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NOTE

## The effects of administering lactic acid bacteria sealed in a capsule on the intestinal bacterial flora of cattle

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### Abstract

We examined the effects of encapsulated lactic acid bacteria administrated orally to lactating cattle on the intestinal flora. A dose of  $3 \times 10^{11}$  colony forming unit (cfu) of freeze-dried *Lactobacillus coryniformis* subsp. *torquens* (JCM1099) encapsulated in an enteric capsule capable of bypassing the rumen was administered for seven days. DNA was extracted from feces 0 and 24 hr after daily administration. Metagenomic analysis showed an increasing trend of the alpha diversity, an index of the species diversity. Furthermore, principal component analysis of intestinal flora revealed that cattle could be differentiated by JCM1099 capsule and suspension administration via principal components 1, 2, and 3. We conclude that administration of encapsulated JCM1099 can alter the intestinal bacterial flora of cattle.

Key Words: dairy cattle, enteric capsule, intestinal flora

Several reports have suggested that lactic acid bacteria (LAB) may provide beneficial microorganisms that improve the composition of

the intestinal bacterial flora, which is essential for maintaining human health<sup>3,20,22</sup>. Recently, LAB have been shown to increase the rate of

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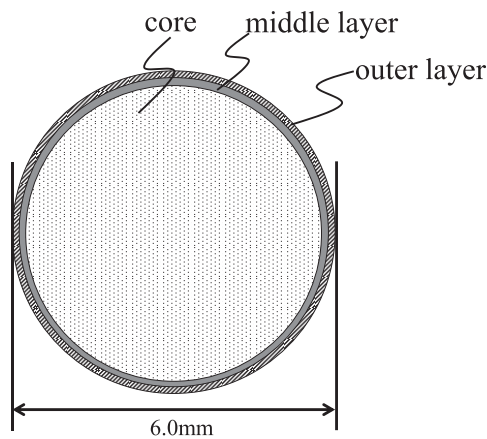
body weight and immunity in farm animals such as hogs, chickens and even fish<sup>15,23,24</sup>). In addition, studies using newborn calves with undeveloped rumen reported that oral administration of LAB as probiotics improved body weight gain and fecal condition<sup>2,17</sup>). On the other hand, in adult cattle, probiotics have been selected to target the rumen instead of gut to improve fiber digestion<sup>5,6,9</sup>). Moreover, Ghorbani *et al.*<sup>10</sup>) reported that direct feeding of microbials including LAB did not affect mean counts of *Lactobacillus* bacteria in feces. This property suggests that the biggest challenge in the use of LAB as probiotics for adult cattle is bacterial death due to a small number of LAB escaping the massive rumen. To overcome this problem, we proposed encapsulating LAB in enteric capsules that have a diameter of 6 mm and specific gravity set at 1.3<sup>21</sup>). Enteric capsules do not dissolve in the neutral to weak acid environment of the rumen and avoids rumination by passing through the rumen quickly, hence permitting the release of their content in the lower gastrointestinal tract<sup>1</sup>). Using these enteric capsules a high concentration of viable, potential probiotics could be directly delivered to the intestine of cattle, which could beneficially alter the intestinal bacterial flora of cattle.

Metagenomic studies of the intestinal flora of humans and livestock were recently conducted using a next-generation sequencer<sup>11,19</sup>). This culture-independent technique can provide a detailed snapshot of bacterial components of the intestinal microbiome found in feces at the time of sampling, but changes over time in the composition of the intestinal flora can be clarified only if fecal samples are immediately collected post-administration at different time points.

In this study, we aimed to examine the effect of administering LAB-containing enteric capsules on the intestinal bacterial flora of cattle. We used a metagenomic analysis to demonstrate that changes in the composition of the intestinal bacterial flora by encapsulated LAB could be estimated.

*Lactobacillus coryniformis* subsp. *torquens*

(JCM1099) isolated from cattle manure was provided by the RIKEN BioResource Centre through the National Bio Resource Project of the Ministry of Education, Culture, Sports, Science and Technology, Japan. JCM1099 was incubated in de Man, Rogosa and Sharpe (MRS; Becton, Dickinson and Company, New Jersey, USA) media at 30°C for 48 hr. Upon reaching the plateau phase, the centrifuged bacterium was rinsed and suspended in phosphate buffered saline (PBS) with 0.5% w/v glycine as protective material for frost damage. Live JCM1099 was freeze-dried (TFD-4-8, TAKARA SEISAKUSHO Co. Ltd., Tokyo, Japan), mixed with starch, and sealed in the capsules as the content. Three-layered, structured enteric capsules with a diameter of 6 mm, specific gravity of 1.3 and approximately 160 mg per capsule were manufactured by Morishita Jintan Co. Ltd. (Osaka, Japan)<sup>4,13</sup>) (Fig. 1). Viable bacterial concentration in the capsules was  $5.1 \times 10^9$  cfu/g-capsule. All animal procedures were conducted according to the guidelines of the animal experimentation department committee of the locally incorporated administrative agency Research Institute of Environment, Agriculture and Fisheries, Osaka Prefecture. Four Holstein cows at  $295 \pm 29$  days in late lactation,  $68 \pm 12$  months old, with an average body weight of  $670.3 \pm 52.9$  kg, lactation numbers of  $2.8 \pm 0.9$  and not pregnant were used for the experiment. They were fed hay and concentrate separately in four installments at 06.30, 08.30, 10.30, and 14.30 hours daily (Table 1). Cows had been given the diet for one month prior to the examination. Water was given ad libitum. Milking was performed twice a day (07.00 and 16.30 hours). Cows 3 and 4 (treatment group) were administered 59 g/head (approximately 370 capsules) of capsules contained freeze-dried JCM1099 with a total viable concentration of  $3 \times 10^{11}$  cfu. The capsules were orally administered in 500 ml of water using a plastic bottle. Cows 1 and 2 (control group) were administered freeze-dried JCM1099 with a concentration of  $3 \times 10^{11}$  cfu suspended in 100 ml of PBS together with



**Fig. 1. Schematic diagram of the three-layer enteric capsule.** Core: a mixture of hydrogenated palm oil and freeze-dried lactic acid bacteria, including titanium dioxide as a density-adjusting agent. Middle layer: composed of hydrogenated oils and titanium dioxide. Outer layer: consists of starch, carrageenan, and so on. Diameter is 6 mm and specific gravity is 1.3.

59 g (approximately 370 capsules) of capsules as placebo, which contained starch instead of LAB. The administration of JCM1099 to cows was carried out daily at the same time for seven days. Feces were routinely collected from the rectum of cows immediately before and 24 hr after daily JCM1099 administration, and 14 days after the first administration to compare with the intestinal flora before and during the administration. Approximately 500 g of feces were transferred to a sterile container and mixed to obtain a homogenous sample, of which 250 mg were used for DNA extraction. A metagenomic analysis using a next-generation sequencer was carried out to estimate the proportion of lactobacilli and changes in the intestinal bacterial flora. DNA was extracted from 250 mg of feces using a Power soil DNA Extraction kit (Mo Bio Laboratories, Inc., California, USA). The V3–V4 region of 16S rDNA was amplified by PCR using a 16S Amplicon PCR Primer set (Forward: 5′- TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG CCT ACG GGN GGC WGC AG-3′ and Reverse: 5′- GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA GGA CTA CHV GGG TAT CTA ATC C-3′)<sup>14)</sup>, which had an Illumina-specific adapter (Illumina, Inc., California, USA). Amplicon PCR

**Table 1. Dietary ingredients and chemical composition**

|                                      | % Dry matter |
|--------------------------------------|--------------|
| Ingredients                          |              |
| Timothy hay                          | 25.0         |
| Oat hay                              | 10.0         |
| Alfalfa hay                          | 10.0         |
| Beet pulp                            | 10.0         |
| Commercial concentrate <sup>a)</sup> | 45.0         |
| Chemical composition <sup>b)</sup>   |              |
| Crude protein                        | 14.8         |
| Estimated TDN                        | 72.7         |

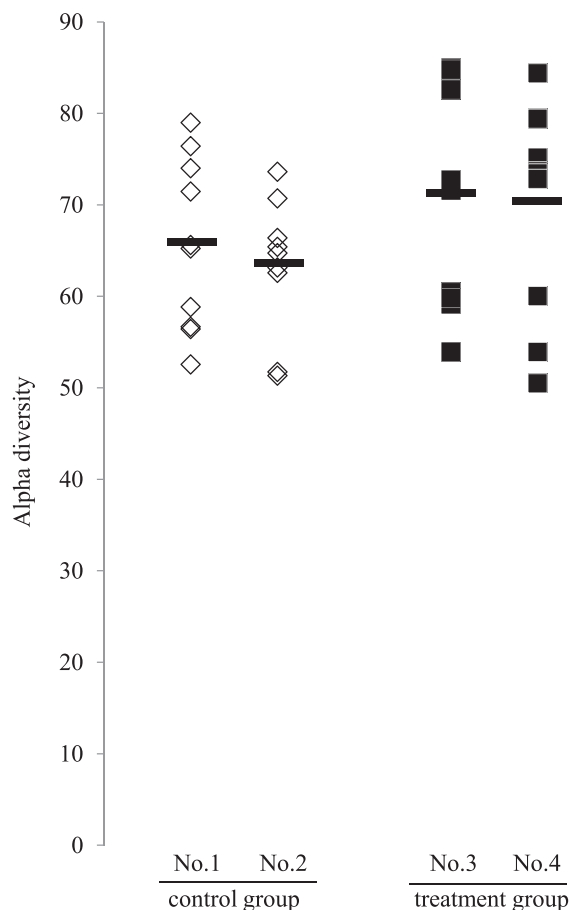
<sup>a</sup>Commercial concentrate containing the following ingredients: heated corn grain, wheat flour, toasted soybean flour, defatted soymeal, rapeseed oil cake, sesame-seed oil cake, corn gluten, wheat bran, sugar beet molasses, calcium carbonate, dehydrated alfalfa meal, calcium phosphate, sodium chloride, and supplement (vitamin A, vitamin D3, vitamin E, ferric sulfate, copper sulfate, zinc sulfate, manganese sulfate, cobalt sulfate, magnesium carbonate, calcium iodate, magnesium oxide and methionine compound)  
<sup>b</sup>TDN, total digestible nutrients. Based on NARO<sup>18)</sup>.

and index PCR were carried out according to a protocol of the 16S metagenomic sequencing library preparation (Illumina, Inc). Amplicon libraries with a different multiplex identification tag sequence were mixed and subjected to high-throughput sequencing using an Illumina MiSeq system (Illumina, Inc.). Obtained FASTQ data were merged into properly paired alignments and trimmed to 150 bp or less with CLC Genomics Workbench (CLC bio Japan, Inc., Tokyo, Japan). A comparative analysis of each sample was conducted using the MG-RAST server (<https://metagenomics.anl.gov/>). Taxonomical attribution of the data was estimated using the ribosomal database project (RDP) ([http://rdp.cme.msu.edu/seqmatch/seqmatch\\_intro.jsp](http://rdp.cme.msu.edu/seqmatch/seqmatch_intro.jsp)). Nucleotide sequence data reported are available in the DDBJ databases under the accession number DRA004106. The alpha ( $\alpha$ ) diversity, an index of the species diversity, was calculated using the MG-RAST server. Statistical analysis of data from each animal was carried out using R statistical software (version 3.1.0; [www.r-project.org](http://www.r-project.org)). Principal component analysis plots were created using the MG-RAST server.

After dismissing outlying values based on the Smirnov-Grubbs's outlier test ( $\alpha = 0.05$ ), the mean values of  $\alpha$  diversity in the groups were compared (Fig. 2). The alpha diversity is one of the indexes of species diversity, and represents species diversity in a given single community. The analysis showed an increasing trend of  $\alpha$  diversity in fecal samples from cattle administered with JCM1099 capsules ( $70.8 \pm 2.6$ ) compared to control ( $64.5 \pm 1.9$ ,  $P = 0.0606$ ). It can be deduced that administering enteric capsules containing JCM1099 tended to increase the number of bacteria species in cattle intestine and diversified the intestinal bacterial flora. This trend was in agreement with that found by a previous study reporting an increase in the  $\alpha$  diversity of the intestinal flora of humans caused by probiotics<sup>7)</sup>.

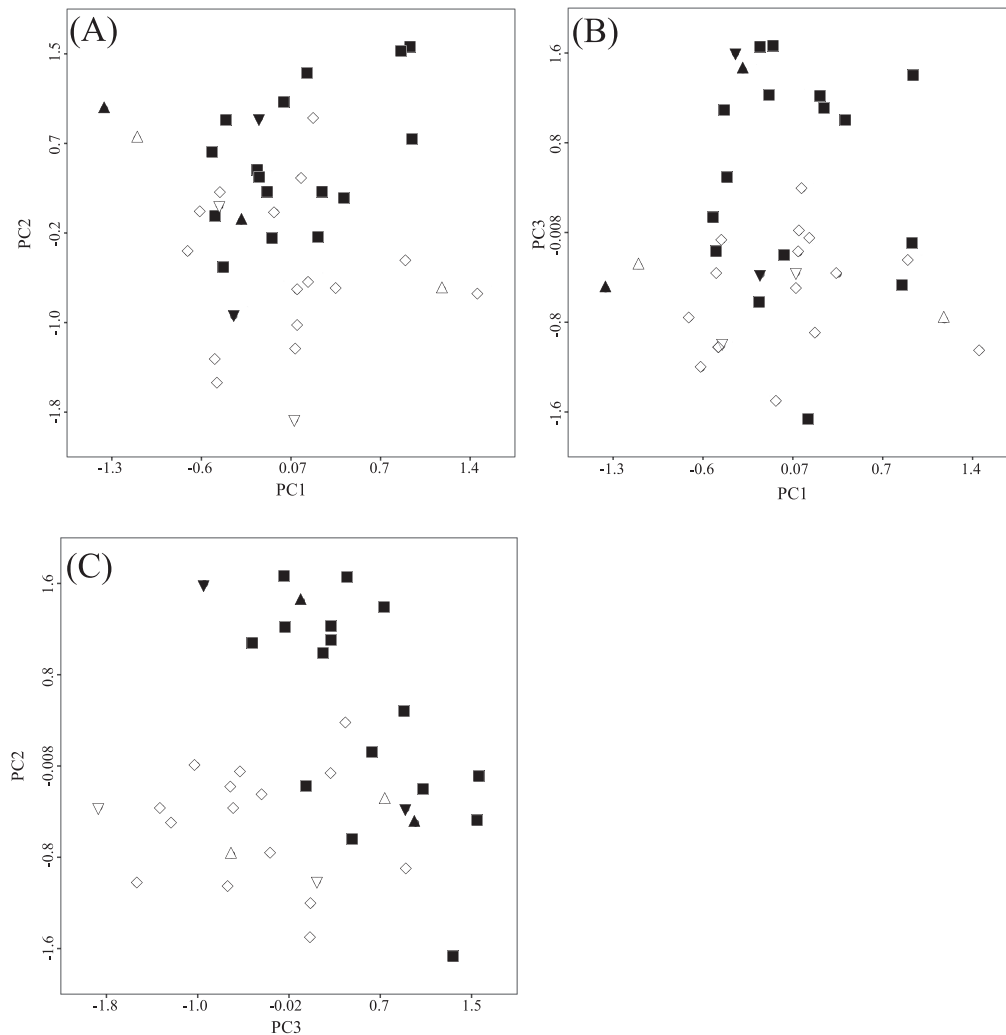
The results of principal component analysis were shown in Fig. 3. The results showed a wide distribution in the plots of principal component 1 (PC1) and principal component 2 (PC2) of fecal samples collected prior to capsule administration (shown as  $\nabla$  of control group and  $\blacktriangledown$  of treatment group), during the administration of JCM1099 suspension (for seven days) (shown as  $\diamond$ ), and 14 days after the first capsule administration (shown as  $\triangle$  of control group and  $\blacktriangle$  of treatment group) (Fig. 3A). In contrast, the data of the treatment group during the administration of encapsulated JCM1099 (shown as  $\blacksquare$ ) were plotted intensively in the upper half of graphic area being differentiated from the other spreading plots of control group (Fig. 3A). Furthermore, individual relationships between PC1 and principal component 3 (PC3), and PC2 and PC3 were differentiated the plots of treatment group from those of others (Fig. 3B and 3C). The fact that samples from JCM1099 capsule-administered cattle can be differentiated from other samples in principal component analysis suggested that the administration of encapsulated JCM1099 caused the intestinal bacterial flora of cattle to retain a certain composition.

In addition to direct interactions between bacteria, the compositional ratio of intestinal



**Fig. 2. The  $\alpha$  diversity in fecal samples of cattle.** The alpha ( $\alpha$ ) diversity was calculated using the MG-RAST server, and the Smirnov-Grubbs's outlier test ( $\alpha = 0.05$ ) was used before calculating the means. Mean values are represented by the horizontal lines.  $\diamond$  cattle administered *Lactobacillus coryniformis* subsp. *torquens* (JCM1099) suspension and empty enteric capsules as placebo (control group);  $\blacksquare$  cattle administered encapsulated JCM1099 (treatment group).

flora is known to vary due to changes in influx of the digestive tract content and interactions with the host<sup>16)</sup>. Since the bacterial composition changes have been reported to have medium- or long-term effects, the persistence of probiotics requires a minimal duration of administration<sup>12,25)</sup>. On the other hand, David *et al.*<sup>8)</sup> reported high responsive change of intestinal flora, such as bacterial phase alteration caused by a single day of diet change. In this study, it can be speculated that administration of JCM1099 had a short-term effect of changes on intestinal bacterial flora of cattle due to introducing LAB, which is consistent



**Fig. 3. Principal component analysis of data obtained from cattle fecal samples collected prior to, during and after the administration of *Lactobacillus coryniformis* subsp. *torquens*.** Each plot was created using the MG-RAST server.  $\nabla$  and  $\blacktriangledown$  indicate samples collected from cows 1-2 (control group) and 3-4 (treatment group), respectively, prior to the start of the experiment.  $\triangle$  and  $\blacktriangle$  indicate samples collected from cows 1-2 (control group) and 3-4 (treatment group), respectively, 14 days after the first capsule administration.  $\diamond$  indicates samples collected during the administration of *Lactobacillus coryniformis* subsp. *torquens* (JCM1099) suspension and empty capsules as placebo, and  $\blacksquare$  indicates samples collected during the administration of encapsulated JCM1099.

with their results of David *et al.*<sup>8)</sup>

In conclusion, the administration of encapsulated JCM1099 resulted in an increase in  $\alpha$  diversity of intestinal flora. Principal component analysis showed a tendency of JCM1099 capsule-administered cattle to be differentiated from JCM1099 suspension-administered cattle. It can be concluded that LAB administered via capsules survive in cattle with fully developed rumen and are released in the lower gastrointestinal tract, which can alter the intestinal bacterial flora. In

addition, our work suggests that enteric capsules have possibility to be used as probiotics, which are helpful in improvement of productivity and prevention of zoonosis. Our enteric capsules could be an effective tool to realize successful delivery of viable microorganism to intestine, which has long been difficult to accomplish. Further study on the effect of delivered substance on microorganism phases, using a larger number of cattle is needed to confirm this possibility.



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