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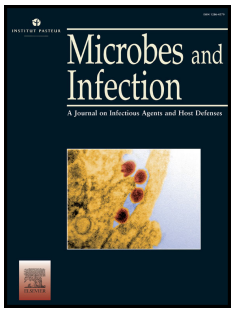

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Rabia Ladjouzi, Bernard Taminiau, Georges Daube, Anca Lucau-Danila, Djamel Drider

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The efficacy of the bacteriocinogenic *Enterococcus faecalis* 14 in the control of induced necrotic enteritis in broilers

Rabia Ladjouzi[a≠*], Bernard Taminiau[a,b], Georges Daube[a,b], Anca Lucau-Danila[a], Djamel Drider[a*]

[a] *UMR Transfrontalière BioEcoAgro INRAe 1158, Université de Lille, F-59000 Lille, France*

[b] *UMR Transfrontalière BioEcoAgro INRAe 1158, Fundamental and Applied Research for Animals & Health (FARAH), Department of Food Sciences, Faculty of Veterinary Medicine, University of Liege, 4000 Liege, Belgium.*

≠ *Present address : UR DYNAMYC 7380, Faculté de Santé, Université Paris-Est Créteil. 94010 Créteil Cedex, France*

***Corresponding authors :** djamel.drider@univ-lille.fr (D. Drider), and rabia.ladjouzi@u-pec.fr (R. Ladjouzi)

ABSTRACT

Purpose: To demonstrate the efficacy of the bacteriocinogenic *Enterococcus faecalis* 14 (*E. faecalis* 14) in the control of induced necrotic enteritis (NE) in broilers.

Methods: Six groups of 504 broilers consisting of an infected untreated control (IUC) group, an infected and amoxicillin treated control (ITC) group, and groups receiving prophylactically (2 groups) or therapeutically (2 groups) *E. faecalis* 14 or its Δ*bac* mutant were used. All groups were challenged with *Clostridium perfringens* 56 to induce NE. To predispose the boilers to develop subclinical NE, a high protein grower diet containing 15% fishmeal and a coccidial inoculum were administered.

Results: NE lesions were observed on D26 in all groups except ITC and those receiving prophylactically and therapeutically *E. faecalis* 14. On D27, only ITC and the group prophylactically treated with *E. faecalis* 14 (T03 T03) were without lesions. Average body weight and daily weight gain remained lower in the treated groups compared to the ITC group, but there was a clear improvement in the period between D21 to D27, especially in the group prophylactically treated with *E. faecalis* 14. Specifically, the daily weight gain (DWG) in this period for group T03, was second highest after the group ITC. Metataxonomic analyses showed a positive effect of *E. faecalis* 14 in maintaining the diversity and richness of the intestinal microbiota, in contrast to ITC group and other conditions.

Conclusions: The results of this *in vivo* study demonstrated the efficacy of the prophylactic administration of the bacteriocinogenic *E. faecalis* 14 in preventing of the NE lesions caused by *C. perfringens*.

Keywords: Necrotic enteritis, Enterocin DD14, innovative measure, AMT, metagenomic analysis, poultry infection, *Clostridium perfringens*

1. Introduction

The post-antibiotic era, which has been heralded for several years, will lead to the end of the anti-infective potential of antibiotics and pave the way for non-antibiotic strategies. In light of this, strategies that have been reported include antimicrobial peptides (AMPs), vaccination, wild and engineered phages, probiotics and fecal microbiota transplantation [1, 2,3].

Bacteriocins are AMPs ribosomally synthesized and endowed with multiple functions [4,5]. They are produced by Gram-positive, Gram-negative bacteria as well as Archaea [6,7]. These bacteriostatic or bactericidal agents are either narrow or broad-spectrum, inhibiting thus taxonomically close bacteria or a wide variety of bacteria by different mechanisms [8, 9, 10,11]. Their *in-situ* production has been shown to allow overgrowth of the producing bacteria over their congeners [12,13]. Bacteriocins are used in the food industry as natural biopreservative agents, consistently preferred over chemical preservatives, to protect products from harmful and spoilage microorganisms [14,15,16]. With the emergence of antimicrobial resistance (AMR), a problem exacerbated by the shortage of antibiotics, thus new strategies and new antibiotics are needed [17,18]. In this context, bacteriocins represent an interesting therapeutic option, and offer numerous advantages over traditional antibiotics, such as their efficacy at nanomolar concentrations [19,20]. In addition, these molecules have the advantage of a rapid pore-forming mechanism, short biological half-life, and sensitivity to proteolytic enzymes, which is likely to minimize the potential development of bacterial resistance [21,22].

E. faecalis 14 produces a leaderless two-peptide bacteriocin active against *Clostridium perfringens*, the causative agent of NE [23, 24]. In its subclinical form, this infection can significantly alter flock performance, while in its acute form it can lead to high mortality. It is noteworthy that NE remains one of the most specific diseases in poultry production, despite

constant advances in nutrition, housing management and genetics [25]. Since the banning of antimicrobial growth promoters, the importance of NE in broilers has increased and the economic losses due to this scourge are estimated to be more than two billion dollars per year worldwide [26,27]. In view of new strategies to control this bacterial infection, we determined the prophylactic and therapeutic potential of *E. faecalis* 14 and its isogenic Δbac mutant strain, deleted in the *ddA* and *ddB* genes coding for the synthesis of bacteriocin, previously obtained [28], against experimentally induced NE in broilers.

The objective was to evaluate the bacteriocin-producing (bacteriocinogenic) strain *E. faecalis* 14 as a means of controlling NE and to compare it with amoxicillin. Treatments were evaluated by scoring individual intestinal lesions, monitoring mortality, individual body weight (IBW), daily weight gain (DWG), daily feed intake (DFI), feed conversion ratio (FCR), and by analyzing the diversity of the gut microbiota.

2. Materials and methods

2.1. Bacteria and their growth conditions

E. faecalis 14 and the isogenic Δ*bac* mutant deficient in the bacteriocin synthesis were grown in GM17 (M17 medium containing 0.5% (w/v) glucose) under semi-aerobic conditions (tubes). The *Δbac* mutant was constructed by allelic exchange using a method based on the conditional replication of the pLT06 vector [28]. *C. perfringens* 56 (Poulpharm, Gent, Belgium) used to induce NE in broilers was grown in Brain Heart Infusion (BHI), under anaerobic conditions. All strains were incubated at 37°C for 24 hours without shaking. BHI and GM17 agar plates were used to determine the number of viable cells (CFU/mL).

2.2. Challenge tests

The different treatments and the critical study events are listed in Table 1 and Fig. 1., respectively. A total of 504 Ross 308 broilers (*Gallus gallus*) from the Vervaeke-Belavi hatchery, Belgium (hatchery number BE31142537-0301) was used in this study. Animals meeting inclusion criteria were selected and randomly assigned to different housing units and divided into six groups, and each group was represented by 3 replicates of 26 birds per pen (total of 78 birds/group). Health condition was scored for each animal as reported [29]. The six groups included: an infected untreated control (IUC) group, an infected treated control (ITC) group, and four infected experimental groups, two of which were treated prophylactically and the other two were treated therapeutically with *E. faecalis* 14 or the isogenic Δ*bac* mutant (Table 1). Regulatory requirements such as vaccination, concomitant medications and therapies, animal removal and necropsy procedures, animal accountability and disposition, and study facility are provided in File S1.

From D12 to the end of the study (D27), all animals were fed a high-protein breeder diet containing 15% fishmeal, and all birds were orally inoculated with 1 mL of a coccidiosis inoculum on D17, which consisted of 76,400 of *Emeria* (*Em*) *acervulina,* 24,000 of *Em. maxima* and 52,400 oocysts per ml. On D17, all broilers were challenged orally with coccidiosis as follows: the animal was properly restrained, the neck was slightly stretched, the beak was opened with the thumb and forefinger, the flexible tube was inserted into the esophagus, and the inoculum (1 mL/animal) was injected. NE was induced by virulent *C. perfringens* 56. From D21 to D24, all groups received ~10^9 CFU of *C. perfringens* 56 orally three times a day. Of note, high protein diets increase nutrient availability to bacteria (e.g. *C. perfringens*) in the gut, promoting their growth and toxin production. Coccidiosis (*Eimeria* spp.) damages the intestinal epithelium, weakening the intestinal barrier and facilitating the translocation and colonization of bacteria in the affected areas, triggering NE. The

combination of both factors (synergistic effect) mimics field conditions where NE outbreaks often occur.

Cultures of *E. faecalis* 14 and the isogenic Δ*bac* mutant strains harvested at 10^9 CFU/mL were administered orally by gavage. The prophylactic test was started 3 days before challenge (T03 and T04), whereas the therapeutic test was started on the second day after inoculation with *C. perfringens* 56 (T05 and T06 for *E. faecalis* 14 and Δ*bac* mutant), for a duration of 3 days for both treatments.

3.3. Management procedures

The study was carried out at Poulpharm (Ghent, Belgium). The poultry house was emptied, cleaned and disinfected. The light programme, temperature, feed and water supply are detailed in file S1.

3.4. Individual body weight (IBW) and daily weight gain (DWG)

IBW was measured per pen on D1 and D12 and individually on D21, D26, and D 27 to determine the development of IBW/growth rate. The average of DWG was calculated from pen weights and animal days for the different study periods using the following formula DWG = (pen weight at Dx - pen weight at Dx-1)/number of animal days (AD); AD = (number of study days in the evaluated study period * number of live chickens at the end of the study period) + the combined number of days that any chicken that died during the period lived in that period.

3.5. Daily feed intake and feed conversion ratio

Total feed weight was measured on D1, D12, D21 and D27 per pen in a feed container (metal bin). Feed intake was calculated for each group over the different study periods, and the DFI average was calculated for the different experimental periods as follows: DFI = (Brutto feed in +

143 netto feed in – brutto feed out) / AD; Loss of feed conversion efficiency can often occur as a result
144 of the negative effect of subclinical NE. Feed conversion ratio (FCR) was calculated for each of
145 the above study periods using the following formula: FCR = DFI/DWG (at the pen level).

146
147 *3.6. Necrotic enteritis lesion scores*

148 On D26 and D27, 13 birds per pen were euthanized and necropsied for scoring of
149 intestinal lesions [30] with a score from 0 (no lesions) to 6 (severe lesions). The same broilers were
150 also scored for typical coccidiosis lesions [31], with a score from 0 (no lesions) to 4 (severe lesions).
151 Here, chickens were scored for *Em. acervulina* and *Em. maxima*.

152
153 *3.7. Ethics*

154 The subclinical NE study was approved by the Poulpharm Ethics Committee under case
155 number P19160-FP (File S1).

156
157 *3.8. Total DNA Extraction*

158 Total DNA was extracted from 250 mg of fecal samples using the QIAamp PowerFecal Pro
159 DNA Kit (Qiagen, Courtaboeuf, France) according to the manufacturer's instructions. The quality
160 and quantity of DNA samples were determined using the Agilent TapeStation 4150 (Agilent
161 Technologies, France).

162
163 *3.9. PCR Amplification and 16S amplicon sequencing*

164 PCR amplification of 16S rDNA and library preparation were performed using the
165 following primers forward (5′-GAGAGTTTGATYMTGGCTCAG-3′) and reverse (5′-
166 ACCGCGGCTGCTGGCAC-3′). Amplicons were purified using the AgencourtAM Pure XP beads
167 kit (Beckman Coulter; Pasadena, CA, USA) and subjected to a second round of PCR for indexing
168 using Nextera XT index primers 1 and 2. After purification, amplicons were quantified using

Quant-IT PicoGreen (ThermoFisher Scientific; Waltham, MA, USA) and diluted to 10 ng/μL. Final qPCR quantification of each library sample was performed using the KAPA SYBR® FAST qPCR kit (KapaBiosystems; Wilmington, MA, USA) prior to standardization, pooling, and sequencing on a MiSeq sequencer using V3 reagents (Illumina; San Diego, CA, USA). A commercial mock community DNA, used as positive control (ATCC MSA 1000, LGC Standards, Molsheim, France), and a negative control (from the PCR step) were included in the sequencing.

3.10. Cleaning and processing of amplified sequences

Raw sequences were processed using MOTHUR v1.47 for alignment and clustering, and the VSEARCH algorithm for chimera detection 7-9 [32]. The standard MOTHUR MiSeq SOP was used for read processing and OTU generation. The 16S rDNA reference alignment and taxonomic assignment were based on the SILVA (v1.38.1) database of full-length 16S rDNA sequences [33]. From 2,616,654 raw sequences, 2,525,946 were retained and 2,312,869 with a median length of 493 nucleotides after searching and removal of chimeric sequences were used. A rarefied table of 10,000 sequences per sample was used for taxonomic assignment and OTU clustering. Good's coverage estimator was used as a measure of sampling effort for each sample, with an average value of 99.77%

3.11. Metataxonomic analyses

Sample ecological indicators (richness estimation-Chao1 estimator, microbial biodiversity-Simpson reciprocal index, and population regularity or equitability derived from Simpson index) using MOTHUR software. Indices differences between treatments have been assessed with ANOVA test using Benjamini-Hochberg False discovery rate (FDR) multiple test correction, using R (p value cut-off = 0.05).

Beta diversity (bacterial community composition) was assessed with MOTHUR using the Bray-Curtis dissimilarity matrix. Difference in microbiota profile between groups (either the sampling date, or the treatment factor) has been evaluated with ADONI2 permutational ANOVA test (999 permutations when possible) in vegan package in R [34]. Non-metric multidimensional scaling (NMDS) analysis, based on the Bray-Curtis dissimilarity matrix enabled to visualize biodiversity between groups. Ordination analysis and 3D graphics were performed using the vegan (https://CRAN.R-project.org/package=vegan, v2.6-4), Vegan3d (https://CRAN.R-project.org/package=vegan3d, v1.2) and rgl (https://CRAN.R-project.org/package=rgl, v1.3.1) packages in R (R: A Language and Environment for Statistical Computing, R Foundation for Statistical Computing, Vienna, Austria, 2015; https://www.R-project.org/, v4.3.1).

3.12. Statistics

Statistical analysis of overall scores was performed using a paired t-test and the analysis of variance by ANOVA. P values < 0.05 were considered to be significant. For mortality statistics, differences were examined using a Cox proportional hazards model with treatment as a fixed effect. For mortality per treatment, post-hoc pairwise comparisons without adjustment method were performed.

4. Results

4.1. General health, clinical signs of coccidiosis and mortality

16 mortalities were recorded, 5 of which occurred within one week of the birds' life. None of the mortalities was related to NE or coccidiosis and no significant differences were observed between the groups. The highest mortality of 3.6% was observed in the T03 and T04 groups, which is noticeably within the range of acceptable mortality in commercial broiler production. For

mortality per treatment, post-hoc pairwise comparisons without adjustment method were performed and showed no significant differences ($P > 0.05$).

4.2. Results of the different treatments on broiler performance parameters

Although the birds were homogeneous in all experimental groups with no significant differences prior to challenge on D1 and D12, the weights of the broilers in the treatment groups were lower compared to the control groups ITC (Table S1). At the end of the study and after challenge, the treated broilers appeared to gain more weight under challenge conditions, especially the T03 group prophylactically treated with *E. faecalis* 14 (Fig.2.A). The DWG in D21 - D27 for the T03 group was the second highest after the T02 ITC group (amoxicillin treatment) (Fig. 2.B, Table S1). On the other hand, groups T04 and T05 had significantly higher IBW on D26 but not on D27 (Table S2). The DFI was significantly higher in the control groups from D12 to D21 and from D21 to D27, while the difference was seen in the T04 group (*Δbac* prophylactic) (Fig. 2. C, Table S3). In the last study period, the FCR was worst in the IUC group, reflecting the occurrence of lesions and lower feed utilization, with most treated groups performing better in this respect, except for the T06 group (*Δbac* therapeutic) (Fig. 2. D, Table S4).

4.3. Necrotic enteritis lesion scores

On D26 and D27, 13 birds each were euthanized and necropsied for scoring of intestinal lesions scores [30]. The Figs. 3 (A & B) shows the incidence, as % per group, and mean NE lesion scores in different groups on two different scoring days. NE lesion scores were assessed on D26 and D27, and Fig. 3 summarizes the scores in the different groups. Subsequently, NE-specific lesion scores were recorded on D26 in animals of all groups except control group ITC and groups T03 and T05. As expected, the highest number of broilers with lesions on D26 was recorded in

IUC group. On D27, no lesions were found in groups ITC and T03, but surprisingly, the highest number of broilers with lesions on D27 was recorded in group treated prophylactically with the *Δbac* strain (Fig. 3A). In turns, the Fig. 30B shows that NE lesions are mainly found in the IUC group, then in the groups treated prophylactically and therapeutically with *Δbac* ($P = 0.391$ and $P = 0.22$, respectively), and to a lesser extent in the group treated therapeutically with *E. faecalis* 14 ($P=0.066$). The two groups with no lesions were the ITC group and the group treated prophylactically with *E. faecalis* 14 ($P=0.007$).

All these results demonstrate the efficacy of prophylactic treatment of NE with *E. faecalis* 14. Treatment with the *Δbac* mutant did not protect broilers against this infection under *in-vivo* conditions, delineating the protective role of EntDD14.

4.4. Coccidiosis lesion scores

The data obtained showed the mean coccidiosis lesion scores for two species included in the inoculum, *Em. acervulina* and *Em. maxima*, scored on D26 and D27. Species-specific lesion scores are shown in stacked bar graphs. Differences were examined using ordinal regression models with treatment as a fixed effect. All groups were compared to IUC (T01). In contrast to NE scores, coccidiosis lesion scores related to *Em. acervulina* were significantly higher in the groups treated with *E. faecalis* 14 prophylactically and therapeutically on both scoring days, whereas lesions due to *Em. maxima* were highest in group T03 on D27. Taking these results into account, the *E. faecalis* 14 prophylactic group is the most predisposed to NE. However, this group showed no lesions specific to NE, supporting the efficacy of the *E. faecalis* 14 strain in protecting broilers against *C. perfringens* 56 under *in-vivo* conditions.

4.5. Effect of treatments on the chicken microbiota

The composition of the gut microbiota was assessed at D10 and D27. At D10, *Lactobacillus* and *Limosilactobacillus* dominated the gut microbiota (Fig. 4A). At D27, a bacterial taxonomic profile was established from the small intestine content showed the prevalence of *Corynebacterium*, *Limosilactobacillus*, *Lactobacillus*, *Ligilactobacillus* and *Brachybacterium* (Fig. 4B). In the groups treated prophylactically or therapeutically with *E. faecalis* 14, the abundance of the genus *Corynebacterium* was lower than in the ITC group, but contained a higher proportion of the genera *Limosilactobacillus*, *Lactobacillus*, *Ligilactobacillus* and *Brachybacterium*.

Beta diversity was visualized using two-dimensional MDS models with samples from D10 and D27 (Fig. 5A). This analysis revealed no significant differences between samples from different groups (ADONIS2 test P value=0.163). Despite a non-significant difference in population richness and alpha diversity (P value = 0.34 and *P* value = 0.44 for Chao1 estimated richness and Inverse Simpson alpha diversity, respectively), a clear advantage was observed in the group treated prophylactically with *E. faecalis* 14 (Fig. 6A & B).

5. Discussion

As a result of the withdrawal of antibiotics as growth promoters, but also because of consumer pressure for antibiotic-free (ABF) or never-use antibiotics (NAE) poultry production, there is a need for sustainable alternatives to prevent disease in commercial poultry farms [35, 26, 27]. To address the problem of AMR, which is partly due to the overuse of antibiotics, LAB, especially those that produce bacteriocins, could be effective alternatives to traditional antibiotics in animal production. In fact, these microorganisms may also benefit from the status of Generally-Recognized-As-Safe or Qualified Presumption of Safety, which facilitates their use [36]. The use

289 of enterococci is questionable because of their dual role in hospital-acquired infections and in the
290 fermentation process of foods such as cheese. However, the use of enterococci in poultry
291 production to control infections attributed to *C. perfringens* has been suggested as a potential non-
292 antibiotic solution in a recent study by Garcia-Vela [37]. Here, the prophylactic and therapeutic
293 properties of *E. faecalis* 14 were evaluated based on its previously reported probiotic properties
294 [23], and *in vitro* anti-*C. perfringens* activity [24]. To determine the efficacy of *E. faecalis* 14
295 against NE etiologic agent, we also used the isogenic *Δbac* mutant that does not produce EntDD14.
296 The results obtained with both strains were compared with each other but also with those obtained
297 with amoxicillin. As shown in Fig. 3, the intestinal lesion scores and their incidences are treatment-
298 dependent. Indeed, at D26, the group with the highest lesion score was T01 obtained for the IUC
299 group. On D27, no lesions were observed in the ITC and T03 groups, which were prophylactically
300 treated with of *E. faecalis* 14. In direct line, Wang et al [38] showed that supplementation with
301 Sublancin 168 was able to control NE induced by *C. perfringens* strain in broilers, and a decrease
302 in *C. perfringens* cecal counts was observed in the group treated with this bacteriocin. Other *in vivo*
303 data showed the ability of griselimycin to cure tuberculosis in a mouse model [39], and Abp118 to
304 eradicate the effect of *Listeria* in mouse and pig models [40]. Taken together, these results illustrate
305 the efficacy of bacteriocins when used *in vivo* models against various pathogens. In a previous
306 report, we found that EntDD14 injected intraperitoneally did not affect the microbiota of mice and
307 did not induce histopathologic changes, in contrast to mice challenged and treated with antibiotics
308 [41]. Interestingly, nisin injected intraperitoneally showed a stable effect on the intestinal
309 microbiota of mice [42]. Rea et al [43] showed that Thuricin CD was able to inhibit several isolates
310 of *C. difficile* without affecting the commensal microbiota in a model of the distal colon [43]. These
311 results show that, unlike antibiotics, bacteriocins do not alter the intestinal microbiota. Here, we

show that the EntDD14-producing strain do not affect cecal *Lactobacillus* diversity, unlike the group treated with amoxicillin. On D26 and D27, coccidiosis lesions due to *Em. acervulina* were important in the groups receiving *E. faecalis* 14 prophylactically or therapeutically, while those due to *Em. maxima* were highest in the T03 group on D27, indicating that *E. faecalis* 14 was effective in reducing the adverse effect of *C. perfringens*. Furthermore, we observed low BW and DWG in the treated groups, but an improving trend appeared in the group treated prophylactically with *E. faecalis* 14.

In conclusion, we demonstrate the ability of *E. faecalis* 14 to reduce the negative effect of NE based on lesion scores when this strain is administered prophylactically and therapeutically to birds. Nevertheless, we could not demonstrate a clear effect on growth parameters in the treated groups, although an improvement appeared at the end of the experiment, indicating the advantage of treatment with *E. faecalis* 14 under infection pressure. Unlike amoxicillin, the addition of *E. faecalis* 14 had no effect on the diversity of the gut bacterial community. To our knowledge, this is the first study to highlight the use of enterococci in the control of NE and the importance of their bacteriocins.

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Data Availability Statement: The data will available on request

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Conflicts of Interest. The authors declare no conflict of interest

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477 **Table 1.** Different treatment groups of this study

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Treatment code	Group name	Treatment	Challenge	Replicates	Broilers per replicate (from D12)
T01	Infected untreated control IUC	-	Yes	3	26
T02	Infected and treated control ITC	Amoxicillin from D20 to D25	Yes	3	26
T03	*E. faecalis* 14 prophylactic	*E. faecalis* 14 – D18, D19 and D20	Yes	3	26
T04	*Δbac* prophylactic	*E. faecalis* Δ*bac* - D18, D19 and D20	Yes	3	26
T05	*E. faecalis* 14 therapeutic	*E. faecalis* 14 – day 22, 23 and 24	Yes	3	26
T06	*Δbac* therapeutic	*E. faecalis* Δ*bac* - D22, D23 and D24	Yes	3	26

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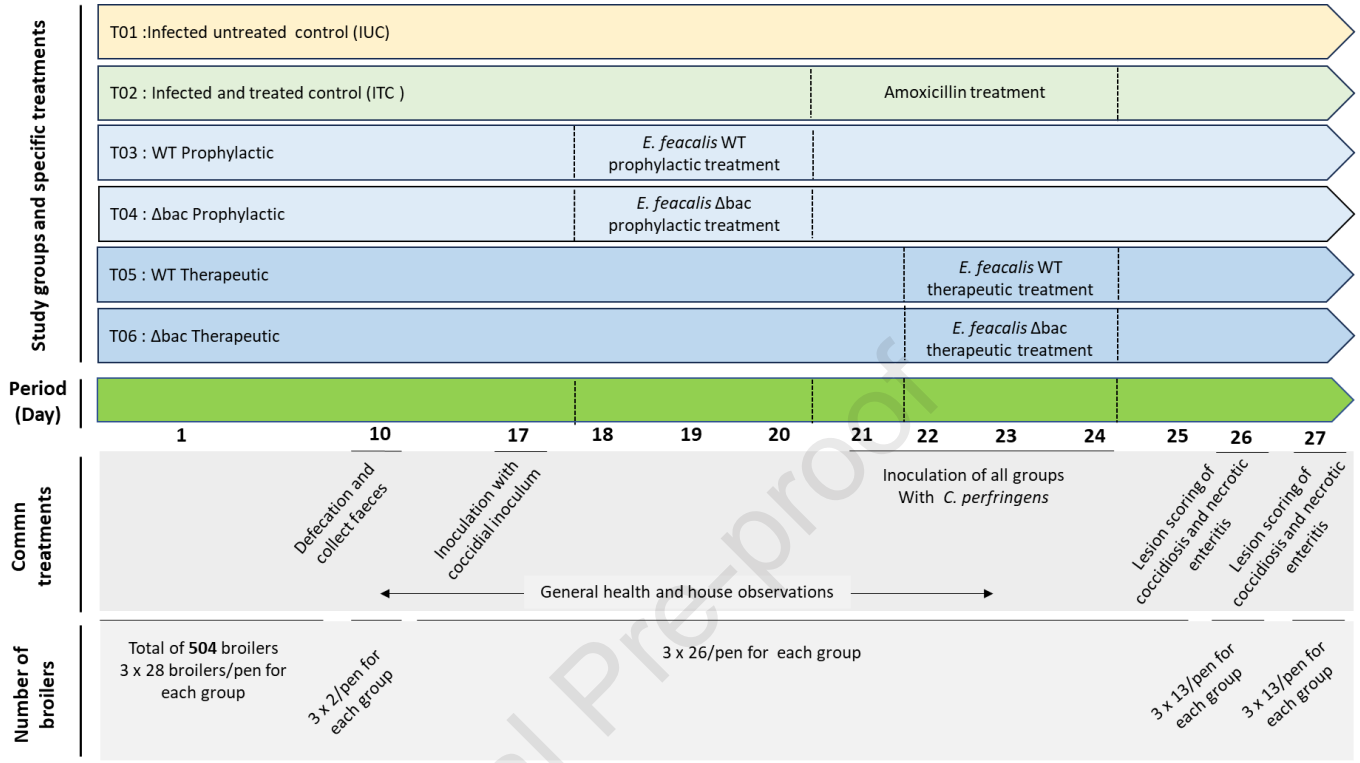

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498 **Fig. 1. Study design and group specific treatments**. WT: *E. faecalis* 14

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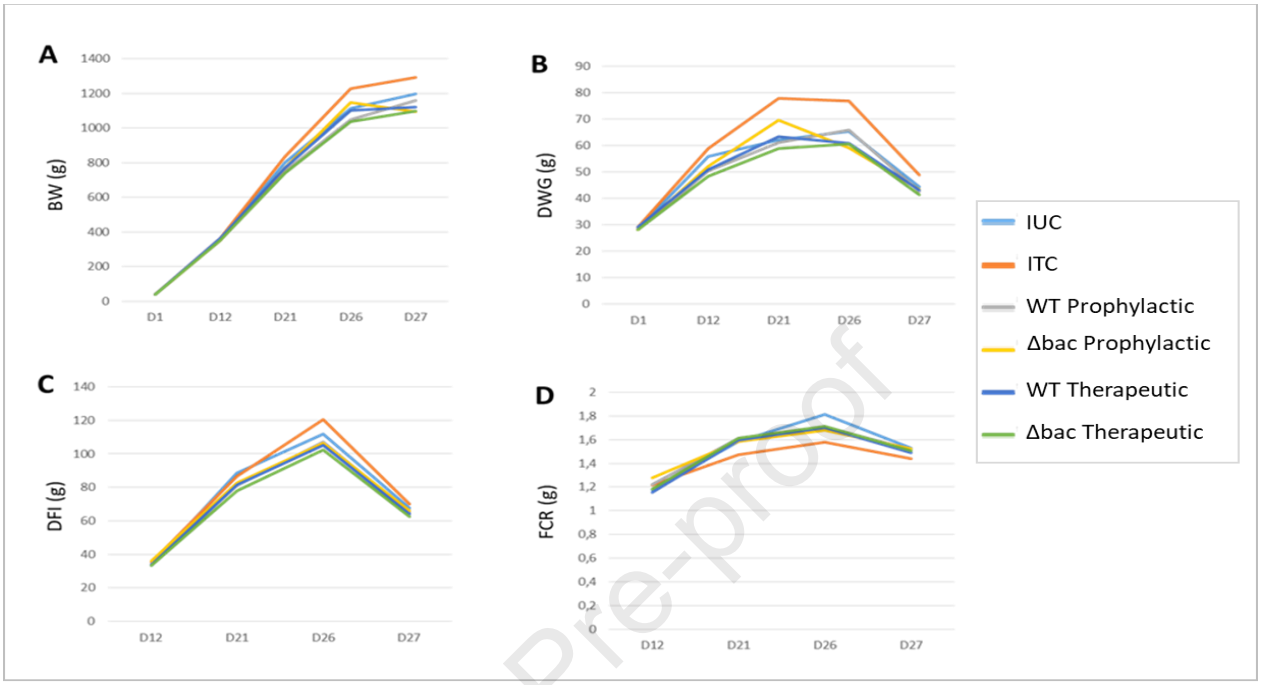

Fig. 2. Results of different treatments on broiler performance parameters. Body weight (A), daily weight gain (B), daily feed intake (C) and feed conversion ratio (D) were calculated per group and for different study periods.

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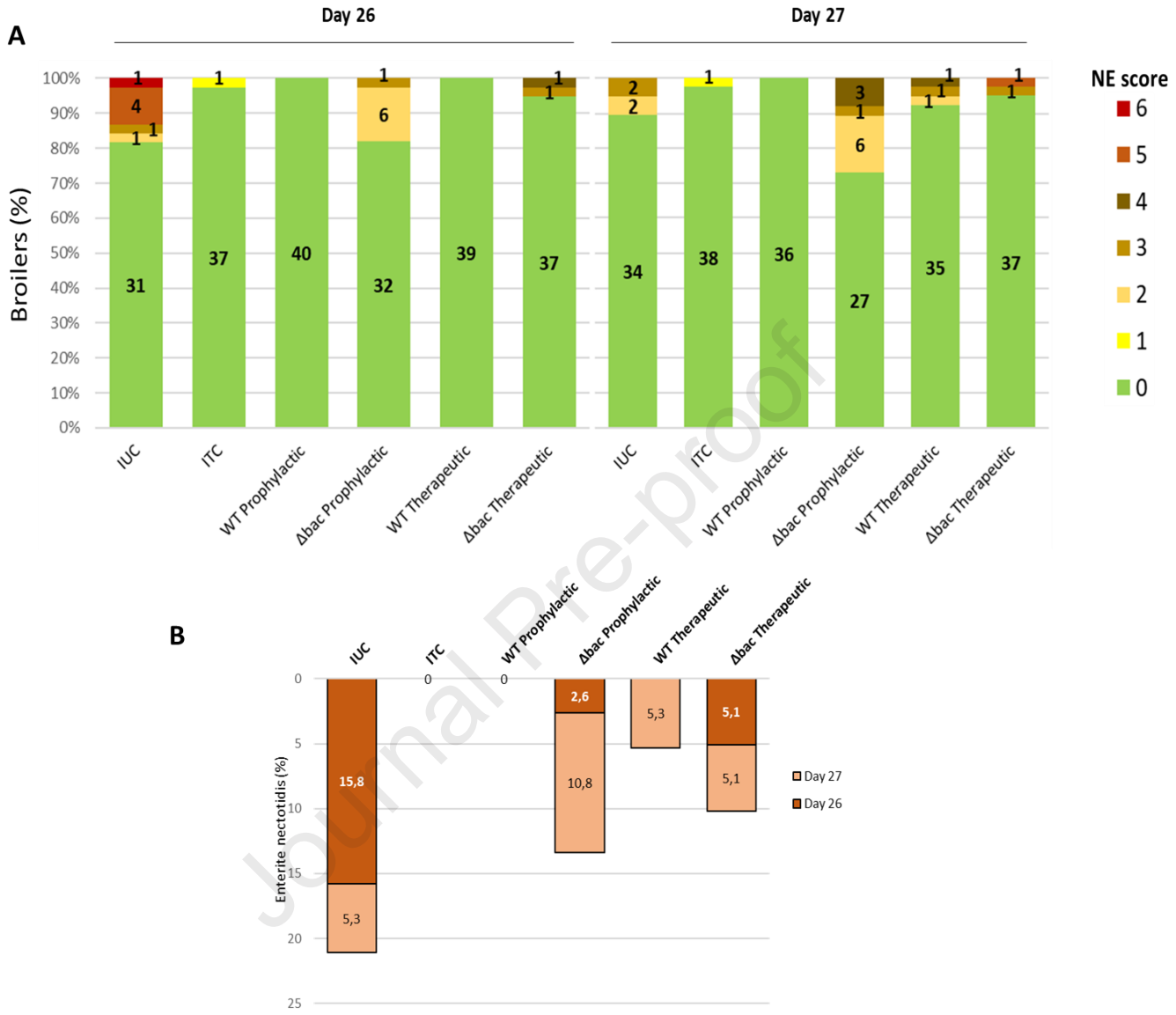

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511 **Fig. 3. Incidence of NE lesions in different groups** on two different assessment days (D26 and

512 D27) (A) and cumulative percentage of NE (regardless of lesion grade) recorded on D26 and D27

513 in different groups (B).

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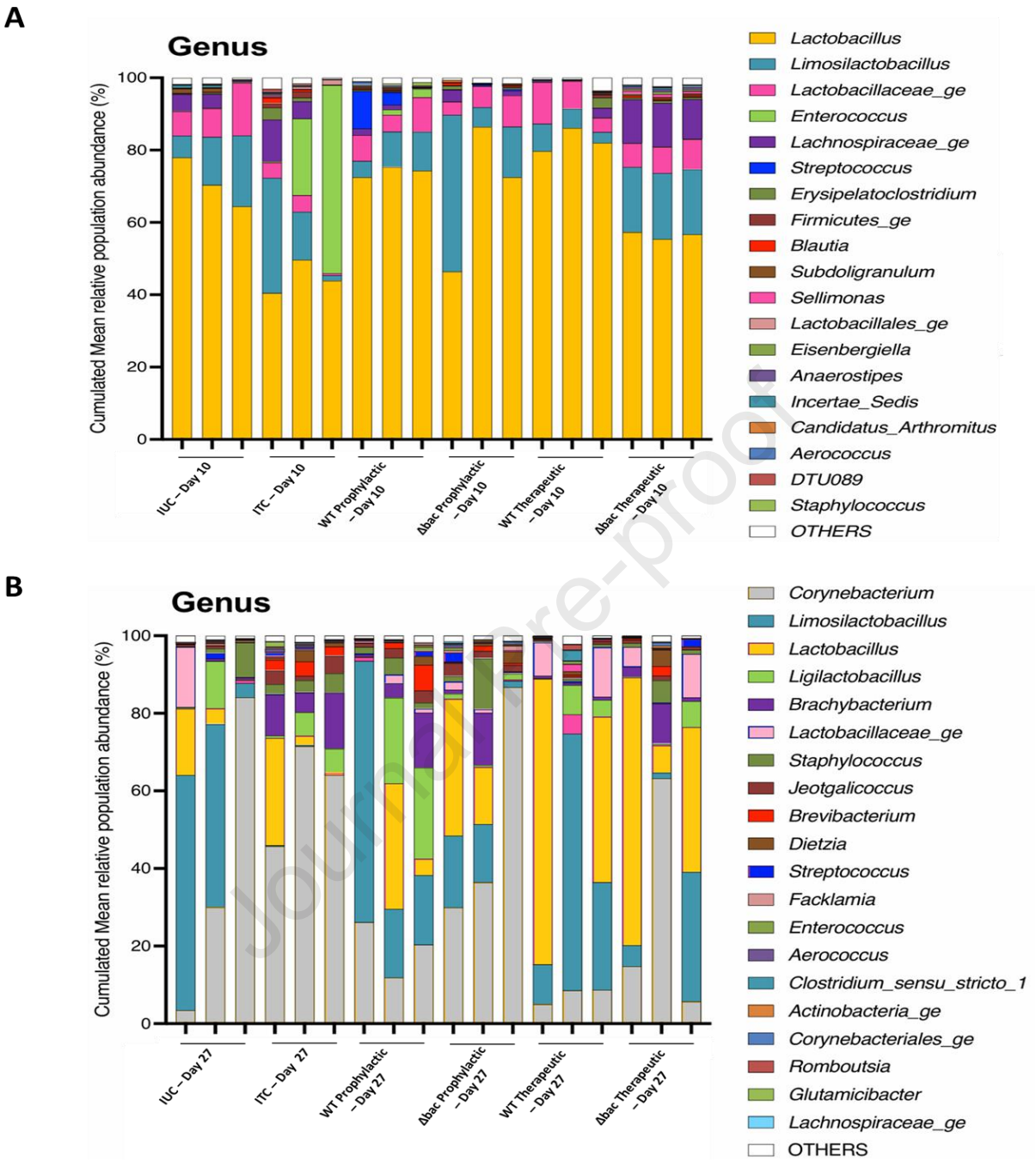

Fig. 4. Stacked bar plot illustrating the relative population abundance of the dominant genera for the D10 faecal samples (A) and the D27 small intestine samples after treatment (B). The OTHER category is the sum of the less dominant population abundances.

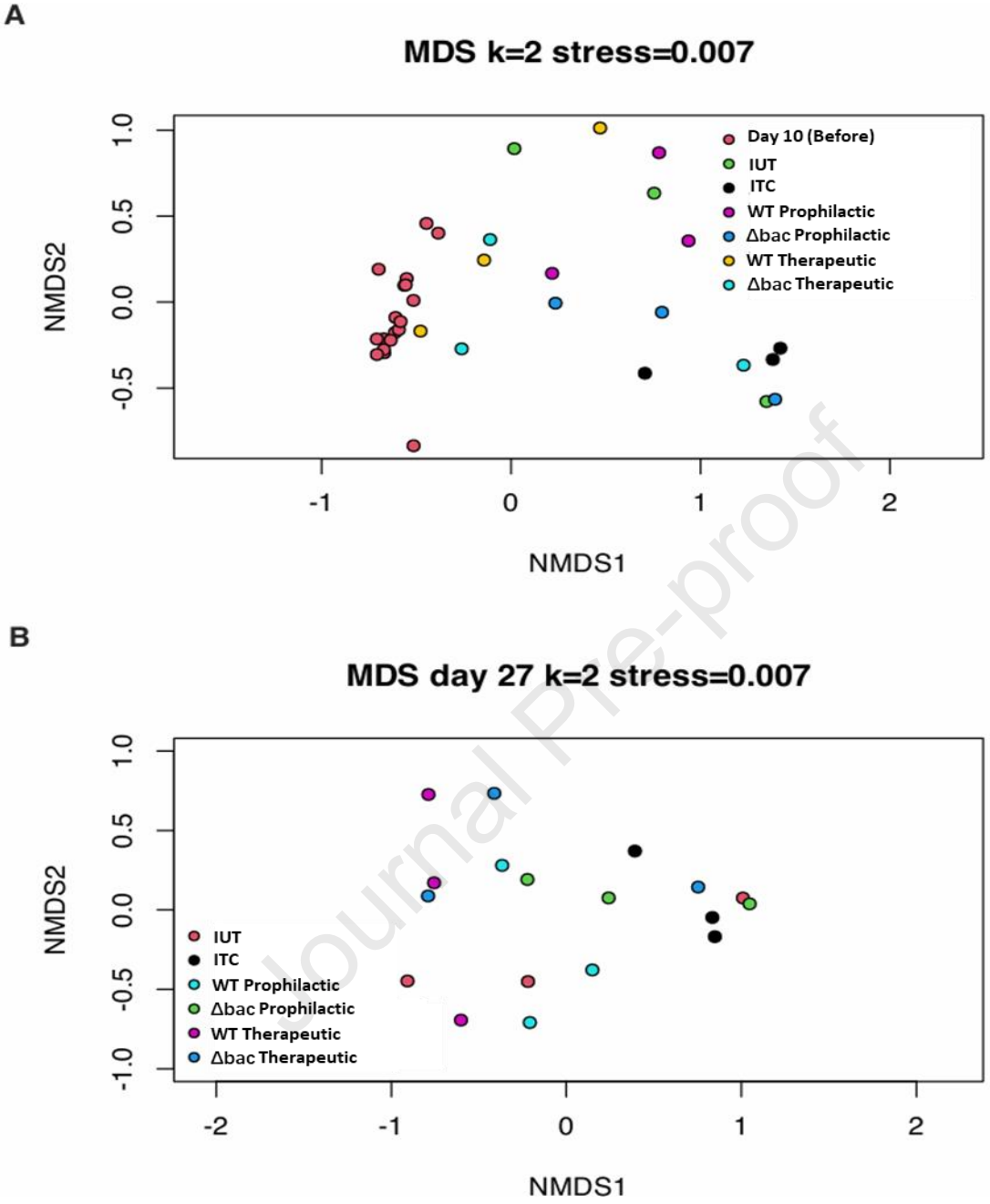

Fig. 5. Beta diversity of microbiota profiles illustrated with Bray-Curtis dissimilarity based non-metric dimensional model for (**A**) D10 feces samples (before) (NMDS model with 2 dimensions, stress = 0.007) and D27 small intestinal samples post treatment, and (**B**) D27 small intestinal samples post treatment only (NMDS model with 2 dimensions, stress= 0.007). Beta diversity clustering of samples has been assessed with ADONIS2 permutational test.

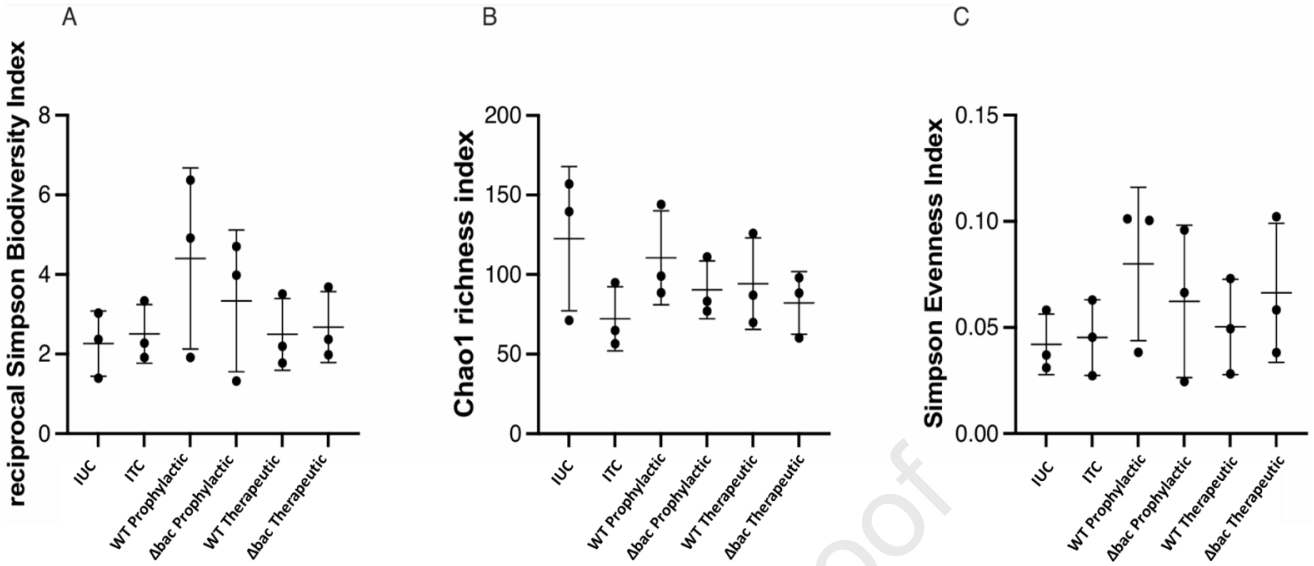

Fig. 6. Ecological indices for the different treatment groups illustrated with dot plots. **A**. alpha diversity measured with the reciprocal Simpson index. **B**. Population richness measured with Chao1 index. **C.** Simpson derived evenness index. For each group, mean values and standard deviation are illustrated with black lines. Differences between treatment has been assessed with ANOVA followed by Tukey post-hoc tests (corrected *P* value cutoff = 0.05).