泌乳中後期牛への活性酵母給与が第一胃内微生物構成および第一胃内発酵に及ぼす影響

[ASJ-2015-0537 FINAL VERSION] Title:

Effects of supplementing an active dry yeast product on rumen microbial community composition and on subsequent rumen fermentation of lactating cows in the mid-to-late lactation period

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Running title:

Yeast for mid-to-late lactation cows

Abstract

The effects of supplementing feed of cows in mid-to-late lactation with an active yeast product (Actisaf Sc 47) were evaluated using 15 Holstein cows in a replicated 3×3 Latin square design. The animals were fed a mixed ration with 33% NDF, consisting of timothy hay (29.8%), a commercial concentrate (70.0%), and commercial calcium triphosphate (0.2%), twice daily to meet 105% of their energy requirement. Yeast supplement was set at 0, 5, and 10 g per day over 21-day periods, each of which consisted of 14 days for adaptation followed by 7 days of data collection. Milking performance, plasma metabolite parameters, rumen volatile fatty acids, lipopolysaccharide, and microbial properties were measured. Although there were no significant differences in feeding and milking performance or blood parameters associated with supplementation, the acetate to propionate ratio in the rumen fluid tended to decrease (P=0.08). The population of *Bacteroidetes*, tended to be less prominent (P=0.07) and the fibrolytic bacterium *Fibrobacter* significantly increased (P<0.05) in the rumen fluid of the yeast 10 g group compared with that of the control. These data suggest that effects of supplementing live yeast to cows in mid-to-late lactation may be limited to microbial composition and fermentation characteristics in the rumen.

Key words: lipopolysaccharide, milking cow, rumen microbial community, volatile fatty acids, yeast.

INTRODUCTION

Efficient dairy cattle production is supported by the function of the rumen, so it is essential for rumen microorganisms to show stable activity. Although the rumen community structure is robust, environmental and stochastic factors, such as diet composition, feeding practices, and farm management, can strongly affect the structure and activities of the gut microbial community (Kocherginskaya *et al.* 2001; Russell & Rychlik 2001; Tajima *et al.* 2001). Disturbance of this ecosystem may lead to impairment of host productivity or sometimes to disease in the host. Direct-fed microbials can be implemented to minimize undesirable changes in rumen fermentation characteristics for the maintenance of host health and productivity under severe conditions (Lettat *et al.* 2012; Allen *et al.* 2013; Uyeno *et al.* 2015). Inoculation with microorganisms capable of hydrolyzing starch or of metabolizing lactate at low pH may also help alleviate the occurrence of rumen acidosis.

The main form of probiotic commonly used in dairy cattle consists of various strains of yeast (*Saccharomyces cerevisiae*), and this has been tested as an additive to ruminant diets (Lascano & Heinrichs 2009; Moya *et al.* 2009). Probiotic yeast strains are sometimes applied to lactating cows to improve milking performance and rumen fermentation efficiency by modulating microbial fermentation pathways. Although previous studies supported the efficacy of yeast supplementation, a conclusive outline has not yet been proposed on the amounts necessary for beneficial results because this may be due in part to milking performance, health status, stage of cows, and dietary characteristics (Chaucheyras-Durand & Durand 2010). It has not yet been evaluated to what extent yeast supplementation to dairy cattle in the late lactating periods affected these characteristics. The present study was performed in cows in the mid-to-late lactating period, i.e., the time at which cows produce milk stably at lower levels than the milking peak, to determine how much supplementing

these cows fed relatively high concentrations (70%) of diet with active yeast was effective for improving their performance (feeding and milking) as well as ruminal parameters.

MATERIALS AND METHODS

Experimental design, dietary treatments, and animal care

This feeding experiment was conducted in five prefectural research institutes (Ibaraki, Chiba, Kanagawa, Toyama, and Ishikawa) under a unified protocol and in accordance with the Japanese Standards Relating to the Care and Management of Experimental Animals. Fifteen Holstein cows (13 multiparous and 2 primiparous) were used in a replicated 3×3 Latin square design with 21-day periods (one Latin square which consisted of three cows per institute). The cows averaged (mean±SD) 164±18 days in milking at the start of the experiment. Each period consisted of 14 days for adaptation followed by 7 days for data collection. The animals were fed a mixed ration with 33% NDF, consisting of timothy hay (29.8%), a commercial concentrate (Platinumix, National Federation of Dairy Co-operative Associations, Tokyo, Japan; 70.0%), and commercial calcium triphosphate (0.2%), twice daily (09.00 and 16.00 hours) to meet with 105% of their energy requirement according to the Japan Feeding Standard (NARO 2006), for example, 22.3 kg DM of feed for 650 kg BW and 35 kg milking, and in another case, 19.2 kg DM of feed for 550 kg BW and 30 kg milking, with free access to water. Dietary treatments were 0 (CON group), 5.0 (Y5 group), and 10.0 g (Y10 group) of a commercial yeast product (Actisaf Sc 47, Lesaffre Feed Additives, Marcq-en-Baroeul, France; $>10^8$ cfu/g) per day with 100 g of rice bran as an extender. We determined these two levels of supplementation according to recommendation for a practical use by the provider (5 g/day/head) and the double amount (10 g/day/head).

Measurements

Daily intakes and refusals of the experimental diets for individual cows were recorded. Body weight was measured weekly before feeding in the morning (09.00 hours). Milk yield was measured at every milking (morning and evening everyday prior to each feeding) on days 15–18 of each period, and then averaged to a daily milk yield. Milk was sampled from six consecutive milkings on days 15-18 and composited. Blood samples were also collected at 13.00 hours on day 20 of each period and were subsequently centrifuged at 3,500×*g* for 30 min at 4°C to collect the plasma, which was stored at –20°C until analysis. To determine rumen organic acid content and microbial community, rumen fluid was collected using a stomack tube at the same timing of blood sample collection, and filtered through four layers of cheesecloth. Aliquots of the filtrates were stored at –20°C until analyzed for organic acids (by mixing 5 mL of the filtrate with 1 mL of 25% [wt/vol] HPO_3) and NH_3 (by mixing 5 mL of the filtrate with 1 mL of 1% [wt/vol] H_2SO_4). To determine microbial composition in the rumen fluid, an additional 7.5-mL fluid sample was mixed with 2.5 mL of phosphate buffered saline plus 10 mmol/L ethylenediaminetetraacetic acid, and immediately frozen.

Chemical analysis

Plasma concentrations of total protein, albumin, glutamic oxaloacetic transaminase, gamma-glutamyl transpeptidase, urea nitrogen, glucose, triglycerides, total cholesterol, and non-esterified fatty acid were analyzed using a Model 7020 automatic analyzer (Hitachi Seisakusho Co., Ltd, Tokyo, Japan). Concentrations of organic acids in ruminal fluid were quantified by high-performance liquid chromatography (Alliance HPLC system; Waters, Milford, MA, USA) using ion-exclusion separation in accordance with Lin *et al.* (2011), and NH_3 determined by the salicylate-nitroprusside-hypochlorite method using a flow injection analyzer. Concentrations of milk fat, milk protein, milk solid-not-fat, somatic cells, and milk urea nitrogen were determined

using an automatic analyzer at each experimental institute. The rumen lipopolysaccharide (LPS) was measured in pyrogen-free laboratory ware. The samples were centrifuged at 4°C and 11,000×*g* for 30 min, and the supernatants were subjected to a kinetic Limulus amebocyte lysate assay (PYROCHROME; Seikagaku Corporation Ltd., Tokyo, Japan). Detailed procedures for sample preparation and method validation have been described previously (Gozho *et al.* 2005).

Microbiological analysis

The prokaryotic cells in 2 mL of the fluid- phosphate buffered saline- ethylenediaminetetraacetic acid mixture were disrupted by glass bead beating, and total RNA was extracted with acid phenol solution followed by purification using an RNeasy mini kit (Qiagen, Valencia, CA, USA) in accordance with the manufacturer's instructions. Solutions of the extracted RNA were stored at –80°C until use. For detection and quantification of the respective bacterial groups, we used five scissor probes applied in previous studies using the same reaction conditions (Uyeno *et al.* 2010). Probes and target groups were as follows: Bac303m (*Bacteroides* and *Prevotella*); Erec482m (*Blautia coccoides* [*Clostridium coccoides* as the former name] – *Eubacterium rectale* group); Fibr225 (*Fibrobacter*); *Arc915m* (Archaea). Sequence-specific cleavage of rRNA fragments and the subsequent calculation to determine the 16S rRNA population of the target group in total 16S rRNAs were performed as described previously (Uyeno *et al.* 2007).

Statistical analyses

Data were analyzed using a Latin-square ANOVA in Stata 13.1 (Stata Corp, College Station, TX, USA) that accounted for treatment as the fixed effect, whereas period, testing site, and cow were the random effects. When significant ($P<0.05$) effects due to dietary treatments were detected,

differences among means were determined using the post-hoc Tukey test. A value of $P<0.05$ on least-squares means was taken to indicate a significant effect of the treatment.

RESULTS AND DISCUSSION

Rumen fermentation and cattle performance

There were no differences in body weight, feed consumption, milk yield, milk fat, milk protein, or milk solids-not-fat among treatments (Table 1). No cattle did exhibit any significant symptom or behavior which is suggested health impairment, therefore each cow was regarded as healthy during the experiment. The metabolic parameters in plasma were not significantly affected by yeast supplementation (Table 2). The results indicated that the expected improvement in production was not observed, which was supported by the lack of changes in blood parameters. The most consistent effects following addition of yeast culture to the diet included improved productivity in lactating cows (Robinson & Erasmus 2009). However, the results of the present study contrasted with those of a cattle study in the pre- and postpartum period, in which dry matter intake (DMI), milking performance, and plasma glucose increased in response to direct-fed microbials supplementation (Nocek & Kautz 2006).

Desnoyers *et al.* (2009) evaluated the effects of yeast supplementation on feed intake, milk production, and rumen fermentation using a quantitative meta-analysis. The results indicated that yeasts were able to exhibit positive effects such as an increase in rumen pH and a decrease in lactic acid, especially in case of higher proportion of concentrate in the diet and to higher intake level. Based on their analysis, an increase in DMI may generally be involved in a positive effect of yeast supplementation and subsequent increase in milk yield and composition. In this regard, because total DMI was limited in the experimental setting, a marked change in milk yield was not observed. With respect to volatile fatty acid (VFA) production (Fig. 1), although not significant, the

concentration of acetate decreased marginally, whereas propionate remained similar in response to yeast supplementation. This resulted in a decreasing tendency in the acetate:propionate (A:P) ratio (P=0.08, Fig. 1[e]). The decrease in A:P ratio but no change in milk composition may be involved in a slight decrease in serum triglyceride, because in this situation milk fat synthesis was unchanged but with a possible decrease in fat deposition. Lactic acid in the rumen liqur was found to be below the detection level (0.1 mmol/L) in all samples.

In a previous study, late-lactation dairy cows fed a relatively forage-rich diet were used to evaluate the effects of yeast supplementation on the rumen environment and in preventing rumen acidosis (consisting of 60% forage and 40% concentrate [DM basis]) (Thrune *et al.* 2009). Cows were fed yeast product at a higher level (10^{10} cfu/d/head) than the present study (>10^{9} cfu/d/head for Y10 as calculated). Although supplementing the diet of late lactation dairy cows with active dry yeast culture did not elicit changes in DMI or milking performance, the supplementation tended to decrease total VFA concentration in the rumen and induced higher ruminal pH in dairy cows. Similarly no increase or decrease in total rumen VFA concentration was observed in this trial (Fig. 1[d]). This suggests that observable benefits of yeasts are likely to be minimal when cattle perform at less than their milking peak while there was a change in rumen ecology.

Microbial community analysis

A notable result obtained from this study was that we succeeded in determining how the rumen community composition changes with yeast supplementation of the diet of cows in mid-to-late lactation (Fig. 2). Overall, the community compositions were similar among groups. However, a low population of *Fibrobacter* increased in response to yeast, suggesting that yeast enhances the activity of fibrolytic bacteria, in accordance with previous *in vitro* and *in vivo* studies (Beauchemin *et al.* 2003; Mosoni *et al.* 2007; Chung *et al.* 2011). On the other hand, although not significant, the population of one of the majority phyla, *Bacteroidetes*, tended to be less prominent in the Y10

group compared with the control group (P=0.07). The decrease in this phyla may suggest an increase in cell death of the gram-negative bacteria, thereafter a tendency in higher LPS levels in the Y10 group than the control (P=0.09, Fig. 1 [f]), given that the endotoxin is usually produced by dead cells of gram-negative bacteria (Miyagawa *et al.* 1979; Plaizier *et al.* 2012). However, this marginal increase in the LPS levels may not be a practical issue in cattle performance, since LPS in the rumen fluid was maintained below a level that is considered to negatively affect rumen function (Gozho *et al.* 2007; Zebeli & Ametaj 2009), which is regarded as around 10^5 EU/mL. Because bacteria belonging to this phylum produce acetate as a result of anaerobic fermentation, the decrease in *Bacteroidetes* may be involved in the decrease in A:P ratio dependent on yeast supplementation. These changes indicated that the live yeast affected rumen microbiota, but its effect was limited to within the rumen and without exerting positive effects in milk production. Recently, the effects of active dry yeast on rumen microbial community structure were determined by 16S rRNA gene-based clustering using a pyrosequencing technique (Pinloche *et al.* 2013). Their evaluation of the effects of yeast on the microbiota indicated that some bacterial groups were more affected than others. For example, the relative abundance of the lactate-utilizing bacteria, such as *Megasphaera* and *Selenomonas*, increased with yeast supplementation as well as the fibrolytic groups, *Fibrobacter* and *Ruminococcus*, confirming improvement of cellulolytic activity as a mode of action of yeast. Furthermore in their study, the addition of yeast provided a marginal decrease in *Bacteroidetes*, as was shown in the present study. The variety of effects may be explained in part by the multiple modes of action of yeast, which have not been determined completely, but changes in rumen fermentation rate and patterns are generally involved. For example, the increase in dietary levels of starch by yeast allows rumen microbes to more effectively metabolize the end product (lactate) of ruminal starch fermentation (Robinson & Erasmus 2009). A less-acidic ruminal environment is beneficial for the growth and fiber-degrading activities of cellulolytic

microorganisms (Hoover 1986). Although rumen pH was not determined in the present study, rumen microbial community analysis, VFA profiles, and health monitoring results of the cattle in this study were in good accordance with the preventive effect of yeast against the decrease in rumen pH (Desnoyers *et al.* 2009). It seems that addition of yeast to the diet supports a change in rumen microbial activity in a common direction, but which groups are specifically affected and whether it affects the health and productivity of the host are largely dependent on conditions, especially feed management. This may be a major reason why such ruminal interventions sometimes have inconsistent outcomes.

In conclusion, supplementing yeast to the diet of dairy cows in the late lactation period affected the rumen via the microbial population and VFA composition when it was fed a high amount (10 g/day), although it did not improve milk yield or quality. Observable benefits of yeasts are likely to be minimal under limited feed supply, thus milking performance could be improved by increasing DMI in a balanced diet.

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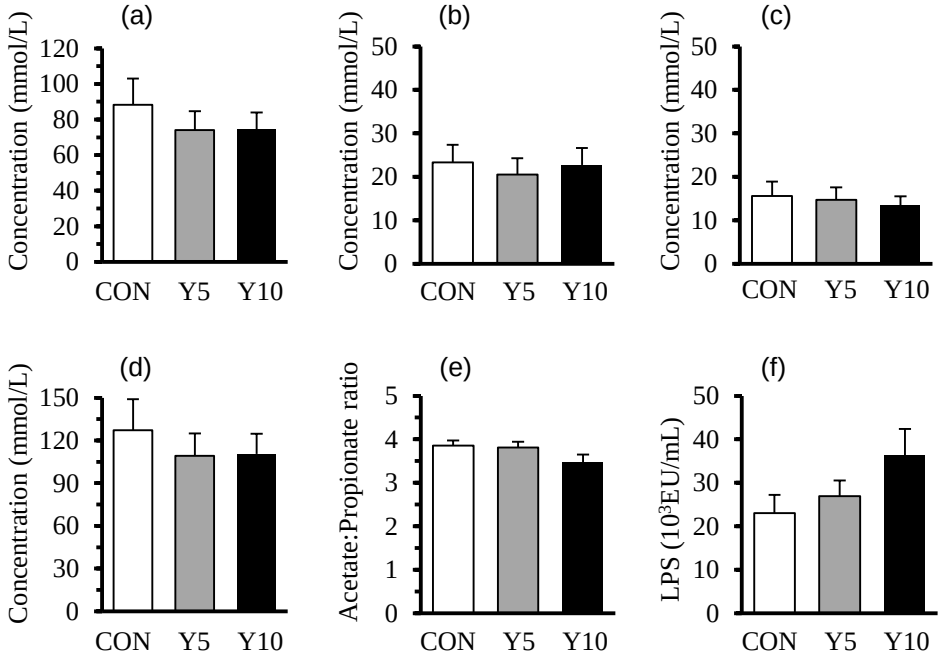
Figure legends

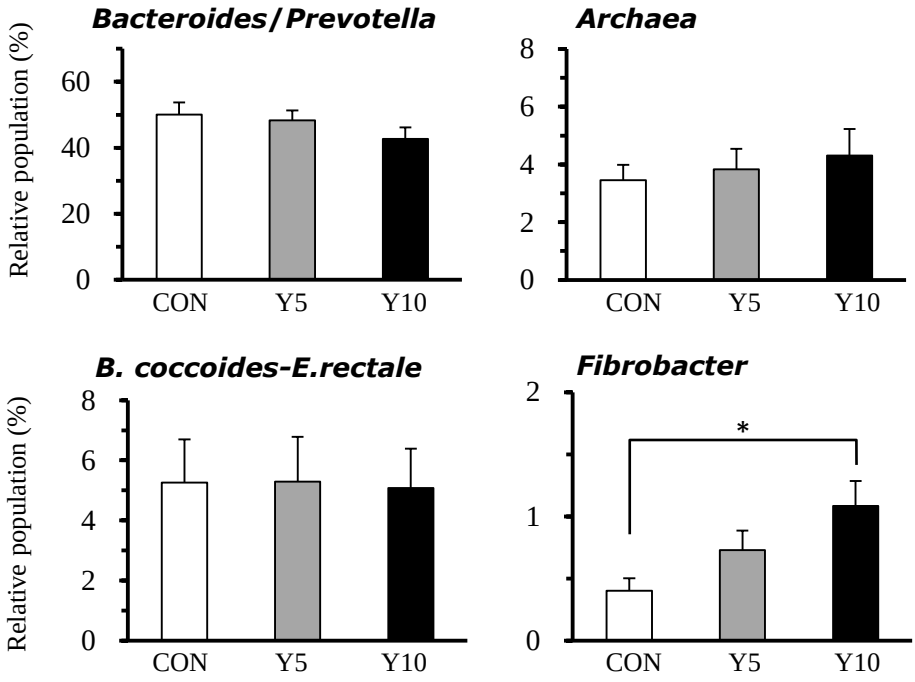
Figure 1 Effects on VFA and endotoxin in the rumen fluid of cows fed a diet supplemented with active yeast. (a) Acetate concentration, (b) propionate concentration, (c) butyrate concentration, (d) total VFA concentration, (e) acetate:propionate ratio, and (f) lipopolysaccharide (LPS) level. Error bars indicate standard error of the mean (SEM). CON, control treatment group; Y5, 5.0 g yeast supplement treatment group; Y10, 10.0 g yeast supplement treatment group.

Figure 2 Effects on ruminal microbial community of cows fed a diet supplemented with active yeast. Error bars indicate standard error of the mean (SEM). Significant differences are indicated by asterisks (*, $P<0.05$). CON, control treatment group; Y5, 5.0 g yeast supplement treatment group; Y10, 10.0 g yeast supplement treatment group.

(Figure 1)


(a)
Concentration (mmol/L)
120
100
80
60
40
20
0
CON
Y5
Y10
(b)
Concentration (mmol/L)
50
40
30
20
10
0
CON
Y5
Y10
(c)
Concentration (mmol/L)
50
40
30
20
10
0
CON
Y5
Y10
(d)
Concentration (mmol/L)
150
120
90
60
30
0
CON
Y5
Y10
(e)
Acetate:Propionate ratio
5
4
3
2
1
0
CON
Y5
Y10
(f)
LPS (10³EU/mL)
50
40
30
20
10
0
CON
Y5
Y10

(Figure 2)


Bacteroides/Prevotella
Relative population (%)
60
40
20
0
CON
Y5
Y10
Archaea
8
6
4
2
0
CON
Y5
Y10
B. coccoides-E.rectale
Relative population (%)
8
6
4
2
0
CON
Y5
Y10
Fibrobacter
2
1
0
*
CON
Y5
Y10

1

Table 1. Effects of supplementing the diet with active yeast on feeding and lactation performance

Parameter	CON group	Y5 group	Y10 group
BW (kg)	653 ± 15	653 ± 16	650 ± 17
DMI (kg/day)	23.3 ± 0.9	23.3 ± 1.2	23.3 ± 1.2
Milk yield (kg/day)	33.9 ± 2.3	32.9 ± 2.4	32.7 ± 2.9
Milk fat (%)	3.75 ± 0.15	3.74 ± 0.25	3.86 ± 0.27
Milk protein (%)	3.38 ± 0.23	3.45 ± 0.22	3.51 ± 0.46
Milk solids-not-fat (%)	8.80 ± 0.08	8.85 ± 0.08	8.90 ± 0.11
Somatic cells (10^4/mL)	65 ± 47	54 ± 28	85 ± 62
MUN (mg/100 mL)	14.0 ± 0.4	13.4 ± 0.9	15.4 ± 1.0

2 BW, body weight; CON, control treatment group; DMI, dry matter intake; MUN, milk urea
3 nitrogen; Y5, 5.0 g yeast supplement treatment group; Y10, 10.0 g yeast supplement treatment
4 group.

5

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Table 2. Effects of supplementing the diet with active yeast on blood plasma parameters of cows

Parameter	CON group	Y5 group	Y10 group
Total protein (g/100 mL)	7.7 ± 0.1	7.9 ± 0.3	7.8 ± 0.2
Albumin (g/100 mL)	3.8 ± 0.1	3.8 ± 0.1	3.8 ± 0.1
GOT (IU/L)	96.1 ± 8.8	129.1 ± 17.8	120.0 ± 12.8
GGT (IU/L)	34.9 ± 4.2	32.9 ± 3.6	38.3 ± 4.0
BUN (mg/100 mL)	17.7 ± 1.2	17.8 ± 1.3	18.0 ± 1.2
Glucose (mg/100 mL)	62.5 ± 1.5	63.8 ± 1.7	62.9 ± 2.2
NEFA (μEq/L)	60 ± 5	64 ± 6	70 ± 8
Triglycerides (mg/100 mL)	7.5 ± 0.4	7.3 ± 0.5	6.9 ± 0.4
Total cholesterol (mg/100 mL)	208.9 ± 8.6	209.3 ± 11.7	203.0 ± 12.8

2 BUN, plasma urea nitrogen; CON, control treatment group; GGT, gamma-glutamyl transpeptidase;
3 GOT, glutamic oxaloacetic transaminase; NEFA, non-esterified fatty acid; Y5, 5.0 g yeast
4 supplement treatment group; Y10, 10.0 g yeast supplement treatment group.

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和文抄録

泌乳中後期牛への活性酵母給与が第一胃内微生物構成および第一胃内発酵に及ぼす影響

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泌乳中後期牛に対する活性酵母給与の効果を明らかにするため、活性酵母給与量を 2 段階に設定した飼養試験を行い、泌乳成績とともに第一胃内微生物構成と発酵特性について調査した。国内公立場所で繋養しているホルスタイン種泌乳中後期牛 15 頭（平均泌乳日数 164 日）を供試し、供試牛は NDF33％となるようにチモシー乾草および配合飼料を処方し、日要求量の 105％を 1 日 2 回給餌した。各個体につき 1 期当たり馴致 14 日、試験 7 日として、対照区（活性酵母無給与）、5g 区（5 g/日給与）、10g 区（10 g/日給与）となる 3×3 ラテン方画で配置した。測定項目は、体重、乾物摂取量、乳量、乳成分、血液生化学一般成分のほか、各期の 20 日目に第一胃液を経口採取し、エンドトキシン活性レベル、有機酸組成、微生物群集構成について測定した。試験の結果、体重、乾物摂取量、乳量、乳成分、血液生化学一般成分および第一胃エンドトキシン活性レベルに区間で有意差は認められなかった。第一胃液中 VFA 濃度にも区間で有意差は認められなかったが、酢酸プロピオン酸比について、対照区と比較して 10g 区で低くなる傾向にあった。また、第一胃内微生物群集では、主要な繊維分解菌である *Fibrobacter* 属の分布量について、対照区と比較して 10g

区で高くなった。以上より、泌乳中後期牛に活性酵母を給与することで、第一胃内の繊維分解菌割合が増加し VFA 組成も変化するが、一方で生産に関する指標には影響を及ぼさなかったことから、より効果が期待できる給与法の検討が必要であると考えられた。