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Development of rumen escapable capsules for cattle

(ウシ用ルーメンバイパスカプセルの開発)

Tomohiro SEYAMA

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Abbreviations

16S	16 small subunit
bp	Base pairs
cfu	Colony forming unit
CP	Crude protein
DDBJ	DNA Data Bank of Japan
DM	Dry matter
DNA	Deoxyribonucleic acid
HDPE	High-density polyethylene
HPLC	High-performance liquid chromatography
LAB	Lactic acid bacterium
MG-RAST	Metagenomics-RAST
N	Newton (= kg m/s ²)
NARO	National Agriculture and Food Research Organization
No.	Number
PBS	Phosphate-buffered saline
PC	Principal component
PCA	Principal component analysis
PCR	Polymerase chain reaction
PMMA	Poly-methyl methacrylate
POM	Polyoxymethylene

PTFE	Polytetrafluoroethylene
rDNA	Ribosomal deoxyribonucleic acid
RDP	Ribosomal data project
S.D.	Standard deviations
TDN	Total digestible nutrient
VB1	vitamin B1 (thiamin)
VB2	vitamin B2 (riboflavin)
VB3	vitamin B3(niacin)

Preface

Milk production of dairy cows has increased due to advances in the genetic improvement. Since it is necessary to balance nutrient intake to meet this increased milk production, a greater burden is placed on the body, including the metabolization of lipids and carbohydrates in the liver. Consequently, metabolic disorders have become more frequent and are now a significant problem for dairy management [26, 41].

To prevent or treat metabolic diseases, orally administration of high energy substances or curative medicines is effective in case monogastric animals. However, cows are polygastric, and of their four stomachs, the first stomach (the rumen) contains 1–100 billion microorganisms per gram. Consequently, even when high energy substances or curative medicines are administered orally, they are diluted in the massive rumen and/or broken down by microorganisms. Thus, intravenous or subcutaneous injections, or a rumen escapable feed should be essential when administering these functional ingredients to cows.

Techniques for delivering high energy substances to improve energy balance, directly to the lower gastrointestinal tract without undergoing degradation in the rumen have been developed (hereinafter referred to as “rumen bypass techniques”). Previous attempts at the establishment of such techniques include a method of kneading amino acids into hydrogenated fat, which is not degraded in the rumen, and a protein denaturation treatment by heating soybean meal [6, 36]. However, these methods can be used to deliver only specific

substances such as amino acids or proteins, and currently, there is no rumen bypass technique that can be used to deliver liquids, microbial materials or curative medicines.

Therefore, in the present study, a new rumen escapable tool for cattle was developed, consisting of a three-layered capsule that can protect the contents from degradation by microorganisms and dilution in the massive rumen (Figure 1). The capsule is manufactured at room temperature so that hydrophilic or heat-sensitive substances can be contained without suffering any harmful heat effect.

These capsules need to avoid physical breakup caused by rumination, prevent chemical and/or microbial degradation in the rumen. Moreover, they also need to reach the lower gastrointestinal tract and then dissolve quickly.

The present thesis consists of three chapters. In chapter I, plastic balls were used to investigate how the specific gravity and diameter of an orally administered spherical material affect the damage on the surface of balls by rumination and delivery to the lower gastrointestinal tract in cows. In chapter II, manufactured capsules contained thiamine hydrochloride was administered to lactating cows to verify that the capsules had escaped the rumen degradation and reached the lower gastrointestinal tract. In chapter III, to examine the effect of administering lactic acid bacterium contained in the enteric capsules, a metagenomic analysis was used to estimate changes in the composition of the intestinal bacterial flora.

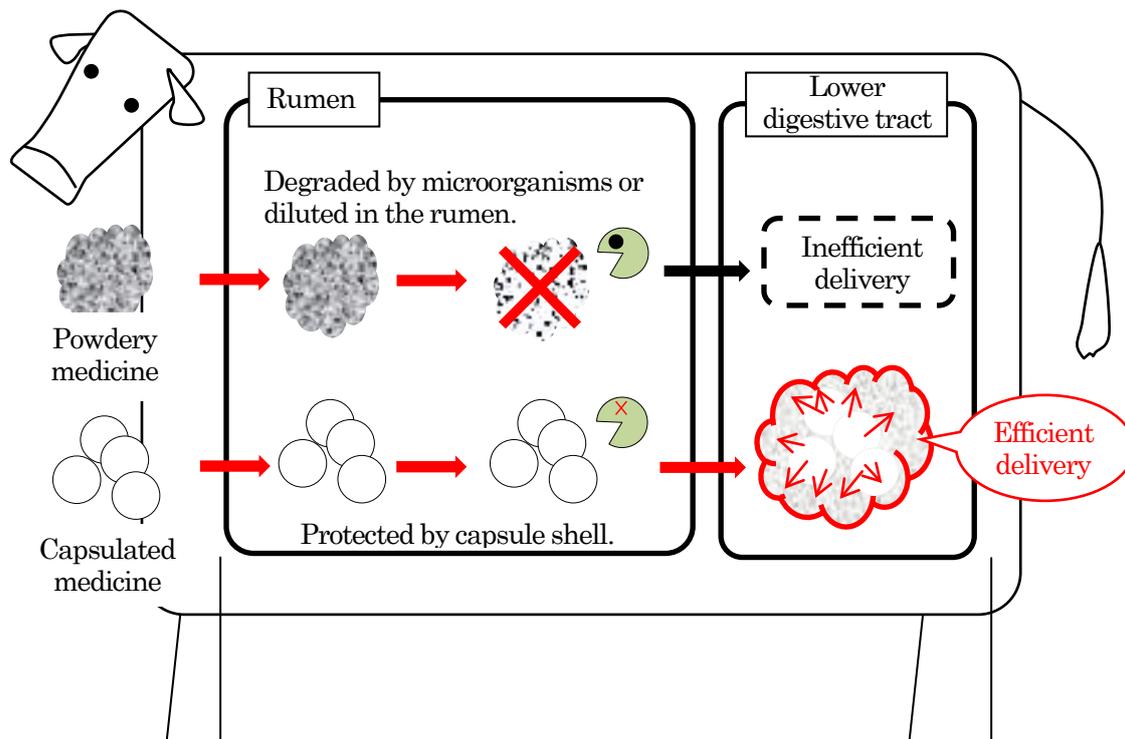


Fig 1. Outline of rumen escapable capsules for cattle used in this study.

Capsules can protect the contents from degradation and dilution in the rumen.

Then, capsules reach the lower gastrointestinal tract and dissolve.

Chapter I

Excretion rates of indigestible plastic balls of different specific gravities and diameters in dairy cattle

Introduction

The use of capsules, which can accommodate diverse contents, would be suitable as a new bypass technique to deliver various materials. These capsules need to avoid physical breakup caused by rumination, prevent chemical and/or microbial degradation in the rumen, and quickly reach the lower gastrointestinal tract. It has been demonstrated that the specific gravity and fragment length of the feed materials are factors that define the rate of passage of ingested material through the gastrointestinal tract [5, 48]. In relation to specific gravity, desBordes and Welch [12] investigated the effect of specific gravity of ingested materials on rumination in Jersey cows. They used indigestible plastic sticks with specific gravity in the range of 0.90–2.15 and reported that the recovery rate of ruminated sticks in feces was highest for specific gravity 1.17. In point of the particle size, Poppi *et al.* [37] investigated how the size of the ingested feed materials affects the outflow in the transition to the reticulum and omasum, and they reported that the critical particle sizes were 1.18 mm and 3.2 mm, respectively [13, 38]. However, the optimal specific gravity or diameter for a spherical container that can quickly pass through the rumen has not yet been evaluated.

In chapter I, to develop capsules as a new rumen bypass technique,

plastic balls were used as models to investigate how the specific gravity and diameter of an orally administered spherical material affect the damage on the surface of balls by rumination and delivery to the lower gastrointestinal tract in cows.

Materials and Methods

Animals and diet

All animal procedures were performed according to the guidelines of the animal experimentation department committee of Local incorporated administrative agency Research Institute of Environment, Agriculture and Fisheries, Osaka Prefecture. Four Holstein cows in late lactation (mean number of days in milk: 330 ± 38 , mean body weight: 711 ± 34 kg, mean calving number: 2.8 ± 0.3) housed in tie stall were used. They were given fermented total mixed ration amounting to 4% on a dry matter basis of the body weight once daily at 14.00 hours (constituents of the ration are listed in Table 1). Water was provided *ad libitum*. The mean dry matter intake was 21.7 ± 1.0 kg. Milking was performed twice a day (07.00 and 16.30 hours).

Table 1. Formula and chemical composition of fermented total mixed ration.

Formula, % as DM basis	
Corn silage	30.0
Italian ryegrass silage	6.0
Alfalfa hay	4.0
Flaked brown rice	19.5
Citrus pulp	5.0
Dried tofu cake	5.0
Wheat bran	9.6
Soybean meal	8.0
Beet pulp	9.5
Soy hull	2.2
Calcium carbonate	0.2
Vitamin premix	0.9
Salt	0.2
Chemical composition	
Dry matter, %	46.2
Crude protein, % DM	14.4
Estimated TDN, % DM	75.3 ^a

a: Based on NARO [35].

DM, dry matter; TDN, total digestible nutrient.

Experimental procedure

Four types of plastic with different specific gravities (Sato Tekkou Co., Ltd, Osaka, Japan): high-density polyethylene (HDPE, specific gravity: 0.95), poly-methyl methacrylate (PMMA, specific gravity: 1.19), polyoxymethylene (POM, specific gravity: 1.41), and polytetrafluoroethylene (PTFE, specific gravity: 2.20) were selected. To determine the optimal size for the capsules, balls with diameters 3.97 mm, 6.35 mm, and 7.94 mm that were molded from the aforementioned types of plastic were used. For each cow, 100 balls of each type, a total of 1,200 balls, suspended in water were administered orally at once using a plastic bottle at 10.00 hours. After oral administration of the plastic balls, all feces for consecutive 120 hr were collected. The feces were picked up from the floor of the tie stall every 12 hr and stocked in containers prepared diurnally. Each container was managed individually in the subsequent processes. After dissolving in tap water, feces were filtered using a 0.5-mm mesh screen, and the solid matter was separated. The solid matter was air-dried and sieved over a 2-mm mesh screen to recover the plastic balls. The recovered plastic balls were identified according to the following procedure: The transparent balls were identified as PMMA, since others were white. The balls that floated on water were categorized as HDPE. The balls that went underwater (POM and PTFE) were weighed individually on an electrical balance after air-drying for 24 hr and were distinguished by their weight per ball. The sizes of the balls could be easily distinguished by appearances. The surface of each plastic ball was checked visually, and balls with bite mark or partial defect were determined to have undergone rumination. Although a few HDPE balls with

partial defect were seen, no defects were observed on the small plastic debris that passed the 0.5- or 2-mm screen filter. Partial defects were observed only on some HDPE balls but not on other kinds of plastic balls. The recovery rate of non-ruminated and ruminated balls was calculated using following equation.

$$rr(\%) = r/a \times 100$$

where rr is the recovery rate, r is the number of recovered non-ruminated or ruminated balls from feces and a is the number of administrated balls.

Results

The cumulative recovery rates of the plastic balls with 4 different specific gravities and 3 different diameters excreted in feces during 120 hr following a single oral administration are shown in Table 2. There was no significant interaction between specific gravity and diameter of the total cumulative recovery rate as shown by results of a two-way analysis of variance. Total recovery rates of the balls with specific gravity 1.19 and 1.41 were almost the same, and they both were significantly higher than that of the balls with specific gravity 0.95 or 2.20, regardless of the diameters. The ruminated rate of the balls with specific gravity 0.95 was significantly higher than that of others, regardless of the diameters. An effect of diameter on the recovery rate of the balls was observed in the group of specific gravity 1.19. Within the group of specific gravity 1.19, the total recovery rate was significantly lower for the balls with diameter 3.97 mm than that for others.

Table 3 shows the cumulative recovery rate over time of plastic balls with diameter 3.97 mm, 6.35 mm, or 7.94 mm. There was no significant difference in the 120 hr cumulative recovery rate for plastic balls with specific gravity 1.19 or 1.41, but cumulative rate of balls with specific gravity 1.19 of diameters 6.35 mm and 7.94 mm was significantly higher than those with specific gravity 1.41, at 24 and 48 hr after administration. In particular, the cumulative recovery rate of plastic balls with specific gravity 1.19 at 48 hr after administration was 80% or higher.

Furthermore, the proportion of indigestible balls with specific gravity 1.19 that were ruminated was 2% or lower, even after 120 hr of accumulation after administration, and the proportion of indigestible balls that reached the lower gastrointestinal tract without being ruminated was 90% and above.

Table 2. Cumulative recovery rate (%) of non-ruminated or ruminated plastic balls with four different specific gravities and three different diameters ingested by lactating Holstein at 120 hr after administration

Diameter	Ball type	Specific gravity			
		0.95	1.19	1.41	2.20
3.97 mm	Non-ruminated	26.8 ± 6.3 ^b	75.3 ± 1.7 ^a	77.3 ± 13.2 ^a	2.5 ± 2.5 ^b
	Ruminated	14.8 ± 3.3 ^a	1.3 ± 0.5 ^b	1.3 ± 0.8 ^b	1.0 ± 1.0 ^b
	Total	41.5 ± 8.2 ^b	76.5 ± 1.9 ^{ax}	78.5 ± 13.7 ^a	3.5 ± 3.5 ^c
6.35 mm	Non-ruminated	18.8 ± 2.1 ^b	92.0 ± 2.9 ^a	79.0 ± 9.1 ^a	3.5 ± 2.9 ^b
	Ruminated	22.3 ± 4.6 ^a	2.0 ± 0.7 ^b	1.3 ± 0.8 ^b	0.5 ± 0.5 ^b
	Total	41.0 ± 5.8 ^b	94.0 ± 2.3 ^{ay}	80.3 ± 9.5 ^a	4.0 ± 2.8 ^c
7.95 mm	Non-ruminated	11.3 ± 2.8 ^b	95.0 ± 3.2 ^a	79.0 ± 11.2 ^a	5.3 ± 3.7 ^b
	Ruminated	12.0 ± 2.5 ^a	1.8 ± 0.6 ^b	1.0 ± 0.6 ^b	1.0 ± 0.7 ^b
	Total	23.3 ± 5.3 ^b	96.8 ± 2.7 ^{ay}	80.0 ± 11.5 ^a	6.3 ± 4.4 ^b

^{abc}Means within rows having different superscripts differ significantly ($P < 0.05$).

^{xy}Means within columns having different superscripts differ significantly ($P < 0.05$).

Values are means ± standard error

Table 3. Daily cumulative recovery rate (%) of non-ruminated or ruminated plastic balls with specific gravity 1.19 and 1.41 and three different diameters ingested by lactating Holstein

Time after administration	Ball type	Diameter	3.97 mm		6.35 mm		7.94 mm	
		Specific gravity	1.19	1.41	1.19	1.41	1.19	1.41
24 h	Non-ruminated		9.0 ± 2.8 ^{ab}	1.5 ± 1.0 ^b	17.5 ± 3.0 ^a	2.0 ± 1.4 ^b	20.5 ± 5.8 ^a	3.0 ± 1.1 ^b
	Ruminated		ND	ND	0.5 ± 0.3	ND	0.5 ± 0.3	ND
	Total		9.0 ± 2.8 ^{ab}	1.5 ± 1.0 ^b	18.0 ± 2.7 ^a	2.0 ± 1.4 ^b	21.0 ± 5.7 ^a	3.0 ± 1.1 ^b
48 h	Non-ruminated		59.5 ± 4.6 ^{ab}	26.3 ± 9.9 ^b	80.8 ± 5.0 ^a	32.8 ± 9.9 ^b	82.0 ± 7.4 ^a	38.3 ± 14.7 ^b
	Ruminated		0.5 ± 0.5	ND	1.0 ± 0.6	ND	1.8 ± 0.6	0.5 ± 0.5
	Total		60.0 ± 4.7 ^{ab}	26.3 ± 9.9 ^b	81.8 ± 5.1 ^a	32.8 ± 9.9 ^b	83.8 ± 7.1 ^a	38.8 ± 15.1 ^b
72 h	Non-ruminated		71.5 ± 3.3	57.8 ± 14.5	90.8 ± 2.5	64.5 ± 10.3	94.0 ± 3.2	61.5 ± 13.2
	Ruminated		1.3 ± 0.5	1.0 ± 0.7	1.8 ± 0.6	0.3 ± 0.3	1.8 ± 0.6	0.8 ± 0.5
	Total		72.8 ± 3.6	58.8 ± 14.8	92.5 ± 1.8	64.8 ± 10.5	95.8 ± 2.7	62.3 ± 13.5
96 h	Non-ruminated		74.0 ± 1.7	70.0 ± 14.2	91.8 ± 2.9	73.5 ± 10.3	94.8 ± 3.2	73.8 ± 11.8
	Ruminated		1.3 ± 0.5	1.3 ± 0.8	2.0 ± 0.7	0.8 ± 0.5	1.8 ± 0.6	1.0 ± 0.6
	Total		75.3 ± 2.0	71.3 ± 14.6	93.8 ± 2.3	74.3 ± 10.5	96.5 ± 2.7	74.8 ± 12.1
120 h	Non-ruminated		75.3 ± 1.7	77.3 ± 13.2	92.0 ± 2.9	79.0 ± 9.1	95.0 ± 3.2	79.0 ± 11.2
	Ruminated		1.3 ± 0.5	1.3 ± 0.8	2.0 ± 0.7	1.3 ± 0.8	1.8 ± 0.6	1.0 ± 0.6
	Total		76.5 ± 1.9	78.5 ± 13.7	94.0 ± 2.3	80.3 ± 9.5	96.8 ± 2.7	80.0 ± 11.5

^{ab}Means within rows having different superscripts differ significantly (P < 0.05).

Values are means ± standard error

Discussion

desBordes and Welch [12] investigated the cumulative recovery rate of indigestible plastic sticks with diameter 1.6 mm, length 5 mm, and specific gravity in the range of 0.90–2.15 for ten days after administration. Their results showed that the total recovery rates of sticks with specific gravities 1.17, 1.42, and 1.77 were significantly higher than those with specific gravities 0.90 and 0.96. The results of our study also show a similar trend. However, desBordes and Welch reported that the ruminated rate of the plastic stick with specific gravity 1.17 was higher than that of sticks with specific gravities 0.90, 0.96, 1.42, 1.77, and 2.15, which is not consistent with our results. This is because of the differences in weight per ball and shape of the feed material, i.e., plastic sticks, which simulated hay, and plastic balls, which simulated capsules. Each indigestible ball was subjected to a buoyant force due to the attachment of air bubbles generated by microbial fermentation in the rumen. Furthermore, the existing position and behaviors of balls in the rumen are known to be affected by the functional specific gravity in the rumen, including the effects of hydration and air bubbles [48]. The plastic balls used in our study had a volume of 33–262 mm³, which is greater than that of the plastic sticks (10 mm³) used by desBordes and Welch. Despite having identical specific gravity values, the weight per ball of the plastic balls was still 3–30 times greater than that of the plastic sticks, and the buoyant force would have less of an impact with an identical amount of air bubbles attached. In the rumen, freshly ingested and partially degraded feed materials move and reside

together in a semi-fluid state [16, 49, 52]. Therefore, differences in the shape of the sticks and balls, including the ease with which they catch on to feed materials, presumably led to interactions with undegraded matter in the rumen that had an impact and produced results that were different from those of the present study.

The size of feed materials has been reported to be an important element that affects the passage and passage time of feed through rumen in some previous studies. In these studies, it was shown that the maximum size of feed, which can transit to the lower gastrointestinal tract, i.e., the critical particle size, is 1.18–3.2 mm [13, 37, 38]. These results are inconsistent with those of the present study, which shows that plastic balls with diameters 6.35 mm and 7.95 mm can easily path through the rumen. On the other hand, Lee *et al.* [27] reported the distribution of undigested corn grains with 4 mm and 8 mm in feces of Holstein steers amounted to approximately 8% of dry matter feces. The size of the corn grains in their study is similar to the size of the plastic balls used in the present study. In addition, Terada *et al.* [45] reported that large amount of undigested corn grain was found in cattle feces, which were sieved with a 4.760 mm mesh screen. They suggested that the passage mechanisms of undigested corn grains were possibly different from that of hay. Hence, it was considered that distinct critical particle size, especially about particles of a certain level of specific gravity and diameter, is not yet been determined.

In the present study, the recovery rate of the plastic balls with a diameter of 7.94 mm was the highest at 96.8%; therefore, the critical particle size of indigestible balls with specific gravities 1.19–1.41 has been demonstrated to be

7.95 mm or above.

Capsules with specific gravity 1.19 or 1.41 (including the contents) and diameters 6.35–7.94 mm could be regarded as optimal tools that can be used to bypass the rumen. In addition, capsules with specific gravity 1.19 can exert quick effect of bypass feeding or those with specific gravity 1.41 are expected to have a sustained-release effect in the lower gastrointestinal tract.

Summary

Plastic balls was used to investigate how their specific gravity and diameter affect excretion rate and rumination in dairy cattle, to develop a capsule that can be used for reaching the lower gastrointestinal tract without physical breakdown and/or degradation in the rumen. Twelve types of indigestible plastic balls composed of a combination of four specific gravities (0.95, 1.19, 1.41, or 2.20) and three diameters (3.97, 6.35, or 7.94 mm) were orally administered to lactating dairy cows, and the balls were collected from feces, until 120 hr post-administration, to evaluate the recovery rate. Recovery rate of the balls with specific gravity 1.19 or 1.41 and diameter 6.35 or 7.94 mm was higher than those with specific gravity 0.95 or 2.20 and diameter 3.97 mm. The cumulative recovery rates at 24 or 48 hr post-administration was higher for balls with specific gravity 1.19 than that for balls with the other specific gravities. These results suggest that specific gravity 1.19 or 1.41 and diameters 6.35–7.94 mm are optimal for use in bypass capsules for administration to cattle. In addition, the passage time of capsules differed between specific gravity 1.19 and 1.41.

Chapter II

Development of three-layered rumen escapable capsules for cattle

Introduction

Advances in the genetic improvement of dairy cows, especially the Holstein variety, have led to large increases in per capita milk production. Since it is necessary to balance nutrient intake to meet this increased milk production, a greater burden is placed on the body, including the metabolization of lipids and carbohydrates in the liver. Consequently, metabolic disorders have become more frequent and are now a significant problem for dairy management [26, 41]. Metabolic diseases, such as ketosis, are primarily caused by a negative energy balance during early lactation [9].

Clinical and latent ketosis are major economic diseases, reducing milk production and also causing reproductive problems, such as an increase in the number of days open [15]. Ketosis is both prevented and treated by the intravenous administration of B vitamins and sugars [43]. Cows are polygastric, and of their four stomachs, the rumen contains 1–100 billion microorganisms per gram. Consequently, even when B vitamins are administered orally, they are broken down by microorganisms, with as little as 52% of thiamine (VB1), 1% of riboflavin (VB2) and 3% of niacin (VB3) escaping the rumen [54]. Thus, intravenous or subcutaneous injections, or a rumen escapable feed must be used when administering these functional ingredients to cows.

Substances that have particularly high potential for decomposition by microbes in the rumen can be protected by coating with oil, enabling them to avoid degradation and reach the abomasum [6]. However, because this method involves the suspension of the administered substance in heat-treated oils followed by solidification through cooling, it is difficult to achieve escaping using hydrophilic substances, such as water-soluble vitamins or medicine, or heat-sensitive substances, such as live microbial agents. More specifically, mixed hydrophilic substances treated in heated oil can be easily separated during solidification process. Also, heat-sensitive substances are inactivated by hot melting oils. Therefore, in chapter II, a new rumen escapable tool for cattle was developed, consisting of a three-layered capsule that can protect the contents from degradation by microorganisms and dilution in the massive rumen. The capsule is manufactured at room temperature so that hydrophilic or heat-sensitive substances can be contained without suffering any harmful heat effect. Encapsulated thiamine hydrochloride indicator was administered to lactating cows to verify that the capsules had escaped the rumen degradation and reached the lower gastrointestinal tract.

Materials and Methods

Animals and diet

All animal procedures conformed to the guidelines of the local ethics committee (Animal Experimentation Department of the Local Incorporated Administrative Agency Research Institute of Environment, Agriculture and Fisheries, Osaka Prefecture). Four Holstein cows of mean (\pm standard deviation) weight 748 kg (\pm 31 kg) and mean 332 days (\pm 60) in milk were assigned to the test in a crossover trial. The test was carried out over two experimental periods. Each trial lasted for two days, and the trial interval was set for seven days. Animals were fed hay and concentrate separately in four installments at 06.30, 08.30, 10.30 and 14.30 daily (Table 4). Water was given *ad libitum*.

Table 4. Dietary ingredients and chemical composition.

	% Dry matter
Ingredients	
Timothy hay	35.0
Oats hay	15.0
Alfalfa hay	10.0
Beet pulp	10.0
Commercial concentrate ^{a)}	30.0
Chemical composition ^{b)}	
Crude protein	12.8
Estimated TDN	68.6

a) 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)

b) TDN, total digestible nutrient. Based on NARO [35].

Capsule preparation

Capsules containing thiamine hydrochloride were manufactured by Morishita Jintan Co., Ltd. (Osaka, Japan) with a diameter of 6 mm, specific gravity of 1.3, approximately 160 mg per capsule itself and a seamless three-layer structure according to the following procedure [2, 4, 23] (Fig. 2): The core liquid suspension consisted of 25% w/w of thiamine hydrochloride and 25% w/w of titanium dioxide to regulate specific gravity, in liquid palm kernel oil having a melting point of 38°C. The middle layer was designed to avoid degradation by stomach acid in the abomasum. It was produced from a liquid suspension consisting of 30% w/w titanium dioxide in hardened palm kernel oil having a melting point of 56.5°C. The outer layer was intended to protect the middle layer from physical damage caused by jostling during transport. The composition of the outer aqueous suspension was as follows: 3.0% w/w kappa-carrageenan, 11.3% w/w cassava starch, 4.7% w/w sorbitol, 0.02% w/w locust bean gum, 1.4% w/w low-methoxy pectin, 0.23%w/w potassium chloride, 0.01% w/w calcium chloride, 0.09% w/w potassium hydrogen phosphate, 0.28% w/w potassium dihydrogen phosphate and 2.3% w/w titanium dioxide. These three types of suspension were applied via an encapsulation machine equipped a triple concentric nozzle. The core, middle and outer layers of the liquid suspension were simultaneously ejected (via the innermost, intermediate and outermost nozzles, respectively) to a flowing-oil chamber. The suspension formed a spherical capsule with three layers via its own surface tension in oil. The obtained wet capsules were dried at 20°C and 22.6% relative humidity on a rotating fluidized bed, thereby solidifying to form

three-layer capsules. The polysaccharide (kappa-carrageenan, cassava starch, locust bean gum and low-methoxy pectin), sugar alcohol (sorbitol) and salts (potassium chloride, calcium chloride, potassium hydrogen phosphate and potassium dihydrogen phosphate) comprised in the outer thin layer of the capsule are commonly used for food additive and authorized feeding to cattle by Incorporated Administrative Agency Food and Agricultural Materials Inspection Center in Japan. The palm kernel oil comprised in the middle layer and core are used for feed of cattle, and also, authorized. Titanium dioxide to regulate specific gravity comprised in the all layers of the capsule is used for food additive in Japan. Wang *et al.* [50] reported that the titanium was accumulated in the spleen and brain when female mice were one time administrated 5 g/kg body weight of fine-sized titanium dioxide suspensions, but showed no acute toxicity. In the present study, orally administrated titanium dioxide was approximately 0.08mg/kg body weight of cattle. The author concluded that the capsule in itself is harmless to cattle and products. In addition, we confirmed that specific gravity of the capsule can be adjusted by using talc (which is authorized feeding to cattle) in exchange for titanium dioxide.

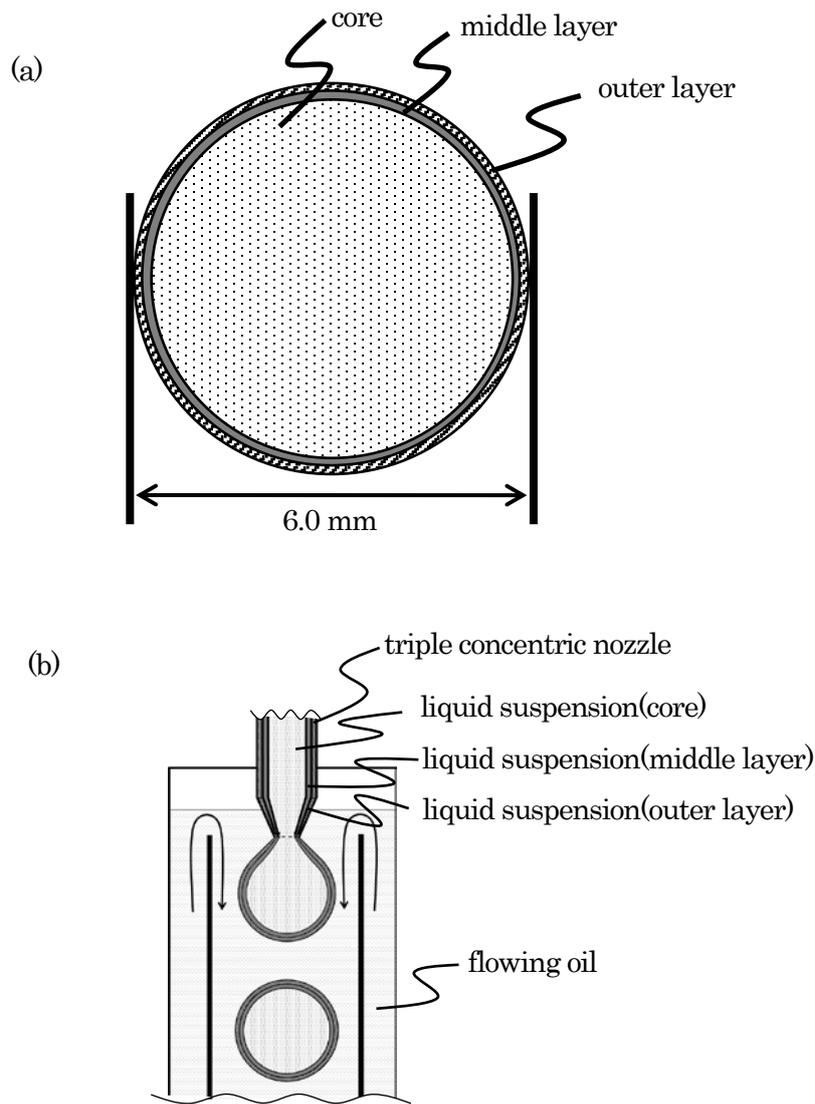


Fig 2. Schematic of (a) the three-layered capsule, and (b) method of manufacture.

(a) Core: a mixture of hydrogenated palm oil and water-soluble vitamins, 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.

(b) Triple concentric nozzle: core, middle, and outer liquid suspension were obtained from respective nozzle. Liquid suspension: three kinds of liquid suspension of core, middle layer, and outer layer. Core liquid included substances intended to be escaped rumen. Flowing oil: carrier of spherical droplets.

***In vitro* incubation of capsules and preliminary administration**

Twenty particles of manufactured capsules per flask were anaerobically incubated by shaking in 100 ml rumen liquor at 39°C for 24 hr in triplicate. Rumen liquor was obtained from lactating cows via rumen catheter and was immediately used for *in vitro* incubation of the capsules. Twenty-four hr later, capsules were recovered using a 2.0-mm screen. Visual inspection established whether or not the capsules had undergone lysis in the rumen liquor. Before the main test, the rumen insoluble capsules were preliminarily administered to cows in order to examine the effect of the breaking strength of the capsules on their solubility in the lower digestive tract subsequent to the rumen *in vivo*. Two cows were orally administered the capsule with five kinds of breaking strength (0.9, 2.5, 4.1, 5.4 and 8.1 newton (N)) every other week. All feces were picked up from the floor after administration for 24 hr, and the status of excreted capsules was checked visually. A rheometer (CR-500DX, Sun Scientific Co., Ltd., Tokyo, Japan) was used to measure the breaking strength of the capsule, and the breaking strength was defined as the breaking load when the capsule broke due to constriction. The measurement was carried out using a pressure sensitive disc-shaped shaft with a diameter of 10 mm, moving at 20 mm/min at 39 °C.

Administration of capsules containing water-soluble vitamins

Thiamine hydrochloride was used to determine whether the capsules successfully escaped the rumen. The capsules contained a core of thiamine hydrochloride (Wako Pure Chemical Industries, Ltd., Osaka, Japan). The test

group was administered encapsulated thiamine hydrochloride, whereas the control group was administered the same amount of thiamine hydrochloride in an aqueous solution. Both the test and control groups were orally administered 30 g of thiamine hydrochloride (approximately 1,500 capsules for test group) per head. A polyvinyl chloride resin installer RUMENFIBE (Meiwa Sangyo Co., Ltd., Kyoto, Japan) was used for oral administration. Administration of both the capsules and solution was forced at 10.00 prior to feeding. Blood samples were collected from the jugular vein every 3 hr, from immediately before administration until 24 hr after administration. All urine was collected by urinary catheterization for 24 hr after administration and weighed after each excretion. Then, 2 ml of urine was immediately stored at -20°C until analysis.

The thiamine concentration was measured using the modifications by Fukuwatari *et al.* [18] to the high-performance liquid chromatography (HPLC) method by Kimura *et al.* [24]. Specifically, for blood samples, 300 µl of whole blood was taken using sodium heparin as an anticoagulant, to which 600 µl of 5% w/v trichloroacetic acid solution was added. This mixture was then centrifuged at $15,000 \times g$ and 4°C. The supernatant was used as an HPLC injection sample following filtration. Following column separation using 0.2 M sodium dihydrogen phosphate/0.3% v/v acetonitrile as the mobile phase, it was mixed with 0.1% w/v potassium ferricyanide and 15% w/v NaOH before measurement using a fluorescence detector. The columns were reversed-phase HPLC columns (COSMOSIL 5C18-MS-II, 4.6 × 250 mm, NACALAI TESQUE, Inc., Kyoto, Japan).

Results

For *in vitro* incubation, there were no lysed capsules in the rumen liquor for 24 hr. When the capsules with a breaking strength of 4.7 N were orally administered to cattle, the capsules were excreted having maintained their spherical shape in feces (Table 5). On the other hand, only fragments of the capsule were obtained from the feces when capsules with breaking strength of 4.1 N or less were administered. So, we used capsules with a breaking strength of 4.1 N in the subsequent experiments.

The results of thiamine hydrochloride level in the blood are shown in Fig. 3. No change in blood thiamine concentrations was found in the control group that was administered an aqueous solution of thiamine hydrochloride. In contrast, in the test group administered thiamine hydrochloride via the three-layer capsules, a marked rise in blood thiamine concentration was found 3 hr following administration. The concentration significantly increased from 12.4 ± 1.03 ng/ml before administration to 54.8 ± 2.21 ng/ml at 6 hr after administration. The elevated blood thiamine concentration was maintained for 9 hr after administration before gradually decreasing. Blood concentrations were 4.1-fold higher in the test group than in the control group 6 hr after administration. Figure 4 shows accumulated thiamine hydrochloride excreted in urine, calculated from urine thiamine hydrochloride level and urine volume after administration of capsules. There was considerable individual variability in the thiamine value, and no difference was found between the test group (801.7 ± 348.2 μ g) and the control

group ($674.6 \pm 184.1 \mu\text{g}$, $P= 0.7993$) at 24 hr after administration.

Table 5. Status of capsules after *in vitro* incubation and preliminary administration.

Breaking strength of a capsule (N)	0.9	2.5	4.1	5.4	8.1
Status of capsules after <i>in vitro</i> incubation	Spherical shape				
Status of capsules in feces	Fragment	Fragment	Fragment	Spherical shape	Spherical shape

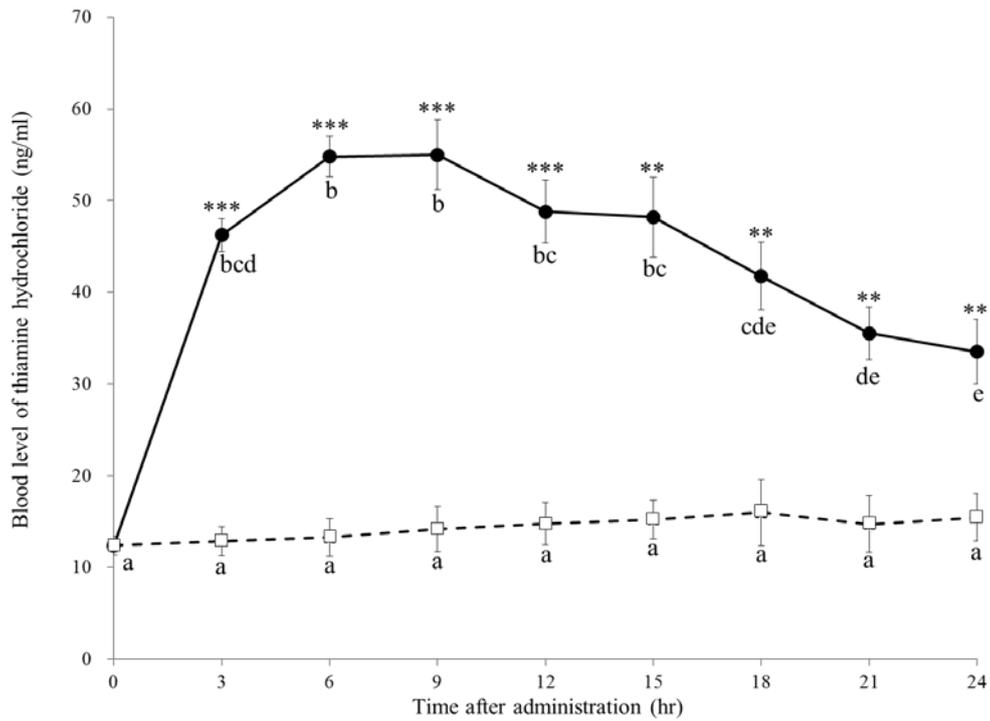


Fig 3. Changes in blood thiamine hydrochloride concentration.

Blood thiamine concentrations were measured before administration and every 3 hr after administration. ●: Encapsulated thiamine hydrochloride-administered group, □: aqueous thiamine hydrochloride-administered group. *: denotes significant differences between the groups at the given time (**, $P < 0.01$; ***, $P < 0.001$). a, b, c, d, e: Significant differences between time periods are noted with different letters ($P < 0.05$). Error bars represent the standard error of the mean.

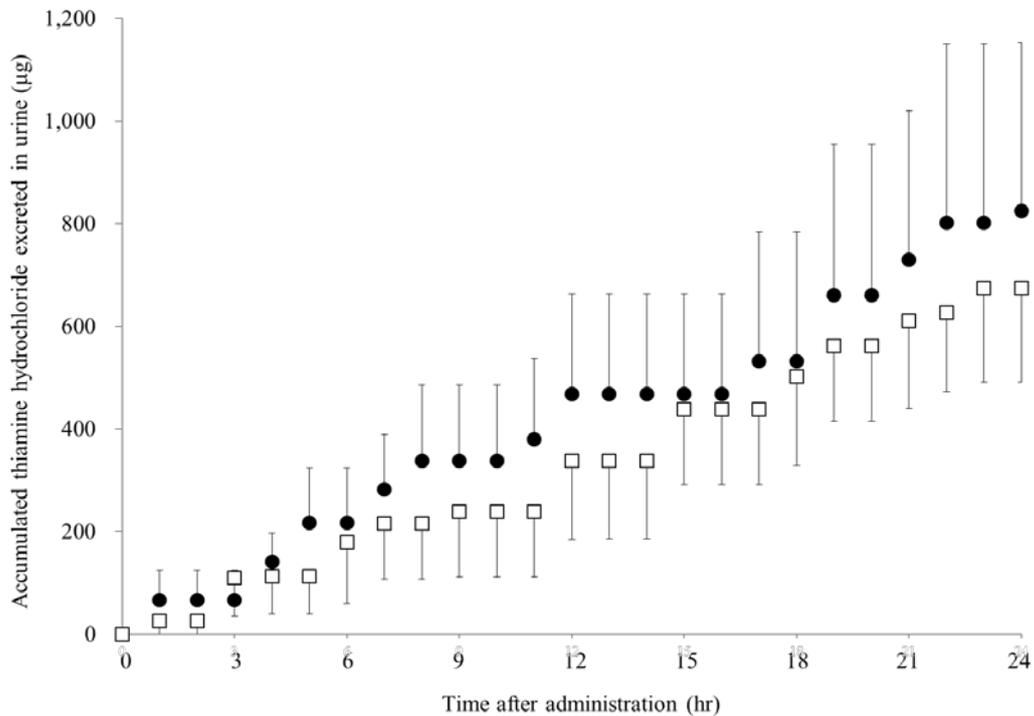


Fig 4. Accumulated thiamine hydrochloride excreted via urine, calculated from the concentration and volume of each urine excretion.

All urine was collected for 24 hr after administration and weighed immediately. Each plot shows average accumulated thiamine hydrochloride in urine for the time elapsed since administration. ●: Encapsulated thiamine hydrochloride-administered group, □: aqueous thiamine hydrochloride-administered group. Error bars represent the standard error of the mean.

Discussion

There were no lysed capsules in *in vitro* incubation, and hence, it was concluded that capsules placed into the rumen retained their shape until flowing out to the omasum. Chapter I showed that the proportion of ruminated indigestible plastic balls (which are similar to the capsules used in this study in shape, size and specific gravity) was 2% or less. According to that finding, almost all the capsules possibly passed through the rumen without being degraded by microorganisms in rumen and/or physical destruction by rumination. In the present study, the capsules were found to be insoluble in rumen liquor *in vitro*. At the same time, no intact capsules were found in feces after the preliminary administration when the breaking strength of capsule was set for less than 4.1 N. These results strongly suggest that the capsules used in the main administration tests in this study escaped from being broken in the rumen, but dissolved in lower digestive tract.

The elevated blood thiamine concentration in the test group suggests that the capsule reached the intestinal tract after passing quickly through the anterior stomach. It has been suggested that solids with a high specific gravity, such as the capsules used in this study, quickly enter the ruminal ventral sacs rather than being limited to the mat formed in the rumen [17]. Additionally, it is known that once solids have entered the ventral sac, they flow through the omasum without reentering the dorsal sac [39]. Hoeller *et al.* [20] reported that the rumen wall of sheep has an extremely low permeability to thiamine and that the

rumen may not be a site of significant absorption of thiamin. Moreover, Miller *et al.* [29, 30] reported that absorption of thiamine occurs mainly in the small intestine. In the results of present study, the blood thiamine levels started to increase 3 hr after administration in the test group, even though thiamine half-life in blood is very short, reported as 4.7 and 7.1 min in fitting two exponential functions [46]. Furthermore, plasma thiamine concentration peaked between 6 and 9 hr after administration before subsequently starting to decline. These results suggest that the capsules moved rapidly to the duodenum without stopping in the rumen; and that almost all the capsules had dissolved and released their contents in a rapid manner, since no capsules lysed in rumen liquor in *in vitro* incubation, as indicated above. The rise of blood thiamine level for the test group seems to be relatively low with respect to the amount of administered thiamine. It is thought to be due to that thiamine is quickly metabolized in blood and its half-life is very short [46].

In contrast, the ruminal retention time of the aqueous solutions, such as those used in the control group, is estimated to be about 10 hr in dairy cows [32]. Assuming that as much as 52% administered thiamine hydrochloride reached the lower digestive tract intact [54], a rise in blood concentrations would be expected to occur 10 hr following administration. However, in practice, this was not observed. This is considered to be due to metabolic action of ruminal microorganisms and/or quick hydrolysis or tissue uptake that somewhat absorbed thiamin, as mentioned above. With regard to urine, there was no difference in accumulated thiamine hydrochloride between control and test groups. Nakajima *et al.* [34] reported that in cattle, thiamine is absorbed from blood by some tissues, such as the brain, heart,

liver and kidney. For this reason, it was concluded that tissue uptake of migrated thiamine from capsules occurred in a matter of hours and that thiamine was not excreted to urine.

The above results show that encasing water-soluble vitamins within a multi-layer capsule structure allows for efficient absorption by cows. The three-layered capsules developed in this study for cows were shown to function as a rumen escapable tool. Additionally, it was demonstrated that the capsules rapidly moved to the duodenum following administration, since peak blood thiamine concentration occurred between 6 and 9 hr after oral capsule administration.

Summary

A new rumen escapable tool is presented for cattle in prospect of developing medical treatment or supplementing trace elements for disease prevention. This tool consists of a three-layered capsule that dissolves in the lower digestive tract, but not in the rumen. The capsule was manufactured by capsule-forming techniques through the use of liquid surface tension. This method does not involve high-temperature treatment, so the capsule can contain not only lipophilic substances but also hydrophilic or heat-sensitive substances. Furthermore, the capsule has a specific gravity of 1.3 and diameter of 6.0 mm, which were previously shown to be appropriate to avoid rumination. The objective of this study was to confirm the effectiveness of the capsule pertinent to rumen escaping. In order to validate rumen escape, capsules containing 30 g of water-soluble vitamin (thiamine hydrochloride) per head were administered to four lactating cows assigned in a crossover trial. In the group administered encapsulated thiamine hydrochloride, blood thiamine levels increased from 12.4 ± 1.03 ng/ml before administration to 54.8 ± 2.21 ng/ml at 6 hr following administration, whereas the level remained at 13.3 ± 2.05 ng/ml in the control group administered via aqueous solution. This indicates that the three-layered capsules passed through the rumen and dissolved in the lower digestive tract, thus functioning as a rumen escapable tool.

Chapter III

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

Introduction

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 [3, 42, 44]. Recently, LAB have been shown to increase the rate of body weight and immunity in farm animals such as hogs, chickens and even fish [28, 47, 51]. In addition, studies using newborn calves with undeveloped rumen reported that oral administration of LAB as probiotics improved body weight gain and fecal condition [1, 33]. On the other hand, in adult cattle, probiotics have been selected to target the rumen instead of gut to improve fiber digestion [7, 8, 14]. Moreover, Ghorbani *et al.* [19] 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 only a small number of LAB escaping the massive rumen. To overcome this problem, the author proposed encapsulating LAB in enteric capsules that have a diameter of 6 mm and specific gravity set at 1.3. 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. 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 [21, 40]. 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 chapter III, the effect of administering LAB-containing enteric capsules on the intestinal bacterial flora of cattle was confirmed. A metagenomic analysis was used to estimate changes in the composition of the intestinal bacterial flora by encapsulated LAB .

Materials and Methods

Lactic acid bacterial strains and capsule preparation

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). Viable bacterial concentration in the capsules was 5.1×10^9 colony forming unit (cfu)/g-capsule.

Animals and diet

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 ± 29 days in late lactation, 68 ± 12 months old, with an average body weight of 670.3 ± 52.9 kg, lactation

numbers of 2.8 ± 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 6). 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).

Table 6. 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 ^{a)}	45.0
Chemical composition ^{b)}	
Crude protein	14.8
Estimated TDN	72.7

^aCommercial 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)

^bTDN, total digestible nutrients. Based on NARO [35].

Treatments and sample collection

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 3×10^{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 3×10^{11} cfu suspended in 100 ml of PBS together with 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.

Metagenomics analysis of intestinal flora

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') [25], which had an Illumina-specific adapter (Illumina, Inc., California, USA). Amplicon PCR 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-through 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). Nucleotide sequence data reported are available in the DDBJ databases under the accession number DRA004106. The alpha (α) 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). Principal component analysis plots were created using the MG-RAST server.

Results

After dismissing outlying values based on the Smirnov-Grubbs's outlier test ($\alpha = 0.05$), the mean values of α diversity in the groups were compared (Fig. 5). The alpha diversity is one of the indices of species diversity, and represents species diversity in a given single community. The analysis showed an increasing trend of α diversity in fecal samples from cattle administered with JCM1099 capsules (70.8 ± 2.6) compared to control (64.5 ± 1.9 , $P = 0.0606$).

The results of principal component analysis were shown in Figure 6. 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 ∇ 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. 6A). 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. 6A). 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. 6B and 6C).

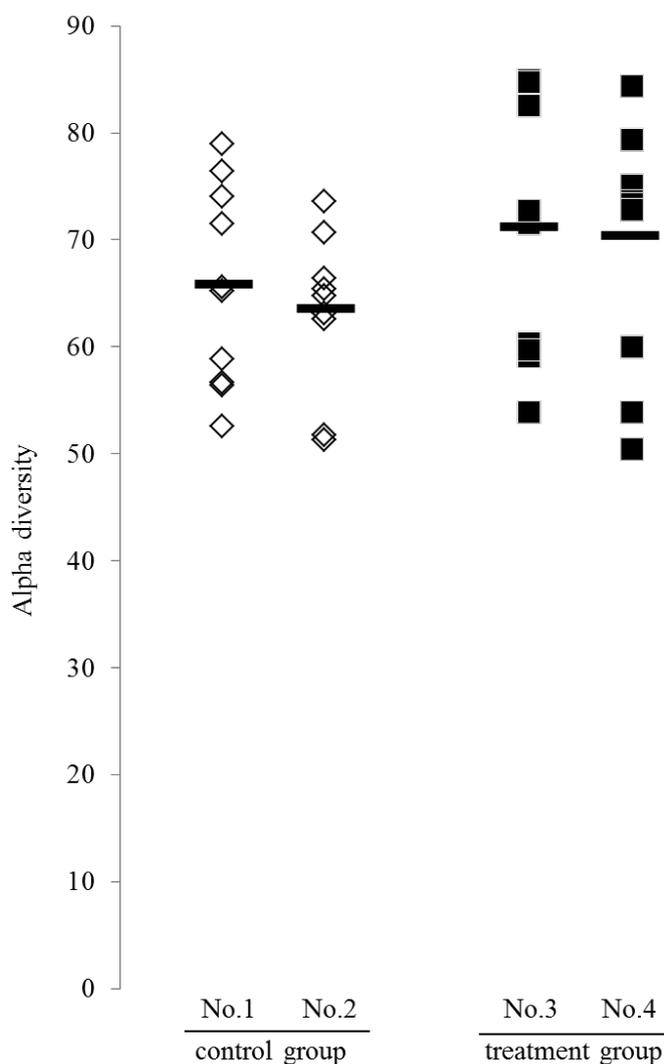


Fig 5. The α diversity in fecal samples of cattle.

The alpha (α) 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).

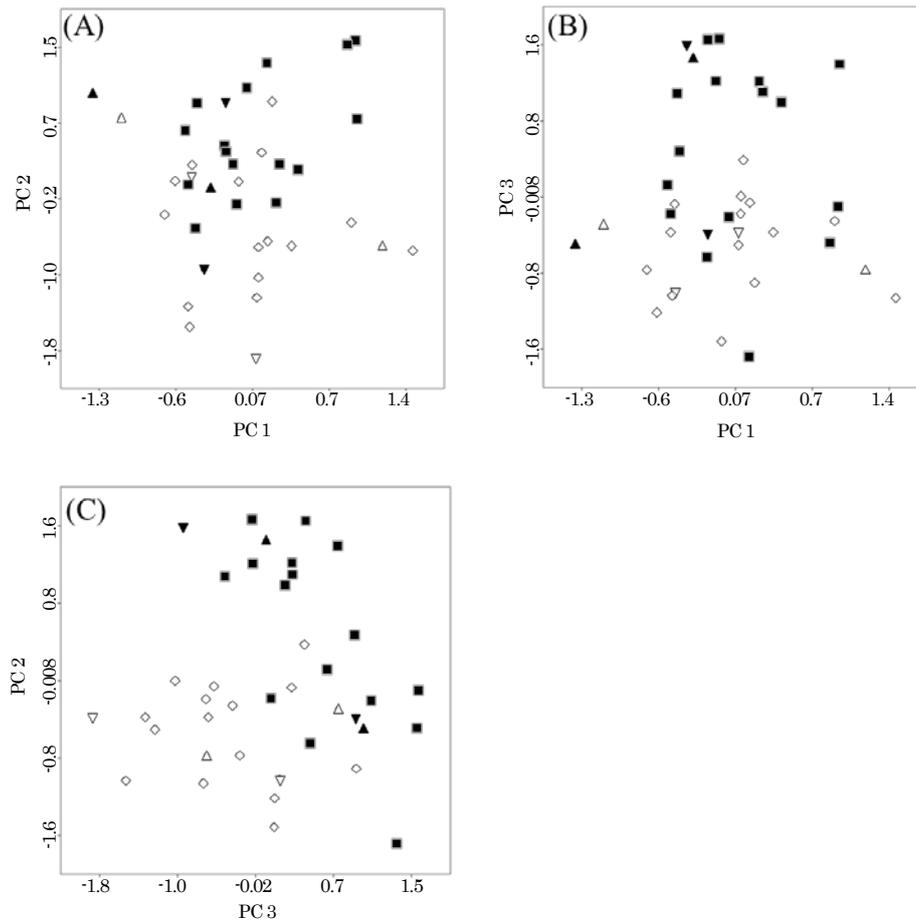


Fig 6. Principal component analysis of data obtained from cattle fecal samples collected prior to, during and after the administration of *Lactobacillus coryniformis* subsp. *torquens*.

Each plot was created using the MG-RAST server. ∇ 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.

Discussion

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 α diversity of the intestinal flora of humans caused by probiotics [10].

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 flora is known to vary due to changes in influx of the digestive tract content and interactions with the host [31]. 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 [22, 53]. On the other hand, David *et al.* [11] 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 with their results of David *et al.* [11].

In conclusion, the administration of encapsulated JCM1099 resulted in an increase in α 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.

Summary

The effects of encapsulated lactic acid bacteria administered orally to lactating cattle on the intestinal flora were examined. A dose of 3×10^{11} colony forming unit (cfu) of freeze-dried *Lactobacillus coryniformis* subsp. *torquens* (JCM1099) encapsulated in an enteric capsule capable of escaping 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. It is suggested that administration of encapsulated JCM1099 can alter the intestinal bacterial flora of cattle.

Conclusion

Cows are polygastric, and of their four stomachs, the first stomach (the rumen) contains 1–100 billion microorganisms per gram. Consequently, even when high energy substances or curative medicines are administered orally, they are diluted in the massive rumen and/or broken down by microorganisms.

In the present study, a new rumen escapable tool for cattle, consisting of a three-layered capsule that can protect the contents from degradation by microorganisms and dilution in the massive rumen, was developed. The capsule is manufactured at room temperature so that hydrophilic or heat-sensitive substances can be contained without suffering any harmful heat effect.

In chapter I, plastic balls as model of capsules were used to investigate how the specific gravity and diameter of an orally administered spherical material affect the damage on the surface of balls by rumination and delivery to the lower gastrointestinal tract in cows. Recovery rate of the balls with specific gravity 1.19 or 1.41 and diameter 6.35 or 7.94 mm was higher than those with specific gravity 0.95 or 2.20 and diameter 3.97 mm. The cumulative recovery rate at 24 and 48 hr post-administration was higher for balls with specific gravity 1.19 than that for balls with other specific gravities. These results suggest that specific gravity 1.19 or 1.41 and diameters 6.35–7.94 mm are optimal for use in rumen escapable capsules for administration to cattle.

In chapter II, manufactured capsules contained thiamine hydrochloride was administered to lactating cows to verify that the capsules had escaped the

rumen degradation and reached the lower gastrointestinal tract. In order to validate rumen escape, capsules containing 30 g of water-soluble vitamin (thiamine hydrochloride) per head were administered to four lactating cows assigned in a crossover trial. In the group administered encapsulated thiamine hydrochloride, blood thiamine levels increased from 12.4 ± 1.03 ng/ml before administration to 54.8 ± 2.21 ng/ml at 6 hr following administration, whereas the level remained at 13.3 ± 2.05 ng/ml in the control group administered via aqueous solution. This indicates that the three-layered capsules passed through the rumen and dissolved in the lower digestive tract, thus functioning as a rumen escapable tool.

In chapter III, to examine the effect of administering lactic acid bacterium containing enteric capsules, a metagenomic analysis was used to demonstrate that changes in the composition of the intestinal bacterial flora could be estimated. 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. It is suggested that administration of encapsulated JCM1099 can alter the intestinal bacterial flora of cattle.

The present study suggests that the three-layered capsule for cattle could enable oral administration of degradable substances in the rumen, which is easier than intravenous injection on-site. Furthermore, capsules could be used in cases where the bypass oil method is not available, such as for medical products or

transmucosally administered vaccines that have little resistance to heat or acid. In addition, it is suggested that enteric capsules have possibility to be used as probiotics, which are helpful in improvement of productivity and prevention of zoonosis. This enteric capsule could be an effective tool to realize successful delivery of viable microorganism to intestine, which has long been difficult to accomplish.

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Summary in Japanese

現代のウシは生産性を伸ばすための遺伝的改良が進んだことから、産乳や産肉に要するエネルギーを、飼料から得られるエネルギーだけで補うことが困難になっている。特に周産期の乳牛では、不足するエネルギーを補うための体脂肪動員に伴う様々な代謝病が問題となっている。単胃動物の代謝病の予防や治療には、医薬品の経口摂取が有効であるが、反芻動物であるウシでは反芻胃、特に第一胃が障壁となって、医薬品を経口投与することができない。

本研究では、成牛で 200L もの容積がある微生物発酵槽になっている第一胃で分解されず、内容物を下部消化管に到達させ得るウシ用カプセルを開発し、その有効性を検証した。

第一章では、ウシの反芻を回避して、下部消化管に到達し得るカプセルの直径と比重を決定するため、カプセルを模した樹脂球の投与・回収試験を実施した。その結果、どの樹脂球直径においても、比重 1.19 と 1.41 の回収率が高く、比重 0.95 と 2.20 は有意に低かった。反芻を受けた樹脂球の割合は、比重 0.95 が他の比重よりも有意に高かった。消化管通過速度の違いとして、樹脂球投与 24 時間後、48 時間後、72 時間後における累積回収率は、比重 1.19 が有意に高かった。以上から、ウシに用いるカプセルとしては、比重 1.19 から 1.41 で、直径 6.35 から 7.94mm が最も適していると考えられた。

反芻を回避できるカプセルが下部消化管で溶解し、内容物を放出していることを確認するため、第二章ではビタミン B1 (チアミン塩酸塩) を内包するカプセルを調製し、ウシに経口投与して、血中チアミン濃度の変化を確認した。その結果、カプセル投与区では、血中チアミン濃度は投与 6 時間後に投与前 ($12.8 \pm 1.3 \text{ng/ml}$) の 4

倍に達した ($52.4 \pm 2.2 \text{ ng/ml}$)。一方、カプセル化せずに同量のチアミン塩酸塩を水溶液で投与した対照区では、投与 6 時間後の血中濃度は $13.3 \pm 2.2 \text{ ng/ml}$ にとどまった。このことから、開発したカプセルは第一胃で溶解することなく、速やかに下部消化管に到達して内容物を放出していることが示された。

上述のように、反芻や第一胃内での溶解を回避するウシ用カプセルを開発することができたが、さらに、従来のウシ用飼料では困難であった「乳酸菌の下部消化管到達」により、腸内細菌相が受ける影響を検証するため、第三章では、乳酸菌カプセルの投与試験を行った。その結果、乳酸菌を生理食塩水に懸濁して投与した対照区と比較して、カプセル投与区では、細菌種の多様性を示す α 多様性が増加する傾向にあった。また、主成分分析では、カプセル投与区と対照区は主成分 1 および 2 で区分できる傾向にあった。以上から、乳酸菌カプセルによって腸内細菌相を変化させ得ることが明らかになった。

動物用医薬品や飼料としての利用には、カプセルの下部消化管への到達率を明らかにしなければならないが、反芻を回避し、内容物を下部消化管に届け得るウシ用カプセルは、従来は困難であった生菌剤や医薬品などの経口投与を可能にする技術であり、新たな治療法や飼養技術の確立に貢献できよう。