Lacticaseibacillus casei ATCC 393

Subjects: Agriculture, Dairy & Animal Science

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Lactic acid bacteria (LAB) are commonly applied to fish as a means of growth promotion and disease prevention. However, evidence regarding whether LAB colonize the gastrointestinal (GI) tract of fish remains sparse and controversial.

Lacticaseibacillus casei colonization crucian carp gastrointestinal tract

1. Introduction

Given the restrictions and prohibitions regarding the use of chemicals and antibiotics, there is an increasing demand for safe, cost-effective, and environmentally friendly feed supplements that possess exceptional benefits for farmed fish such as phytogenics, prebiotics and probiotics [1]. One of therapeutic benefits of probiotics are that they can colonize or temporally colonize gastrointestinal (GI) tract and thereby modulate the intestinal microbiota via competitive adherence and exclusion, resulting in the production of beneficial substances for the host [2][3]. Colonization is one of the most important characteristics when evaluating the application of probiotics in animal rearing. LAB are one of the most widely used and studied bacteria in aquaculture, but their colonization in the intestinal tract of fish remains highly debated. Tian et al. [4] stated that *Lacticaseibacillus casei* CC16 can colonize the intestines of common carp. Other papers have reported that *Pediococcus acidilactici* (Bactocell®, Lallemand Inc., Montreal, QC, Canada) [5], *Bacillus paralicheniformis* FA6 [6], *Lactiplantibacillus plantarum* G1 [7], *Lacticaseibacillus casei* ATCC 393 [8], *Latilactobacillus sakei* CLFP 202 [9], *Lactococcus lactis* CLFP 100 [9] and *Leuconostoc mesenteroides* CLFP 196 [9] can also colonize the GI tract of goldfish, grass carp, shabout fish and rainbow trout. However, some papers have shown that probiotic strains, including *Lactobacillus*, in the GI tract rapidly decreases following the withdrawal of supplementation [10][11][12][13][14][15][16], indicating their transient nature. Meanwhile, Ringø et al. [17] raised the following question: “Are probiotics permanently colonizing the GI tract?”.

Colonization was defined by Conway and Cohen as the indefinite persistence of a particular bacterial population without the reintroduction of that bacterium [18]. Most bacterial cells are transiently present in the GI tract of aquatic animals, with the continuous intrusion of microbes from water and food [19]. Commercial feed or homemade feed are usually unsterile except for specific pathogen free (SPF) or gnotobiotic animals [20]. Considering the widespread existence of lactic acid bacteria (LAB) and *Bacillus*, it is rational to speculate on their existence in aquafeed. The transient microbes in the GI tract enter water with feces and can then be reintroduced to that same GI tract. However, in probiotic colonization-related studies, little attention has been paid to the influence of microbes originating from feed and water, resulting in a conclusion that ignores the prerequisite for colonization,

i.e., that it occurs “without the reintroduction of that bacterium”. In addition, the monitoring time for the persistence of probiotic microbes in the GI tract has often been insufficient, and there has been an absence of transit markers for evaluating the clearance time for transient microbes [21].

Colonization is a very important characteristic for screening additive strains and studying the mechanisms of probiotic action, but is associated with several significant challenges. First, the target bacteria being found in the water and diet can interfere with the colonization study. Second, lacking suitable methods for colonization study, some molecular methods such as 16S rRNA amplicon technology based on DNA samples cannot tell whether the bacteria are alive or dead. Third, once the probiotic supplementation has ceased, the proportion of the target strain may remain at a very low level [22], requiring a detection method with higher sensitivity for viable cells.

L. casei (Lc) is one of the species commonly used in aquaculture [4][17] and has shown some beneficial properties when applied to fish [23][24]. However, whether bacteria colonize the GI tract of fish has been unclear.

2. Effect of 100% Water Renewal on Interfering Bacteria

During the baseline period, no cultivable Lc or thermophiles were detected in the rearing water (<1 cfu/mL). During the administration period, 0–9 × 10^2 cfu/mL of Lc and 0.1–8 × 10^3 cfu/mL of Gs were detected in the rearing water. No Lc was detected following the cessation of bacterial supplementation and 100% water renewal up to the end of the experiments. Several Gs colonies were occasionally detected in the first week, whereas no Gs were detected after the second water renewal during the post-administration period.

3. Effect of Sterilizing the Feed with ^{60}Co Irradiation

The bacterial content of the commercial aquafeed is shown in **Table A2**. There were general heterotrophic bacteria at 10^4–10^6 cfu/g of the commercial diet, LAB at 10^2–10^4 cfu/g and thermophiles at 10^2–10^4 cfu/g. Using 16S rRNA gene sequencing identification, it was found that the general heterotrophic bacteria were mainly species of the genera *Bacillus* (including *Bacillus licheniformis* and *Bacillus subtilis*), and others include *Enterobacter*, *Parabacillus*, *Pantoea,* etc. The LAB were *Pediococcus*, *Enterococcus* and *Bacillus coagulans*. The thermophiles included mainly *Geobacillus*, *Parageobacillus*, and *Bacillus*. None of these bacteria were detected after ^{60}Co irradiation sterilization.

4. Selective Culture for LAB and Gs

The pH of MRS medium was adjusted to 5.4–5.5 for the selective culture of Lc. The MRS agar with a pH of 5.4–5.5 had high specificity for Lc growth, except for the occasional presence of some fungi and motile bacteria that failed to subculture in the rearing water and the gut at very low doses. There was no significant difference between the regular MRS and the 10% GI tract homogenate MRS (pH 5.4–5.5) (**Figure 1**). In other words, the improved MRS

agar had a high specificity and sensitivity and was, thus, able to detect the LAB strains used in our study of the GI tract homogenate.

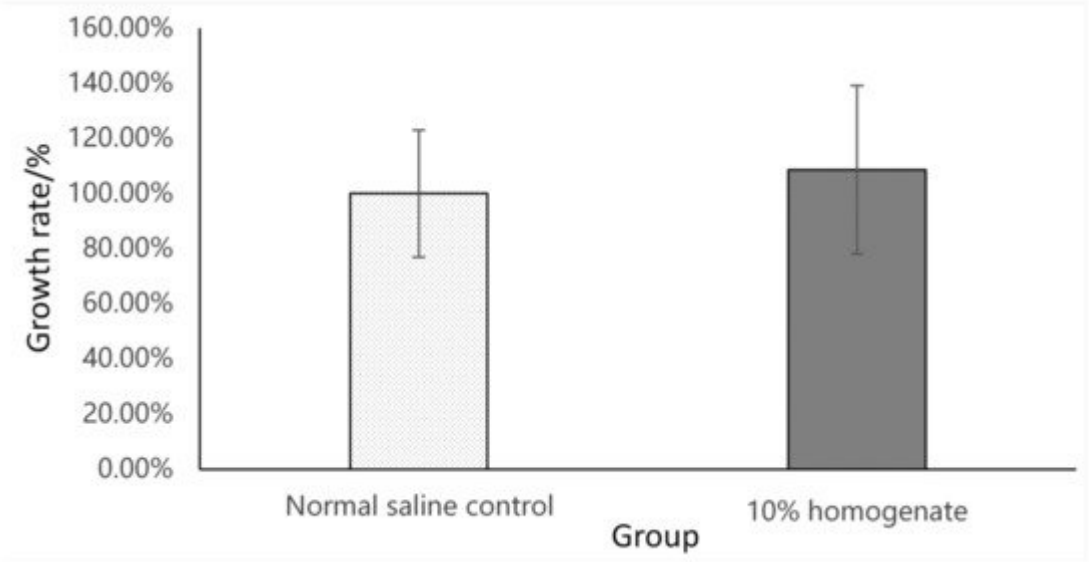

Figure 1. Comparison between the growth rate of *L. casei* in the normal saline control and 10% GI tract homogenate (n = 9) on the MRS plate.

The growth rate of Gs at 57 °C was 83.78% ± 26.80% (**Figure 2**) when suspended in the 10% GI tract homogenate, which was slightly lower than that of the normal saline control. However, there were no significant differences between the two groups (p > 0.05).

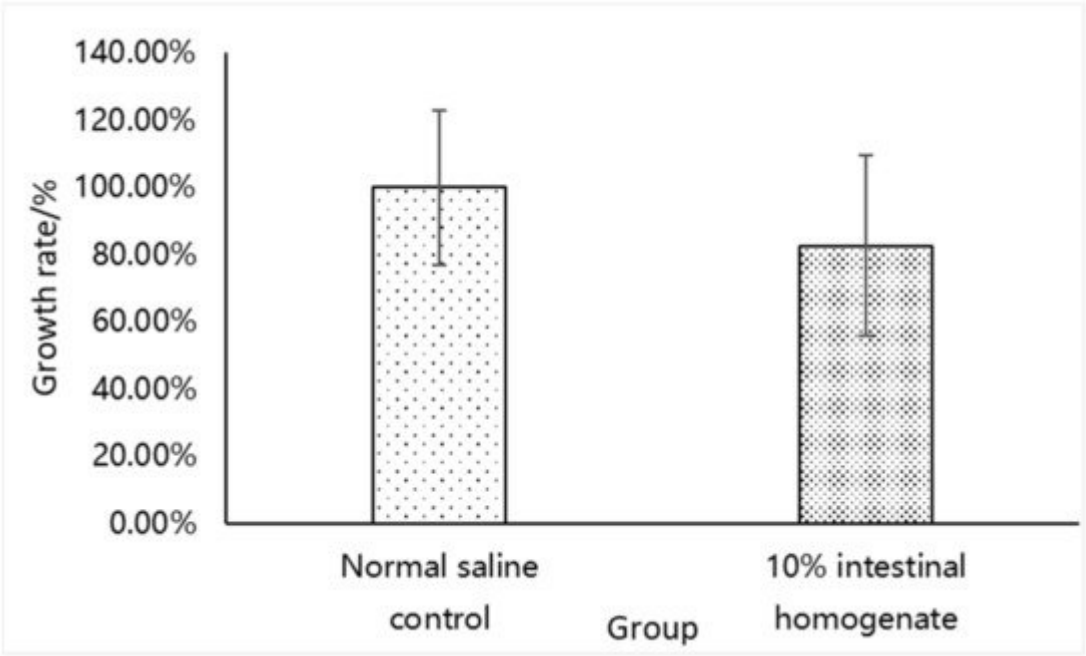

Figure 2. Comparison between the growth rate of Gs in the normal saline control and 10% GI tract homogenate (n = 9) on the NA plate.

5. The Concentration of Lc Changes in the GI Tract of Crucian Carp

The concentration of Lc and Gs in the GI tract decreased dramatically after the cessation of both bacteria supplements (**Figure 3**). In the first 3 days, the Lc concentration decreased from 2.6×10^5 (5.43log) to 20.67 (1.32log) cfu/gastrointestine, and Lc could not be detected in the GI tracts of two out of nine fish. Seven days after the cessation of the mixed diet, Lc could not be detected in any of the sampled fish (< 2 cfu/gastrointestine), although Gs was remained detectable up to day 11 (7/9). As can be seen from **Figure 3**, Lc was eliminated from crucian carp gastrointestine faster than Gs.

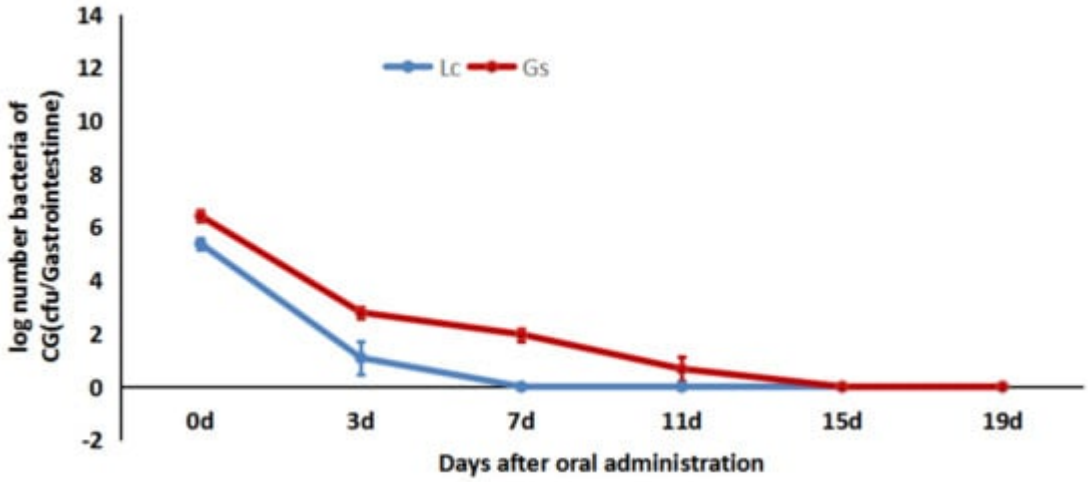

Figure 3. Kinetics of Lc and Gs elimination in the GI tract of crucian carp (*n* = 9).

6. Relative Abundance Changes of Lc and Gs in the Crucian Carp Gastrointestine

Gastrointestinal content samples, collected at five time points during the three periods (from day −7 to day 21), were analyzed using a 16S RNA gene sequencing technique, and the results are shown in **Figure 4**. Lc was detected at very low abundance in the gastrointestine before the administration of the mixed diet (Day–7). It is not surprising that Lc became the major taxon in terms of abundance (36.75% ± 3.59%) after the administration of the mixed diet (day 0), whereas 7 days after the cessation of the mixed diet, the relative abundance of Lc decreased to 0.11% ± 0.03%. Fourteen days later, the relative abundance of Lc decreased to a very low level again, even lower than that of the control group (**Figure 4** and **Figure 5**).

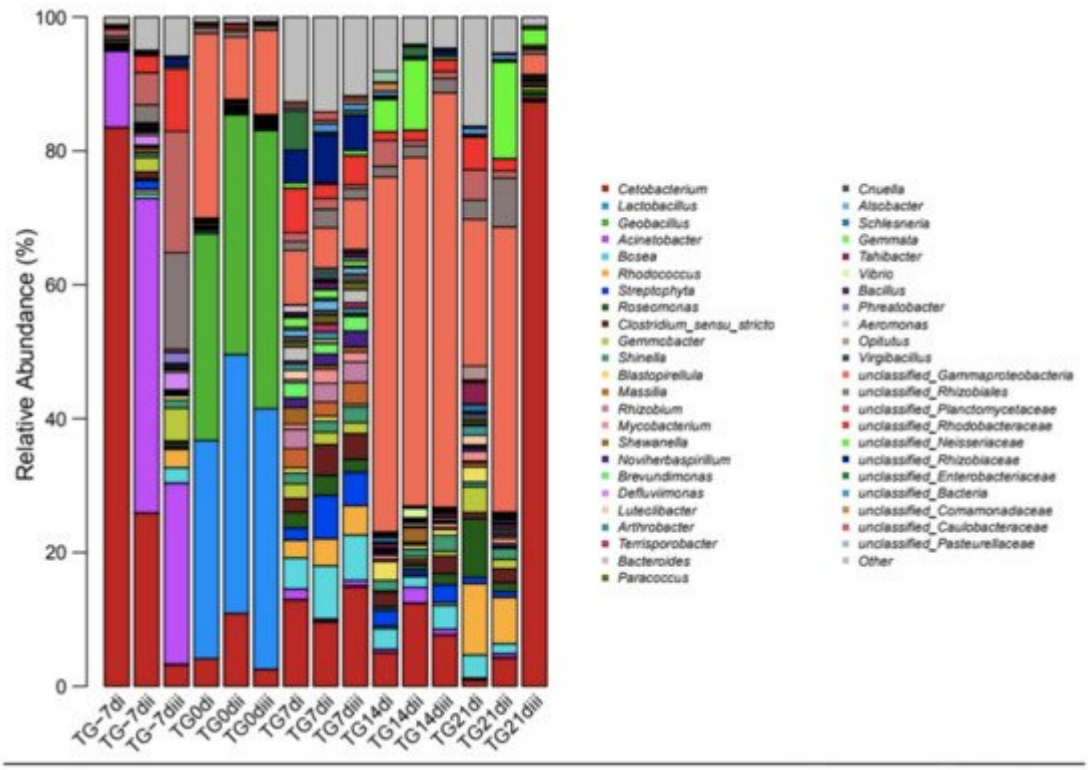

Figure 4. Bar plot illustrating the relative higher abundance bacterial genera for the individual fish. TG: treatment group: −7, 0, 7, 14 and 21 d represent the sample time points; i, ii, and iii represent individual triplicates within a group.

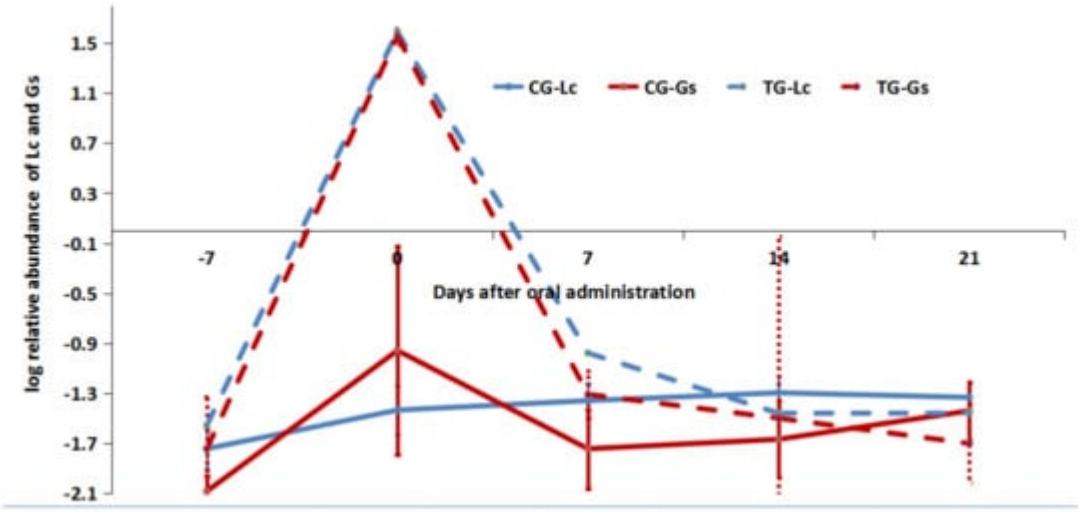

Figure 5. The changes in relative abundance of Lc and Gs in the CG and TG from day −7 to 21.

The relative abundance of Gs had the same trend as that of Lc (see **Figure 4** and **Figure 5**). At day 0, the relative abundance of Gs was 36.12% ± 5.31%, which was similar to that of Lc (**Figure 5**), but the number of viable Gs was eight times that of Lc (**Figure 6**). At day 7, although the relative abundance of Lc was 0.11% ± 0.03%, which was higher than other time points (except day 0), there was no viable Lc in the GI tract. We speculate that inactive Lc have reentered the GI tract because of the first incomplete replacement of the rearing water, and the same issue

might also exist with the Gs. Viable Gs was detectable up to day 7, which is consistent with the results in Experiment 1. Regarding the control group, the relative Lc and Gs abundance remained at a very low level during the whole experiment, and no viable Lc and Gs were detected.

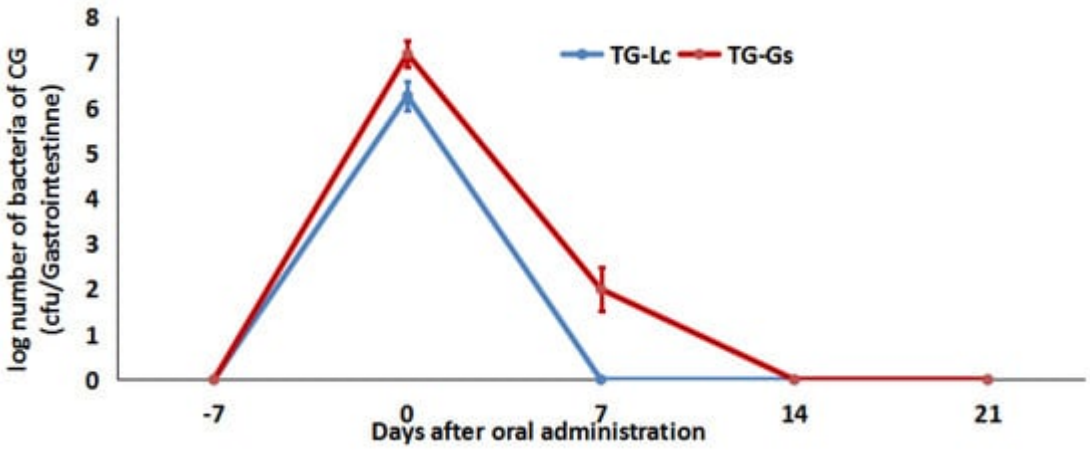

Figure 6. The changes in viable Lc and Gs bacteria in the TG from day −7 to 21.

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