruminococcus* (7.17%), *Blautia* (7.02%), *Johnsonella* (4.39%), *Lactobacillus* (1.80%). The relative abundances of *Salmonella* and *Enterobacter* were observed less than 0.01% of the total gut bacterial composition. Whereas, the gut microbiota genus in ST-infected mice exhibited distinctively with significantly higher abundances of *Salmonella* (5.27%) and *Enterobacter* (3.72%), but lower abundances of *Bacteroides* (9.98%), *Blautia* (5.16%), *Johnsonella* (3.34%), and *Lactobacillus* (0.17%) were observed. Other gut microbial genus-level

noticeable differences between ST infected mice and control included reduced *Anaerobranca*, *Anaeroplasma*, *Butyrivibrio* and raised *Akkermansia*, *Desulfobacter*, *Enterococcus*, *Klebsiella*, *Leptolyngbya*, *Natronincola*, *Staphylococcus*, and *Tolumonas*, *Trabulsiella*. Compared with secondary control, probiotic pretreatments notably increased the relative abundances of *Bacteroides*, *Blautia*, *Escherichia*, *Johnsonella*, and *Lactobacillus* as well as lowered *Salmonella*, *Enterobacter*, *Klebsiella*, *Tolumonas*, and *Trabulsiella*. Particularly, LC^{+mcra} pre-administration in mice modulated the *Salmonella* and *Enterobacter* relative abundances back to control levels in cecum and significantly escalated their relative abundances of *Bifidobacterium* (0.12%), *Blautia* (8.43%), and *Lactobacillus* (9.18%).

The overall cecal bacterial species diversity was observed to be lowered with ST infection in mice but promoted by LC^{+mcra} pre-treatment and protection (Fig 9). Specifically, compared with primary control, the mice group infected with ST exhibited significantly reduced gut intestinal microbial diversity at species level which was indicated by various alpha-diversity indexes including Chao-1, Fisher-alpha, Margalef's richness, and Simpson (numerically higher), and Shannon. However, the one-week daily pre-administration/prevention with LC^{+mcra} instead of with wild-type LC before ST challenging caused a notably increased bacterial species diversity in cecum compared with secondary control group and even higher in comparison with primary control group.

Discussion

The probiotic strain LC^{+mcra} with 7-fold upregulation in its expression level of *mcra* gene coding linoleate isomerase has been found with prominently significant 21-fold higher rate

300 in total linoleic acids production per bacterial cell [20]. In a previous study, we revealed *in*
301 *vitro* that LC^{+mcra} could competitively exclude the growth and adhesive activity of both ST
302 and EHEC [20] and at meanwhile, suppress their vital virulence gene factors. Moreover,
303 though effectiveness of probiotics in combatting enteric bacterial pathogens is still
304 controversial, several researchers have suggested that their secondary metabolites such as
305 CLA might enhance their overall *in vivo* health-beneficial functions [14,23–25]. Here in
306 the current study, we systematically and in-depth investigated the double effects of both
307 *Lactobacillus* and CLA on murine gut health. According to our results, 1-week consecutive
308 consumption of LC^{+mcra} through oral administration efficiently prevented/mitigated the
309 following *Salmonella* infection. Although probiotic administration through water might
310 generate variance of bio-availability in mice gut, it is worth mentioning that early-staged
311 oral probiotic gavage possesses high risk in potential induced injury in 3-week-old mouse
312 esophagus. The bacterial fecal shedding serves as a key indicator about the gut intestinal
313 colonization [26], correspondingly we observed reduced ST/EHEC in both fecal content
314 and intestinal fluids. Though similar studies conducted based on EHEC were not systematic
315 and completed, *Salmonella* colonization was claimed to be restricted by functional fatty
316 acids oral supplements *in vivo* [25,27–29], in which the virulence gene factors of
317 *Salmonella* were suggested to be manipulated [30,31].

318 On the other hand, probiotic itself was addressed to be capable of reducing
319 intestinal pathogens through physical repellence and colonization resistance [32–36].
320 Fortunately, all these studies mentioned above supported our *in vivo* findings in which
321 either wild type or genetically engineered *L. casei* remarkably diminished ST/EHEC
322 colonization in cecum, jejunum, and ileum. Whereas, LC^{+mcra} displayed more intensive

323 reductions considering the extraneous strengthening effects implemented by its over-
324 promoted CLA production [14]. In fact, CLA has been documented and linked with
325 antimicrobial active against several enteric bacterial pathogens including *Salmonella*
326 though the specific mechanism are still under study [23,37]. Most importantly, the *in vivo*
327 examination based on BALB/cJ mice model justified the protective roles of LC^{+mcra} on
328 combating enteric bacterial pathogens, following and matching with previous *in vitro*
329 outcomes relied on various pathogenic bacterial strains [20,38,39].

330 In most cases, *Salmonella* infections are associated with diarrhea, weight loss,
331 dramatic alterations in composition of blood cells, as well as death [12,40–42]. Accordantly
332 we detected 10^5-10^7 CFU intestinal colonization of ST induced salmonellosis and caused
333 around 8% weight loss, 52% higher level of RBC, 19% and 71% lower levels of WBC
334 (especially neutrophils and lymphocytes) and PLT, and severe cecal inflammation in the
335 survival mice. The physical, hematological, and gut intestinal abnormalities mentioned
336 above in our *in vivo* examination contributed in the 40% death rate of mice challenged with
337 enteric bacterial pathogen ST. However, probiotics in secreting different types of
338 functional fatty acids initiate attenuation in over-reactive gut inflammation through anti-
339 inflammatory activities [14,20,22], which correlates with the LC^{+mcra} (CLA) mediated
340 relative up-regulation of murine intestinal anti-inflammatory cytokine genes from mice
341 under salmonellosis found in our study. Therefore, apart from the direct colonization
342 competition and repellence, daily administration of probiotics, especially LC^{+mcra}, also
343 prevented regular salmonellosis symptoms and maintained the overall physical and gut
344 health condition of mice through mediating immuno-modulation. If in future study, several
345 other tissues including kidney, liver, lung, et al. could be examined for LC^{+mcra} pre-

346 treatment on prevention of ST systemic infection.

347 To address concerns from the host's point of view, the maintenance of intact and
348 operative gut intestine physiological condition is crucial in both metabolism and symbiotic
349 intestinal microbiota composition [3,43–45]. In our study, LC^{+mcra} and its byproduct CLA
350 prevented ST-induced elimination of goblet cells, villi, and microvilli as well as the
351 inflammatory infiltrations between circular folds in cecum, which maintained the overall
352 functions in terms of intestinal nutrients absorption and profoundly raised the survival rate
353 (0 death) in mice. As a matter of fact, CLA has been previously connected with colitis and
354 inflammatory bovine disease recovery [46,47], but the specific mechanisms are still under
355 discovery. Here our findings based on CLA are in support of these researches and suggest
356 a protective mechanism from both bacterial colonization and host histology sides.

357 A balanced gut microbial ecosystem serves as the crucial defense against
358 colonization and infection with enteric pathogens [14,48,49]. *Salmonella* infection could
359 have negatively impact on gut intestinal microbiome composition by diminishing the
360 abundances of Firmicutes including *Lactobacillus* and *Bifidobacterium*, and
361 simultaneously favoring the dominance of Proteobacteria inducing follow-up opportunistic
362 infections [50–52]. In our study, we observed the raised abundances of *Salmonella* and
363 *Enterobacter* with overall reduced bacterial species diversity following ST challenge in
364 mice, whereas LC^{+mcra} pre-administration successfully prevented the negative shifting of
365 gut microbiota composition induced by ST infection. As a matter of fact, CLA-containing
366 diets were reported to alter the fatty acids metabolism and developing homeostatic gut
367 microflora [53,54]. The healthier intestinal microbial distribution shaped by CLA-
368 producing probiotic daily consuming, in terms of higher abundances of *Lactobacillus*,

369 *Bifidobacterium*, and *Blautia* as well as microbial species diversity/richness, strengthened
370 the first-line gut intestinal defense system against multiple pathogenic bacterial infections,
371 may possess a tight association and be the explanation of reduced bacterial pathogen
372 colonization and inflammation in mice gut.

373 Based on previous research, EHEC oral challenge on distinct mouse models can
374 result in various levels of colonization, morbidity, and mortality [55]. Specifically, EHEC
375 dose as low as 10^2 CFU led to cecal colonization and death in germ-free mice [56,57]
376 whereas for conventional mice model like BALB/c, considerably higher dose of EHEC
377 was requisite in order to cause diseases [58,59]. In some cases, infectious dose of EHEC
378 less than 10^{10}-10^{11} CFU failed to even introduce cecal colonization [60,61], which parallel
379 with our findings. Based on the current study, 10^7 CFU EHEC orogastrically challenge on
380 BALB/cJ mice induced 10^2-10^4 CFU/g intestinal fluid colonization on cecum, jejunum,
381 and ileum but failed to motivate any visible physiological abnormalities or mortality in
382 mice. This could be explained by the relative resistance in BALB/c mice towards EHEC
383 through shorter shedding duration and producing higher serum/fecal levels of O157-
384 specific IgA [55,60]. On the other hand, LC^{+mcra}, as we observed *in vitro* [20] and predicted
385 for *in vivo*, stood out in reducing the colonization level of EHEC as well as preventing from
386 kidney histological abnormalities and weight loss in BALB/cJ mice. Further research
387 dependent on germ-free or compromised commensal flora mouse model might be
388 substantial in revealing how LC^{+mcra} involved in defending host from EHEC pathogenesis
389 and post-infectious complications.

390 To conclude, the current study has demonstrated a substantial influence of CLA
391 over-producing probiotic strain, LC^{+mcra} exerted on *Salmonella* and pathogenic *E. coli*

infections in conventional mice. Specifically, mice orally given LC^{+mcra} daily for one week minimized EHEC colonization and protected themselves from ST-facilitated serious salmonellosis which was observed by notably reduced fecal shedding and intestinal colonization of ST, amelioration on acute inflammation, and prevention on hematological and histological abnormalities. In depth metagenomic analysis revealed that LC^{+mcra} pretreatment modulated mice cecal bacterial community with increased diversity which are predominated with comparative higher Firmicutes and lower Proteobacteria. The outstanding protective roles of LC^{+mcra} against ST and EHEC infection plus its profound effectiveness over wild-type LC may provide a promising option for prophylaxis on pathogenic *Salmonella* and diarrheagenic *E. coli* infections and reduce enteric bacterial infections.

Materials and methods

Ethics statement

Mice *in vivo* experiments were performed in ABSL2 facilities in Department of Animal and Avian Sciences, University of Maryland in accordant with protocol #R-NOV-17-55 approved by the Institutional Animal Care and Use Committee (IACUC). The best effort was made for minimizing the suffer of animals. To ensure animal welfare, mice were monitored and recorded for physical appearance and body weight once/day on a daily basis during experimental period. Animals were euthanized by CO_2 exposure in a chamber for 5 minutes until all evidences of cardiac function and respiration were absent.

Bacterial strain and growth conditions

415 *Lactobacillus casei* (LC, ATCC 334) and our laboratory generated linoleic acid over-
416 expressed *L. casei*, LC^{+mcra} [20,38] were used as probiotics while *Salmonella enterica*
417 serovar Typhimurium (ST, ATCC 14028) and enterohemorrhagic *Escherichia coli*
418 EDL933 (EHEC, ATCC700927) were chosen as enteric bacterial pathogens in this study.
419 Both *Lactobacillus* strains were grown on MRS agar at 37 °C for 24 h in the presence of
420 5% CO_2 (Forma™ Scientific CO_2 water jacketed incubator, Thermo Fisher Scientific Inc.,
421 Waltham, MA, USA). ST and EHEC were grown on LB agar (EMD Chemicals Inc.,
422 Gibbstown, NJ, USA) for 18 h at 37 °C under aerobic conditions (Thermo Scientific
423 MAXQ 4450, Thermo Fisher Scientific Inc., Waltham, MA, USA).
424
425 **Mice model and animal experiments**
426 The 3-week-old BALB/cJ Mice (approximately 8-10 g) were purchased from The Jackson
427 Laboratory (Bar Harbor, ME USA) and reared in static micro-isolating cages with cellulose
428 Bio-Performance bedding and huts as environmental enrichment. Teklad standard rodent
429 diet and regular tap water were provided for mice feeding and drinking, respectively. A
430 total of 90 mice (45 male and 45 female) were used for each trial. Following a completely
431 randomized method, 90 mice were randomly assigned to 9 groups (designated A1 to C3)
432 resulting in 10 mice per group; two cages were assigned to each group with a total of 5
433 mice per cage. Mice cages were changed weekly, and each individual mouse was weighed
434 and monitored with health examinations daily. At the end of the second, third, and fourth
435 week, 3, 3, and 4 mice from each group respectively, were randomly selected and
436 euthanized with CO_2 inhalation in euthanasia chamber for organ samples collection.
437

438 **Feeding probiotic to BALB/cJ mice and challenging with ST and EHEC**

439 Overnight culture of LC or LC^{+mcra} in MRS broth were diluted in fresh 5 mL MRS broth
440 at 1:50 and allowed for 3 h further growth. The bacterial cells in exponential phase were
441 harvested following centrifugation at 3,000 × *g* for 15 min, PBS washing, and resuspension
442 in 1.0 mL PBS. A final concentration of 10^{11} CFU/mL was adjusted with PBS and used to
443 feed mice. The design of *in vivo* mouse trial was summarized in Table 1. Probiotic (either
444 10^9 CFU/mL LC or LC^{+mcra}) cells were maintained in water bottle fill with regular tap
445 water for group B and C and feed to mice from Day 1 to Day 7. Control mice, group A,
446 was fed with regular tap water only.

447 Overnight culture of ST and EHEC bacterial cells in LB broth were diluted in fresh
448 5 mL LB broth at 1:50 and allowed for 3-4 h further growth at 37 °C. The exponential
449 phase bacterial cells were harvested and washed by centrifugation at 3,000 × *g* for 15 min
450 and resuspended in 1.0 mL of PBS. A final concentration of bacterial cells was adjusted to
451 10^8 CFU/mL in PBS. On Day 7, an aliquot of 100 µL ST or EHEC suspension containing
452 approximately 10^7 CFU was fed to mice in groups 2 or 3 respectively, with oral gavage,
453 and the mice were reared thereafter for another 3 weeks. Mice in group 1 was orogastrically
454 fed with 100 µL PBS and served as control.

455

456 **Sample collection and processing**

457 In order to estimate the bacterial fecal shedding, fecal samples were collected from each
458 mouse in sterile Whirl-Pak bags using sterile spoons at Day 1, 2, 3, 6, 7, 8, 9, 14, 21, and
459 28 for PBS serial dilution and plating on specific agar plates (MRS agar for *L. casei*, XLT-4
460 agar for ST, MacConkey agar for EHEC) [62]. In order to investigate the bacterial

461 colonization in mice gut, intestine, ilium, jejunum, and cecum from each euthanized mouse
462 were separated and harvested. Then the ilium, jejunum, and cecal fluids were serial diluted
463 with PBS, followed by plating on specific agar plates. Specifically, MRS agar for *L. casei*,
464 XLT-4 agar for ST, MacConkey agar for EHEC were used, respectively.

465 Mice cecum was kept in RNA Later for further RNA extraction, cDNA reverse
466 transcription, and inflammation-related gene expression level analysis. For hematological
467 analysis, the blood samples from each mouse was collected from heart in VACUETTER®
468 Heparin tubes (Greiner Bio-One, Monroe, NC, USA) and further analyzed with a ProCyte
469 Dx® Hematology Analyzer (IDEXX, Westbrook, ME, USA) according to the
470 manufacturer's instructions.

471

472 **RNA extraction, cDNA synthesis, and Quantitative RT-PCR for evaluation of**
473 **targeted gene expressions**

474 Extraction of mice intestinal RNA was carried out using TRIzol® Reagent (Life
475 Technologies Co., Carlsbad, CA, USA) following previous methods [63]. The cDNA
476 synthesis was performed according to the manufacture's instruction of qScript cDNA
477 SuperMix. The PCR reaction mixture containing 10 μL PerfeCTa SYBR Green Fast Mix
478 (Quanta Biosciences, Beverly, MA, USA), 2 μL of each 100 nM primer, 2 μL of cDNA
479 (10 ng), and 4 μL of RNase-free water was amplified using an Eco Real-Time PCR system
480 with 30 sec denaturation at 95 °C, followed by 40 cycles of 95 °C for 5 sec, 55 °C for 15
481 sec, and 72 °C for 10 sec. All the relative transcription levels of target genes were estimated
482 by comparative fold change. The CT values of genes were normalized to the housekeeping
483 gene, and the relative expression levels of target genes were calculated by the comparative

484 method [64]. Quantitative RT-PCR was carried out in triplicate.

485

486 **Histopathology analysis**

487 Intestinal tissue samples were taken from mice after euthanization and were stored in
488 neutral buffered formalin (4% formaldehyde; pH 7.4) at 4°C for further processing. Once
489 the samples were removed from fixative, they were dehydrated with increasing
490 concentrations of ethanol, cleared in xylene, and embedded in paraffin. Microtome (LEICA
491 RM2065, Leica Biosystems, Buffalo Grove, IL, USA) was used to harvest 5 μm thick
492 paraffin sections followed by heat fixing at 37 °C overnight. Then the slices were stained
493 with hematoxylin and eosin and mounted with DPX mounting medium 13512 (Electron
494 Microscopy Sciences, Hatfield, PA, USA). Histological observations were performed
495 under a light microscope (BA210E, Motic Asia, Hong Kong, China).

496

497 **Metagenomic sequencing and analysis**

498 Mice cecal contents were harvested and 5 samples from each group of control, ST infection,
499 LC pretreatment followed by ST infection, or LC^{+mcra} pretreatment followed by ST
500 infection were randomly selected for metagenomics analysis. Microbial genomic DNA
501 extraction was carried out using QIAamp Fast DNA Stool Kit (QIAGEN, Valencia, CA,
502 USA) following instructions from the manufacturer. The variable V3 and V4 regions of
503 microbial 16S rRNA gene were targeted for phylogenetic classifications. DNA libraries
504 were prepared for equimolar-pooling using Nextera DNA Library Preparation Kit and
505 Nextera Index Kit (Illumina, San Diego, CA, USA) according to the manufacturer's
506 instructions. Paired-end sequencing (2 × 300 bp) was conducted on Illumina MiSeq using

MiSeq v3 600-cycle kit (Illumina, San Diego, CA, USA). Sequence data was processed through MiSeq Reporter - BaseSpace for FASTQ Workflow generation followed by taxonomic classification based on Greengenes database (http://greengenes.lbl.gov/). Demultiplexing was performed using only perfect index recognition (mismatch = 0) followed by removing PhiX reads. 16S sequence length below 1250 bp or with more than 50 wobble bases was filtered, and all entries classified with no genus or species were also filtered. The relative abundances and alpha-diversity indices were calculated using 'vegan' R package and plotted in Excel.

Statistical analysis

All data were analyzed by the SPSS software. Comparison among multiple mice groups were performed with the one-way analysis of variance followed by Tukey's and Bonferroni's tests. For all tests, significant differences were considered on the basis of P values below a significant level of 0.05.

Acknowledgements

The authors would like to thank Dr. Vinod Nagarajan, Zabdiel Alvarado Martinez, Arpita Aditya, for their assistance during animal experiments. We also thank Dr. Rachel Dennis and Jasmine Mengers from department of Animal and Avian Sciences in the University of Maryland for their guide on histopathological studies.

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750

751 **FIGURE LEGENDS**

752

753 **Fig 1. Comparative weight gain and loss in mice across different groups.** Mice groups
754 were assigned with the following manner: (A) ST infection, (B) ST infection and LC 1-
755 week pre-treatment, (C) ST infection and LC^{+mcra} 1-week pre-treatment, (D) EHEC
756 infection, (E) EHEC infection and LC 1-week pre-treatment, and (F) EHEC infection and
757 LC^{+mcra} 1-week pre-treatment. Each dot indicates individual mouse weight and horizontal
758 bars at each time point indicate averaged weight of mice in accordant group.

759

760 **Fig 2. Effect of LC^{+mcra} on reducing colonization of ST in mice gut intestine.** The
761 bacterial numbers of ST at 14, 21, and 28 days in ileum (A), jejunum (B), cecum (C), and
762 feces (D) from ST-infected mice with no probiotic treatment, LC, or LC^{+mcra} 1-week pre-
763 treatment were investigated in triplicate. Different letters ('a' through 'c') at single time
764 point are significantly different ($p < 0.05$) in the numbers of ST among control and
765 treatments.

766

767 **Fig 3. Effect of LC^{+mcra} on reducing colonization of EHEC in mice gut intestine.** The
768 bacterial numbers of EHEC at 14, 21, and 28 days in ileum (A), jejunum (B), cecum (C),
769 and feces (D) from EHEC-infected mice with no probiotic treatment, LC, or LC^{+mcra} pre-
770 treatment were investigated in triplicate. Different letters ('a' through 'c') at single time
771 point are significantly different ($p < 0.05$) in the numbers of EHEC among control and
772 treatments.

773

774 **Fig 4. Comparison on colonization levels of LC and LC^{+mcra} in mice gut intestine.** The
775 bacterial numbers of specific *L. casei* at 14, 21, and 28 days in ileum (A), jejunum (B),
776 cecum (C), and feces (D) from mice daily administered with LC or LC^{+mcra} for one week
777 were investigated in triplicate. Asterisk (*) at single time point are significantly different
778 ($p < 0.05$) in the numbers of gut colonized or fecal shedding wild-type LC and LC^{+mcra}.
779
780 **Fig 5. Cecum histopathology in mice.** Representative H&E-stained cecum sections from
781 experimental groups were showed in panels (A-C & G-I captured under 100×; D-F & J-L
782 captured under 100×): (A&D) control mice, (B&E) intestinal villi and microvilli reduction
783 in ST-infected mice with 1-week LC pre-treatment, (C&F) normal intestinal histology in
784 ST-infected mice with 1-week LC^{+mcra} pre-treatment, (G&J) moderate depletion of goblet
785 cells and villi/microvilli in ST-infected mice, (H&K) massive elimination of goblet cells
786 and villi/microvilli in ST-infected mice, (I&L) intestinal inflammation and infiltration at
787 circular folds in ST-infected mice (arrows).
788
789 **Fig 6. Differential expression levels of mice cecal cytokine genes.** The relative log fold
790 changes in expression of IL-1β (A), IL-6 (B), IL-10 (C), INF-γ (D), TGF-β (E), and TNF-
791 α (F) genes from cecum tissue cells collected from mice control, under ST infection, pre-
792 treated with wild-type LC and challenged with ST, or pre-treated with LC^{+mcra} and
793 challenged with ST were examined in triplicate. Different letters ('a' through 'd') at single
794 time point are significantly different ($p < 0.05$) among control and treatments.
795
796 **Fig 7. Mice cecal microbial community phylum-level structure.** Bacterial distributions

797 at phylum level in cecal contents from individual pooled dataset were depicted in terms of
798 (A) control mice providing placebo, (B) mice infected with ST, (C) mice daily administered
799 with LC for one week followed by ST challenge, and (D) mice daily administered with
800 LC^{+mcra} for one week followed by ST challenge.
801
802 **Fig 8. Mice cecal microbiota composition at genus level.** Bacterial genus-level
803 community composition in cecal contents from consolidated pool of dataset was compared
804 among different mice groups. Overall 30 bacterial genera were targeted based on their
805 relative abundances and importance in gut microbiome. The total relative abundances of
806 all targeted 30 genera varied from 43 to 46% in different mice groups.
807
808 **Fig 9. Bacterial diversity at species level in murine cecum.** The assessment of alpha-
809 diversity including Observed number of taxa species (A), Chao-1 (B), Fisher's alpha (C),
810 Margalef's richness (D), Simpson index (E), and Shannon index (F) was determined and
811 analyzed among different mice groups. Standard deviations among individual group
812 members were provided. Different letters ('a' through 'c') are significantly different ($p <$
813 0.05) among control and treatments.
814
815
816
817
818
819

TABLES

Table 1. Mice groups, numbers per group, and their treatment/infection.

Group (#)	Mice (n)	Probiotic Treatment (daily during 1st week)			Pathogen challenge (beginning of 2nd week)		
		PBS	LC	LC^{+mcra}	PBS	ST	EHEC
A1	10	+	-	-	+	-	-
B1	10	-	+	-	+	-	-
C1	10	-	-	+	+	-	-
A2	10	+	-	-	-	+	-
B2	10	-	+	-	-	+	-
C2	10	-	-	+	-	+	-
A3	10	+	-	-	-	-	+
B3	10	-	+	-	-	-	+
C3	10	-	-	+	-	-	+

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Table 2. Hematological changes and comparison of mice in different groups

Day	Red Blood Cells (10^6/mm^3)				White Blood Cells (10^3/mm^3)				Platelets (10^5/mm^3)			
	Control	Infection	LC	LC^{+mcra}	Control	Infection	LC	LC^{+mcra}	Control	Infection	LC	LC^{+mcra}
14	10.15±0.14*^{b}	13.03±4.20^{a}	10.84±0.55^{b}	10.60±0.46^{b}	3.48±0.35^{a}	2.95±0.80^{b}	3.12±0.61^{a}	3.55±0.42^{a}	7±3^{a}	2±2^{c}	5±2^{b}	7±3^{a}
21	10.34±0.21^{b}	14.89±3.87^{a}	10.72±0.65^{b}	10.51±0.33^{b}	3.65±0.25^{a}	2.96±0.40^{b}	3.13±0.69^{a}	3.54±0.38^{a}	8±1^{a}	2±1^{c}	5±2^{b}	8±3^{a}
28	10.29±0.13^{c}	15.64±4.37^{a}	11.84±2.14^{b}	10.71±0.53^{c}	3.59±0.22^{a}	2.90±0.49^{b}	3.26±0.30^{a}	3.51±0.29^{a}	7±2^{a}	2±1^{c}	4±2^{b}	8±2^{a}

* Means with different letters (a-c) for each type of blood cell in different groups at individual time point are significantly different at $p<0.05$

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Table 3. White Blood Cells (WBC) counts of mice in different groups

WBC	Group	Day		
		14	21	28
Neutrophils (K/µL)	Control	1.31±0.40*[a]	1.34±0.40[a]	1.50±0.21[a]
	Infection	0.78±0.36[b]	0.41±0.22[b]	0.27±0.14[b]
	LC	1.02±0.30[ab]	1.07±0.37[ab]	1.08±0.48[ab]
	LC^{+mcra}	1.27±0.56[a]	1.16±0.11[ab]	1.20±0.23[ab]
Lymphocytes (K/µL)	Control	2.19±0.22[a]	2.50±0.13[a]	2.44±0.41[a]
	Infection	1.18±0.25[b]	0.92±0.15[b]	0.73±0.18[b]
	LC	2.05±0.78[a]	2.03±0.13[ab]	1.82±0.26[a]
	LC^{+mcra}	2.37±0.37[a]	2.53±0.15[a]	2.46±0.52[a]
Monocytes (K/µL)	Control	0.04±0.01[b]	0.04±0.02[b]	0.04±0.02[c]
	Infection	0.16±0.04[a]	0.14±0.06[a]	0.19±0.04[a]
	LC	0.05±0.02[b]	0.06±0.03[b]	0.08±0.03[b]
	LC^{+mcra}	0.05±0.02[b]	0.05±0.02[b]	0.05±0.01[c]
Eosinophils (K/µL)	Control	0.06±0.04[b]	0.07±0.03[c]	0.07±0.03[b]
	Infection	0.16±0.03[a]	0.16±0.04[a]	0.17±0.05[a]
	LC	0.09±0.04[b]	0.11±0.01[b]	0.09±0.03[b]
	LC^{+mcra}	0.07±0.03[b]	0.06±0.03[c]	0.06±0.04[b]
Basophils (K/µL)	Control	0.01±0.01[c]	0.01±0.01[c]	0.01±0.01[c]
	Infection	0.03±0.01[a]	0.03±0.02[a]	0.04±0.02[a]
	LC	0.02±0.01[b]	0.02±0.01[b]	0.02±0.01[b]
	LC^{+mcra}	0.01±0.01[c]	0.01±0.01[c]	0.01±0.01[c]

* Means with different letters (a-c) for each type of WBC in different groups at individual time point are significantly different at $p<0.05$

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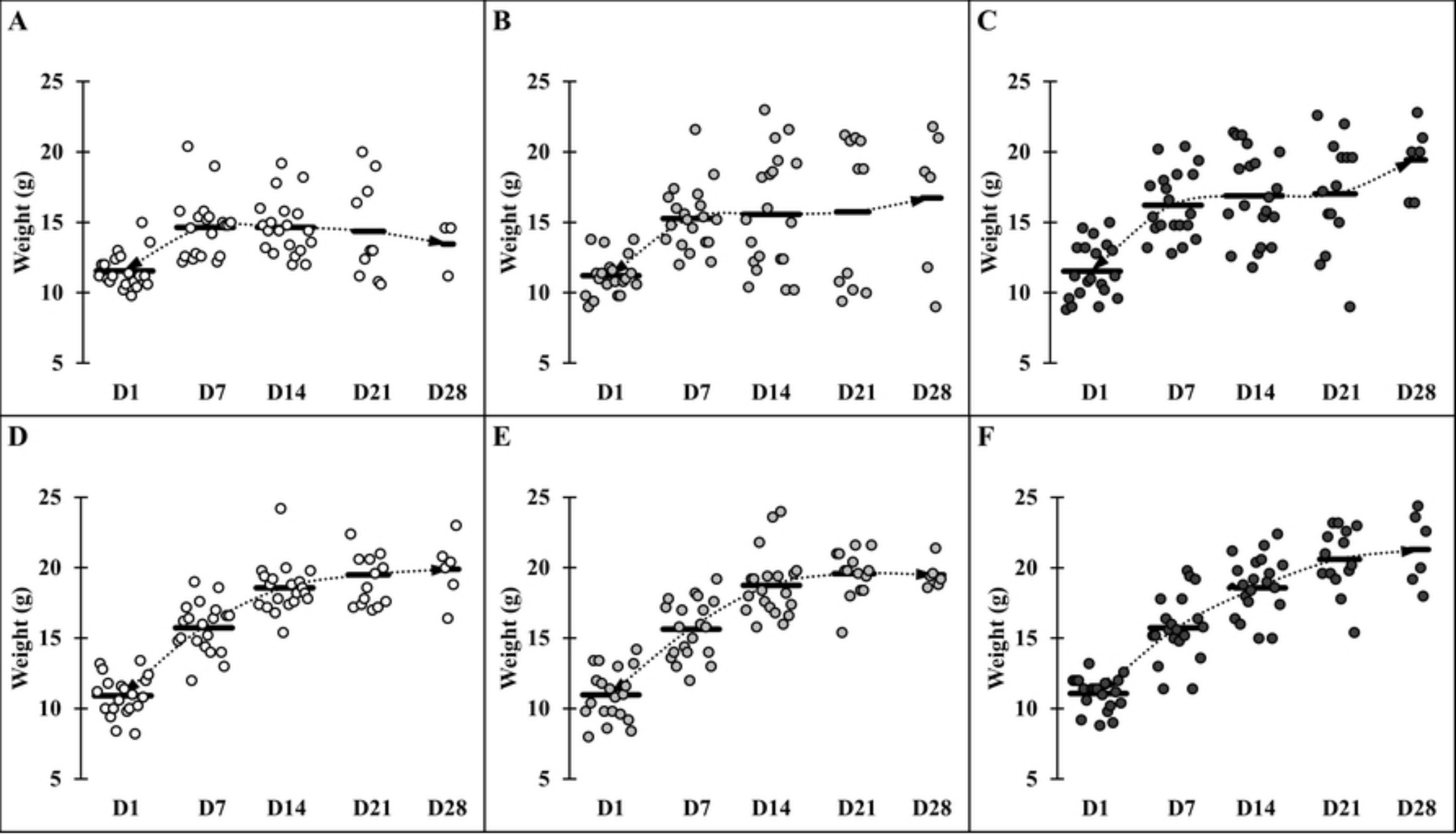
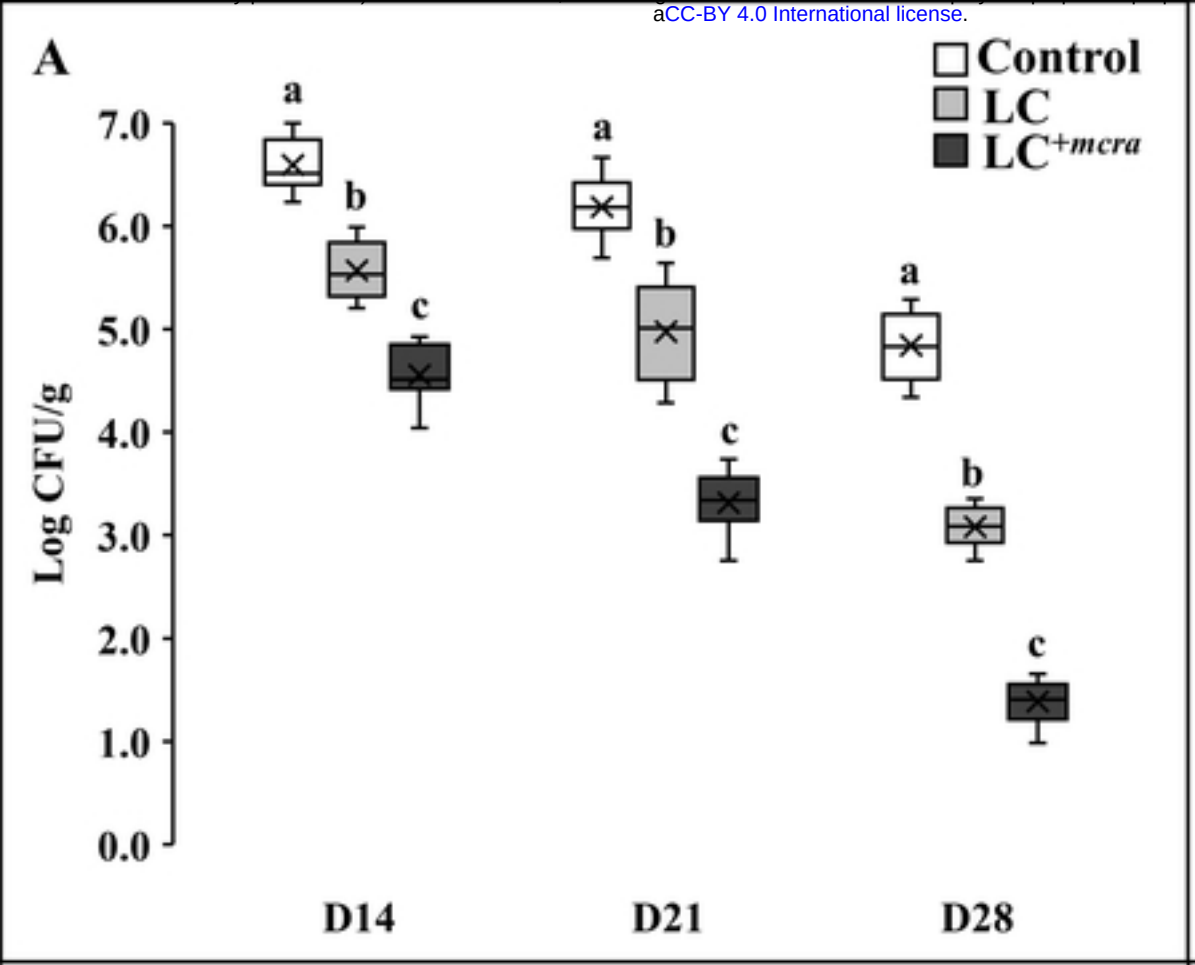
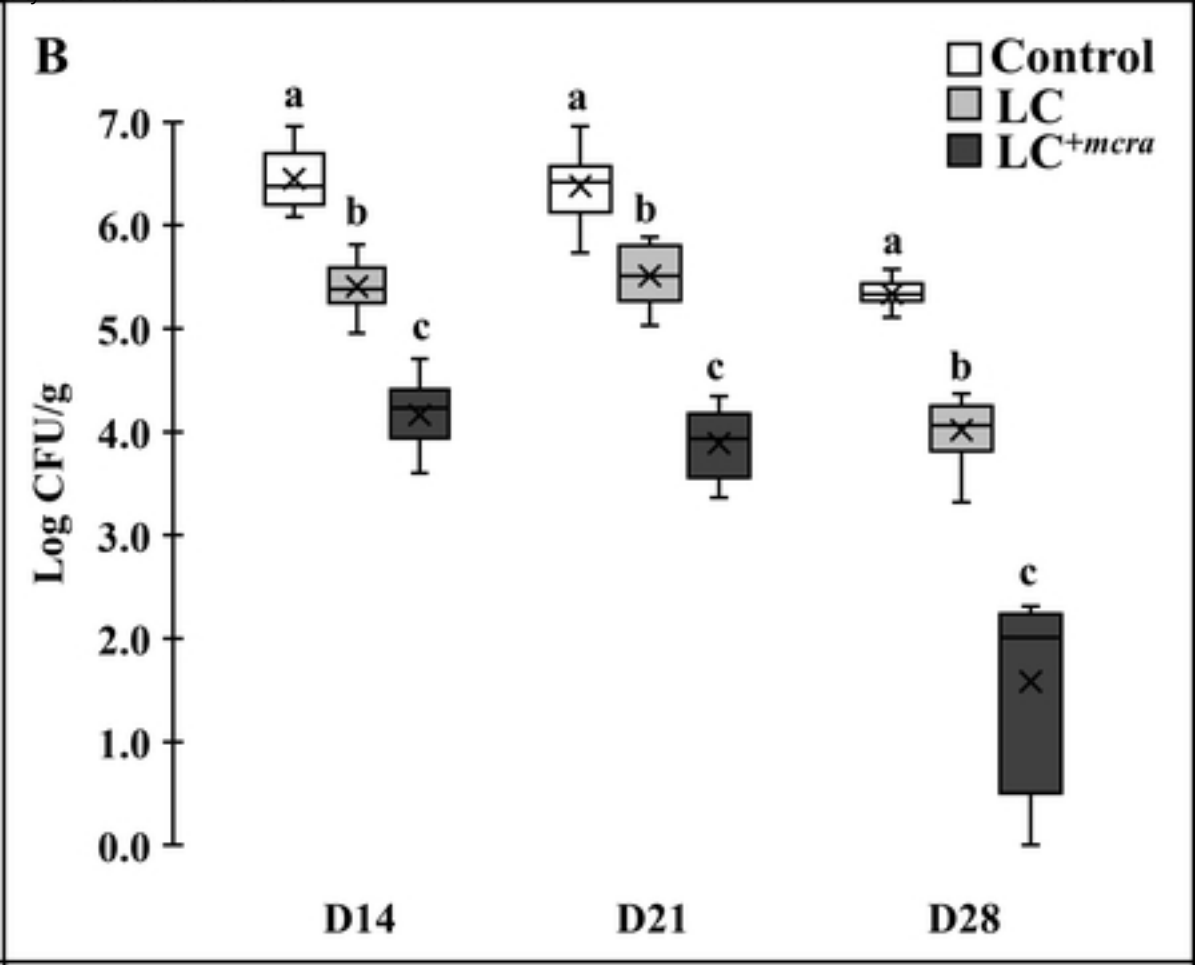
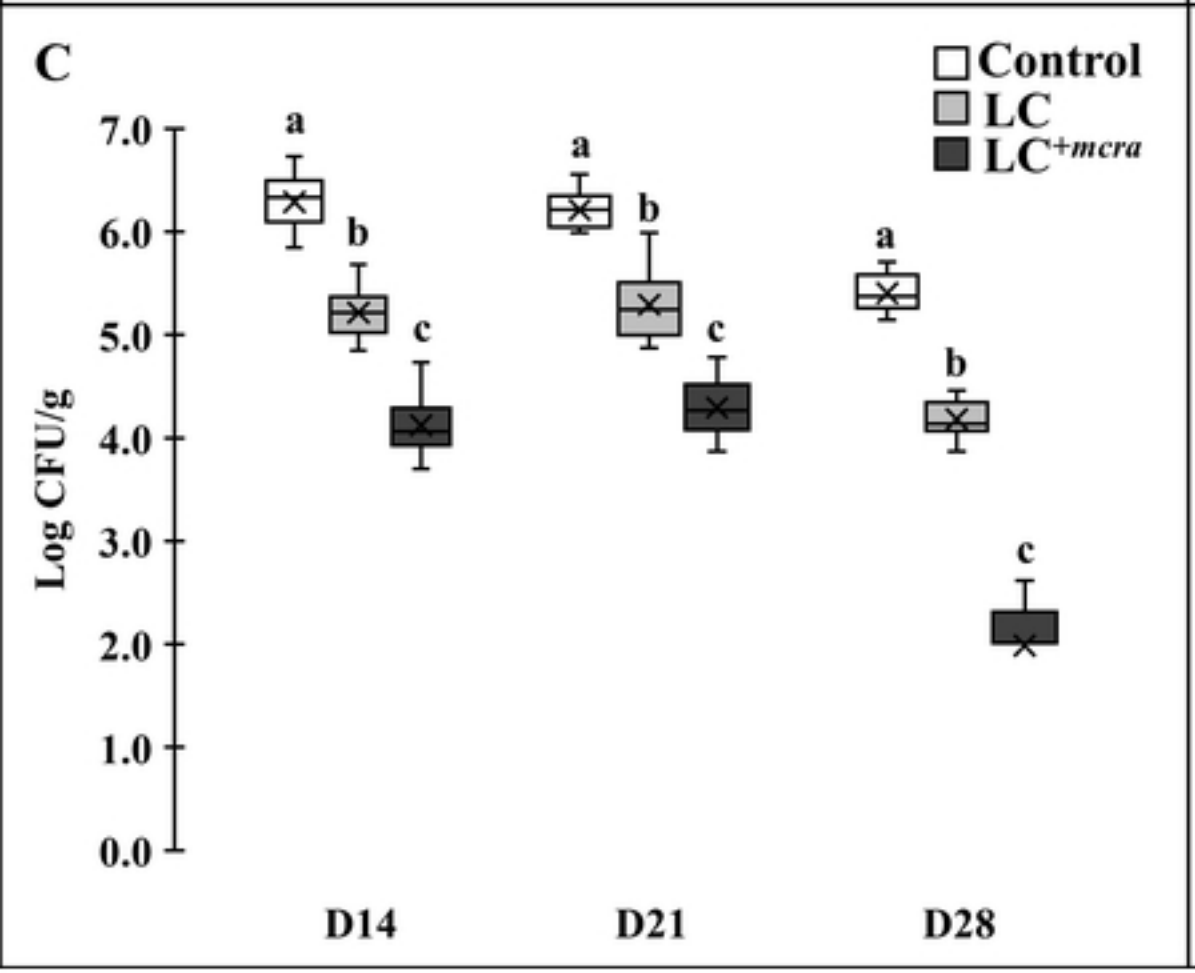


Figure1







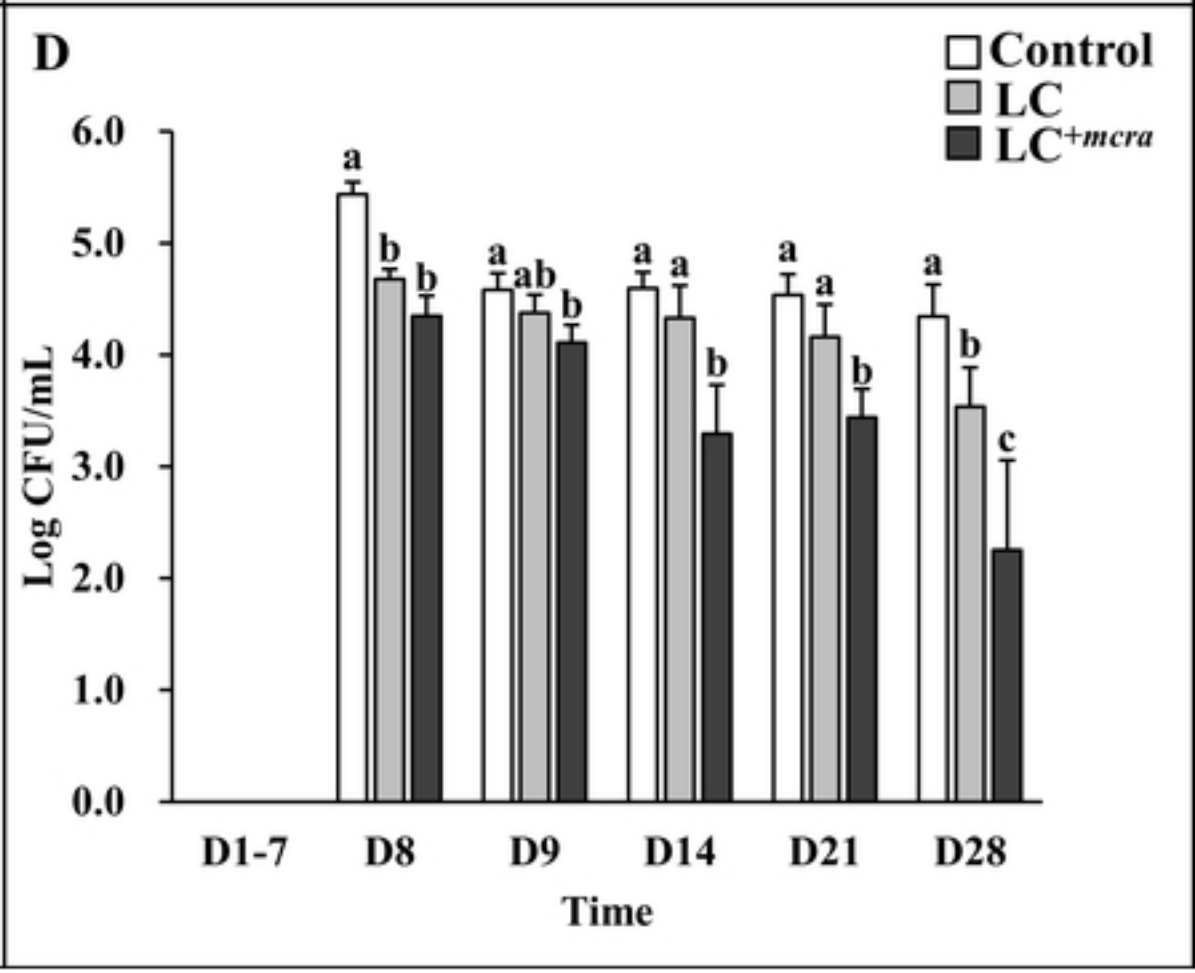


Figure2

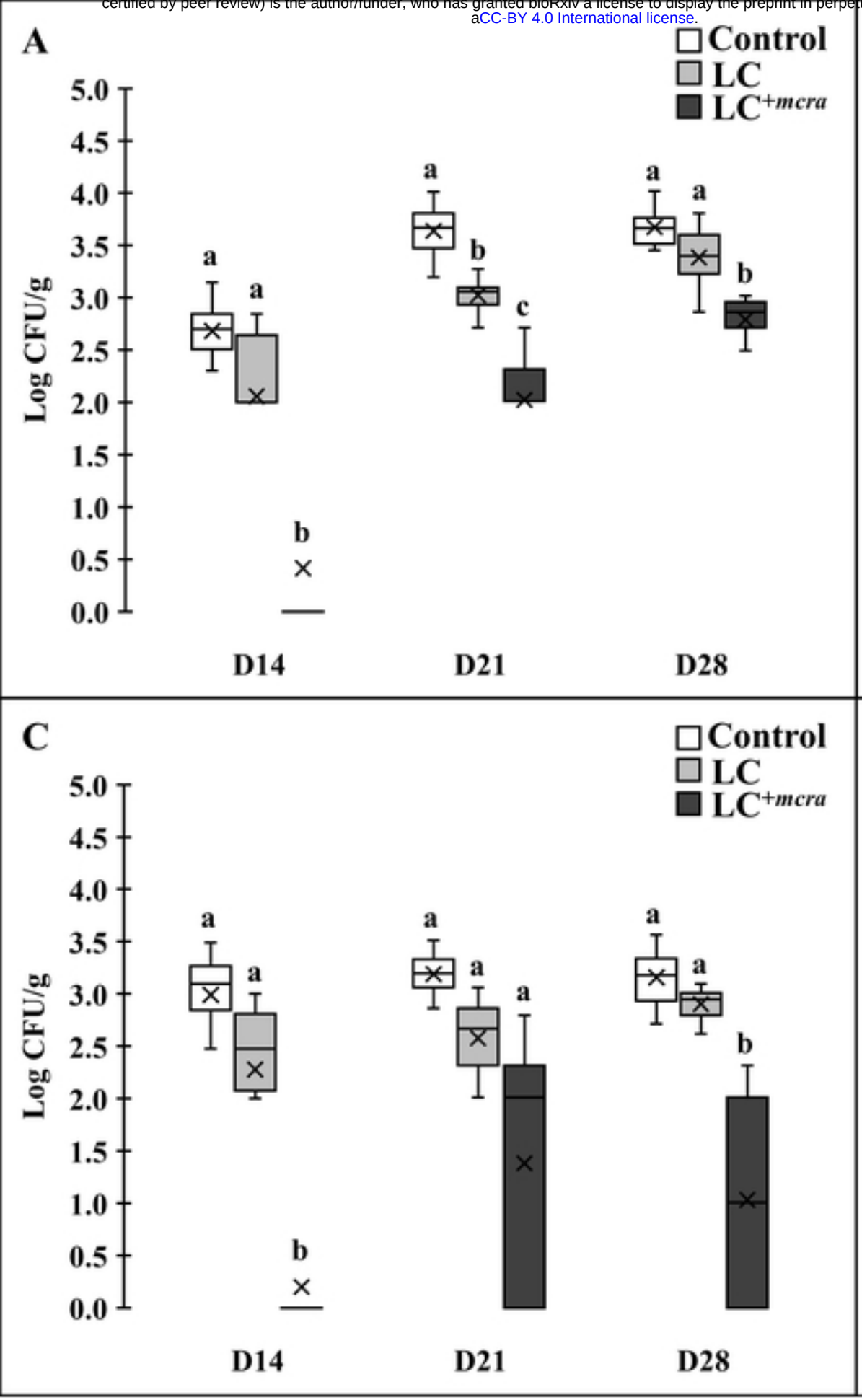


Figure3

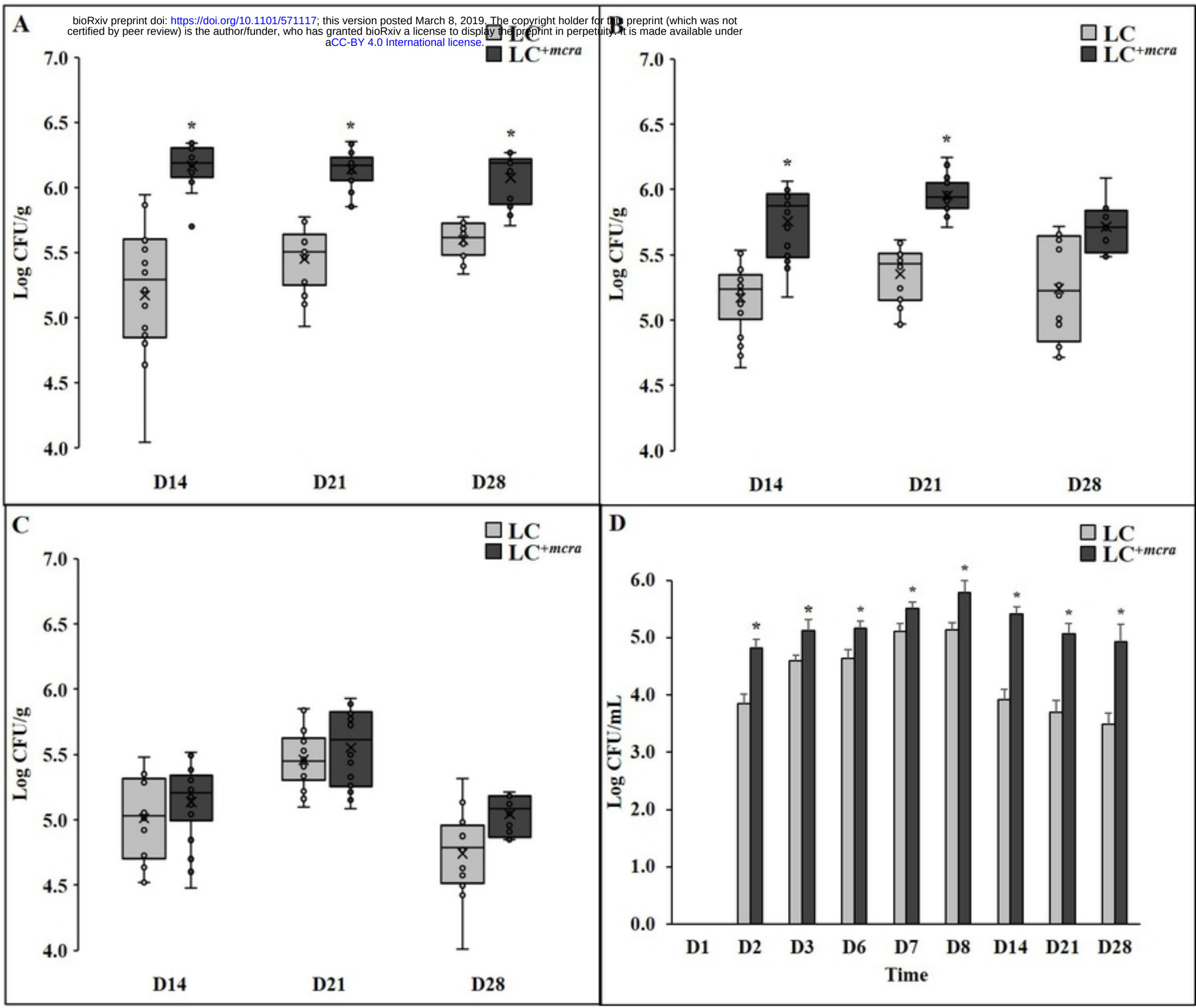


Figure4

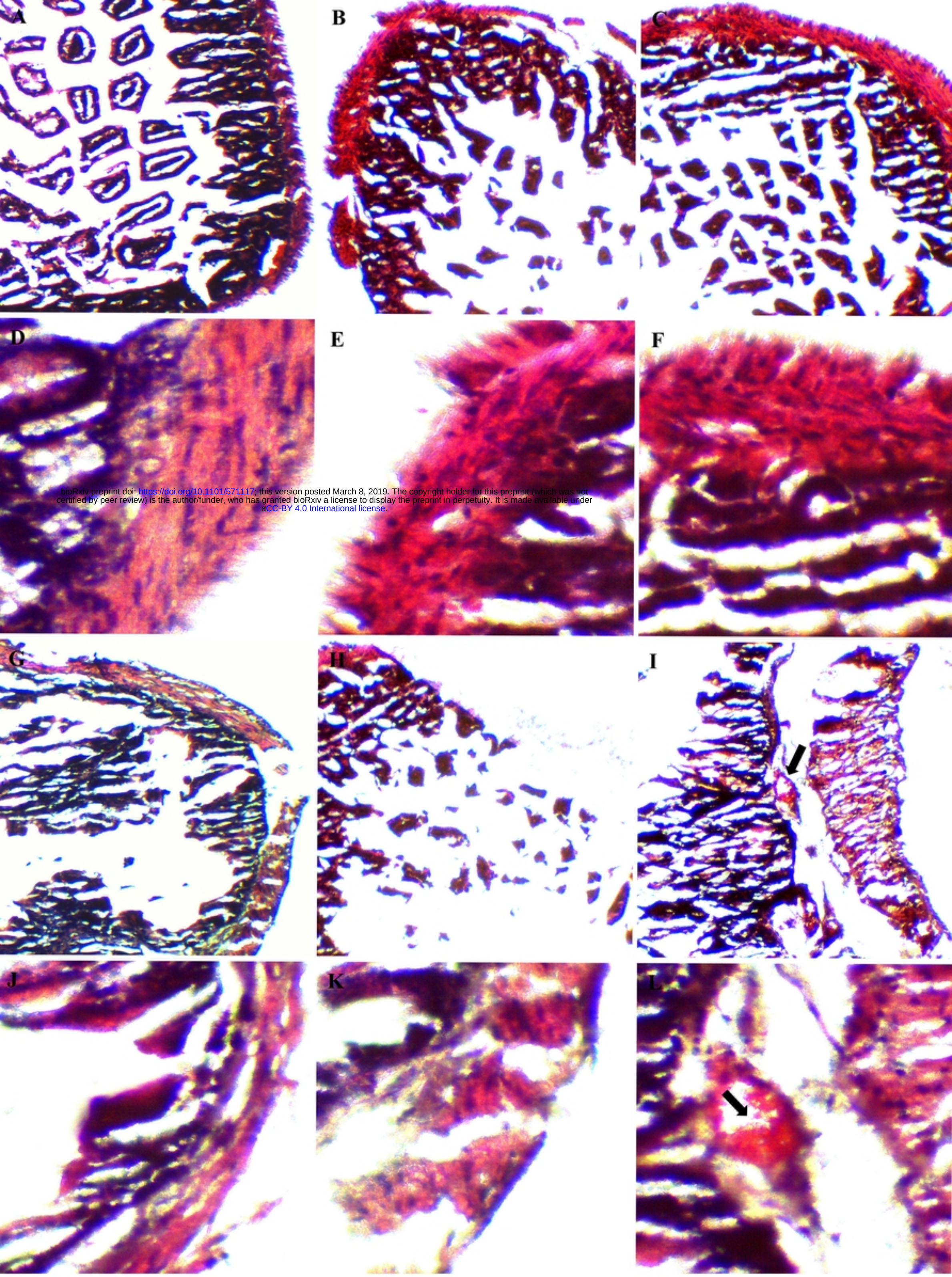


Figure5

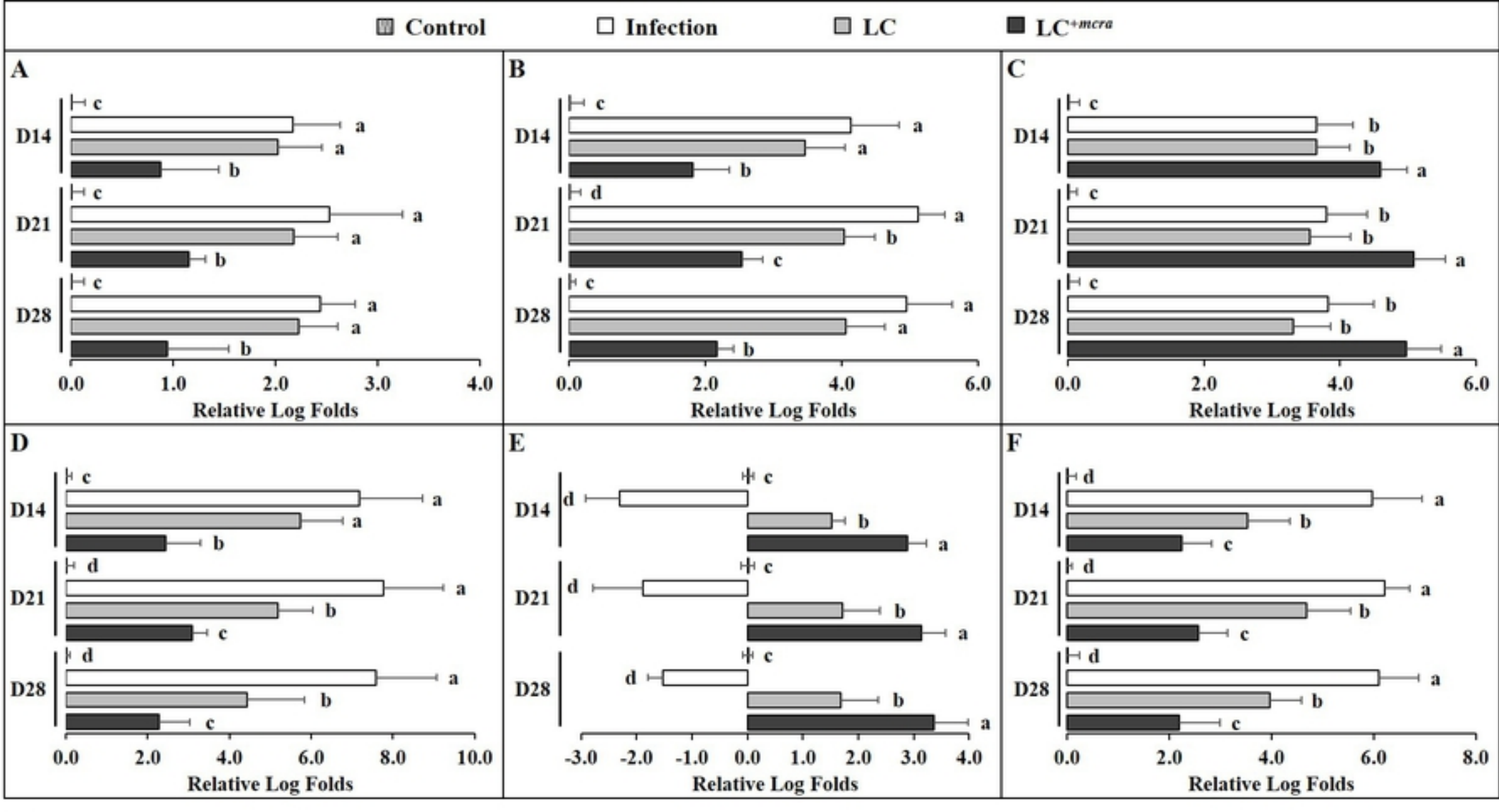

Control
Infection
LC
LC+mcra
A
B
C
D
E
F
D14
D21
D28
Relative Log Folds

Figure6

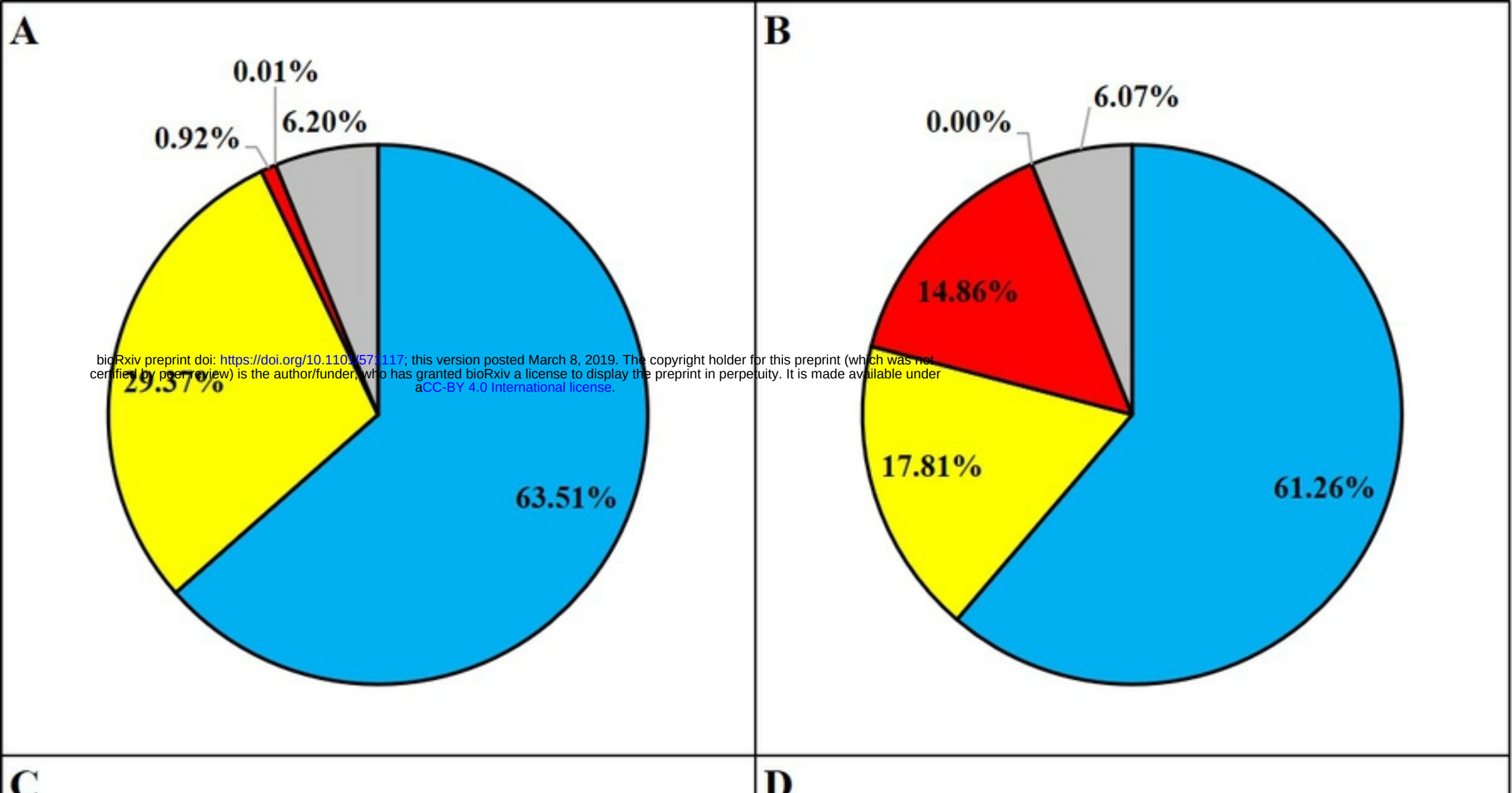


Figure7

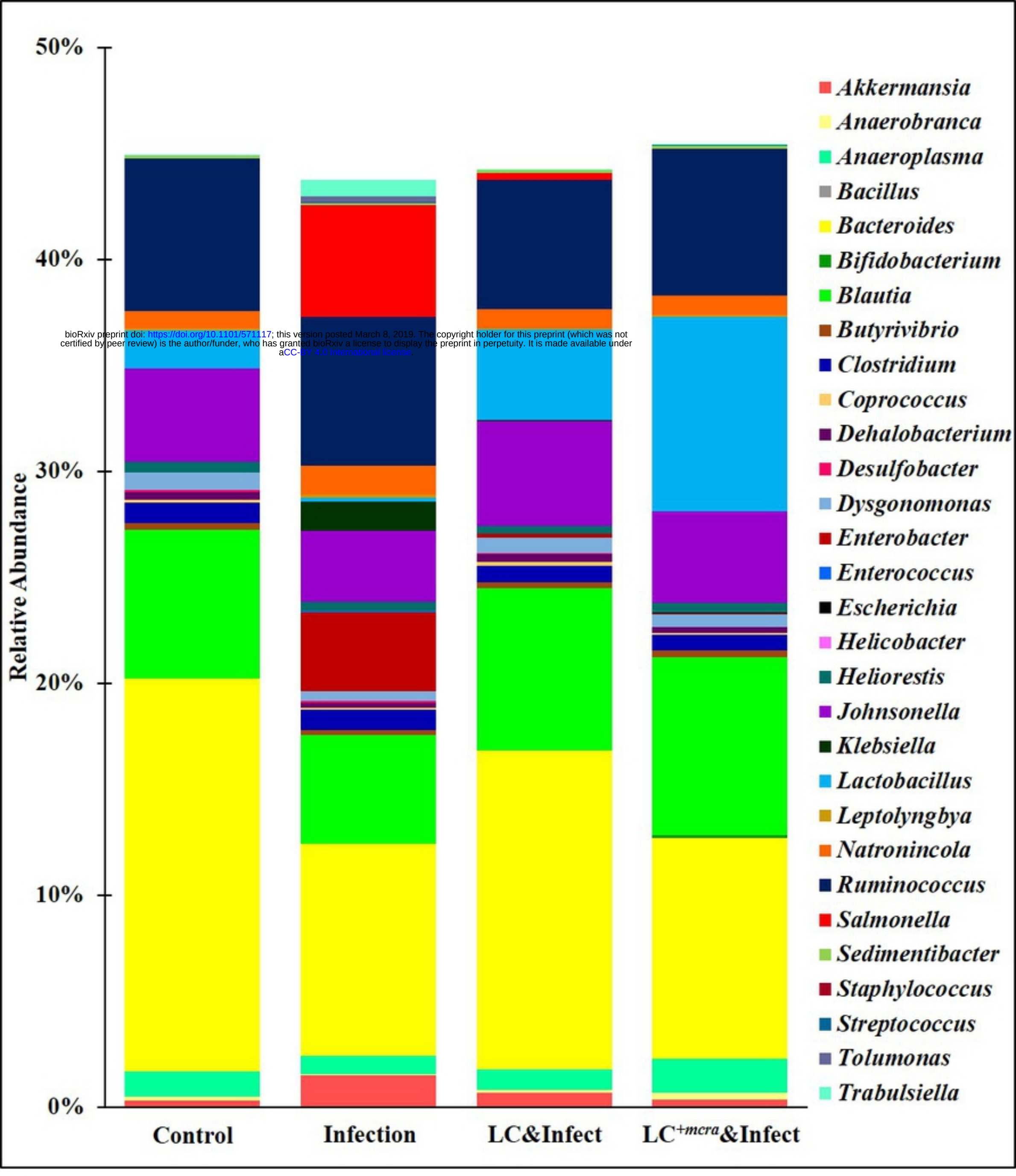


Figure8

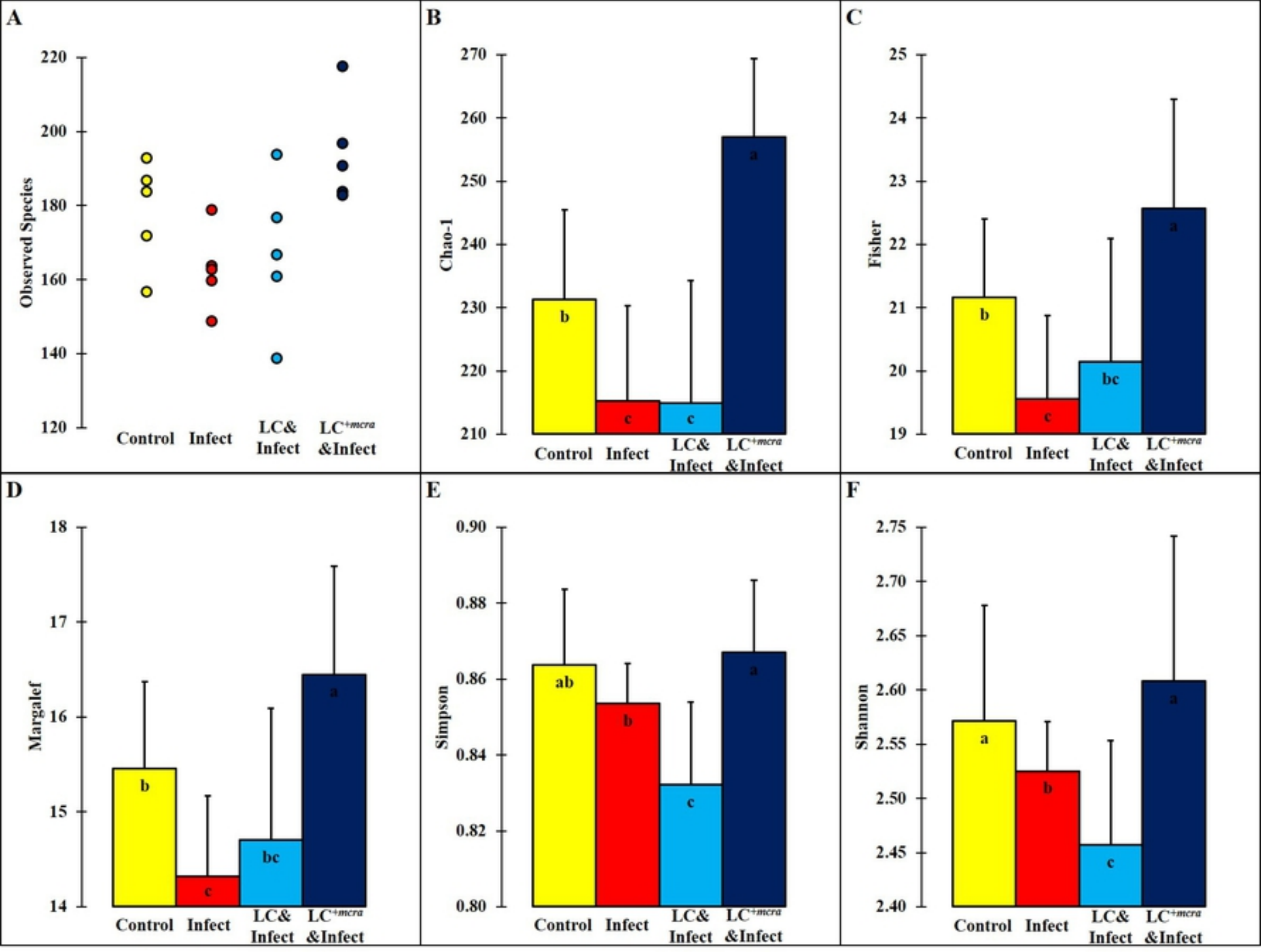

A
Observed Species
220
200
180
160
140
120
Control
Infect
LC& Infect
LC+mcra &Infect
B
Chao-1
270
260
250
240
230
220
210
a
b
c
c
C
Fisher
25
24
23
22
21
20
19
a
b
c
bc
D
Margalef
18
17
16
15
14
a
b
c
bc
E
Simpson
0.90
0.88
0.86
0.84
0.82
0.80
a
ab
b
c
F
Shannon
2.75
2.70
2.65
2.60
2.55
2.50
2.45
2.40
a
a
b
c

Figure9