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Title:

Effects of cellooligosaccharide or a combination of cellooligosaccharide and live *Clostridium butyricum* culture on performance and intestinal ecology in Holstein calves fed milk or milk replacer

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ABSTRACT

The effects of oral administration of a prebiotic (cellooligosaccharide [CE]) and a combination of a probiotic (a commercial *Clostridium butyricum* strain) and prebiotics (referred to as symbiotics [SB]) on performance and intestinal ecology in Holstein calves fed milk replacer (MR) or whole milk were evaluated. Forty female calves (experiment 1) and fourteen male and female calves (experiment 2) were used in this study. Calves were fed MR (experiment 1) or whole milk (experiment 2) necessary for daily weight gain of 0.3 kg based on birth weight in two daily feedings and weaned at 46 d. Calves were divided into a CE feeding group, SB feeding group (only in experiment 1), and control group. The CE and SB groups were fed CE at 5 g/day before weaning and 10 g/day postweaning. Only the SB group received 10^8 colony-forming units (CFU) of *Cl. butyricum* culture per day. Commercial calf starter was offered for ad libitum intake. Health and feed intake of the animals were monitored daily, and body weight were measured weekly. Fecal samples were analyzed for determination of bacterial community composition by an RNA-based method (sequence-specific SSU rRNA cleavage method) and for organic acid profiling. In 49-day experiments, feed intake, daily gain, and occurrence of diarrhea of the calves were unaffected by either CE supplementation or SB supplementation, and all calves were healthy during each experiment. The fecal bacterial community compositions and the organic acid profiles were not different among groups in experiment 1. In experiment 2, the level of the *Cl. coccoides–Eu. rectale* group was higher in the feces of CE group than controls at 4 weeks of age and fecal butyric acid concentration was higher (8.0 vs. 12.2 [mmol/kg feces], $P < 0.05$) at that time. There were no differences in prebiotic bacteria (the genera *Lactobacillus* and *Bifidobacterium*) between groups at this time point. These results suggested that CE and *Cl. butyricum* supplementation have less effect on the

performance of healthy calves fed MR. However, prebiotic supplementation seems effective for modulation of the intestinal bacterial community of calves when administered with whole milk.

Keywords:

Prebiotic

Calves

Milk replacer

Gastrointestinal tract

Bacterial community

1. Introduction

Diarrhea is regarded as a major problem in preweaned dairy calves (Cowles et al., 2006; Hill et al., 2005), and prevention of diarrhea is important to promote the growth of calves. Antibiotics have been widely used in milk replacer (MR) in the USA and Japan (Kobashi et al., 2005; Sawant et al., 2005) to improve performance and reduce scours in dairy calves. However, as the use of antibiotics in animal feed was prohibited in the European Union in 2006 as part of an initiative to promote the prudent use of antibiotics, there is increasing interest in alternatives (Berge et al., 2009).

A detailed understanding of indigenous intestinal microflora is a way to address diarrhea, as the microflora is involved in host nutrition, mucosal defense, and host immunity, and therefore influences the performance of the animals (Gibson et al., 2004; Zoetendal et al., 2004). The intestines of newborn animals and humans are sterile, but microbial colonization of the gastrointestinal tract begins immediately at birth (Favier et al., 2002). Thereafter, a complex and dynamic microbial ecosystem with high densities of living bacteria is established in the large intestine as animals grow to maturity. Molecular-based monitoring of the intestinal bacterial communities of calves has revealed that the community undergoes dynamic changes during the first 12 weeks of life, including reduction of health-promoting bacteria such as lactobacilli and bifidobacteria from the community in the early stage of life in cattle (Uyeno et al., 2010a). It is considered effective for healthy calf rearing to optimize the enteric flora by increasing the number of potentially beneficial microorganisms.

One potential measure to enhance the impact of these beneficial bacteria is the use of oligosaccharides. Non-digestible oligosaccharides have been used in calf diets to eliminate harmful bacteria from the intestine and help improve the health of the animals (Heinrichs et al., 2003; Quigley et al., 1997). It is possible that these compounds affect

91 the viability of this beneficial bacterial group, and as a result strengthen gastrointestinal
92 function. Cellooligosaccharide (CE), consisting of glucose with beta-1-4 linkages, is a
93 commercially available oligosaccharide. We have reported that CE feeding in milk-fed
94 calves resulted in improvements in daily gain (DG) and feed efficiency during the
95 postweaning period (Hasunuma et al., 2011). However, little information is available
96 and further investigations are required to determine how the commensal bacteria
97 composition of calves changes with the administration of CE, particularly in the
98 preweaning period at which probiotic bacteria decrease the populations.
99 The objective of this study was to evaluate the effects of oral administration of
100 CE or *Clostridium butyricum* cells and CE (referred to as symbiotics [SB]) in the
101 preweaning period on feed intake, DG, fecal bacterial community compositions, and
102 organic acid profiles of Holstein calves fed MR or whole milk, both of which are
103 practical liquid feed for calves.
104
105 **2. Materials and methods**
106 *2.1. Animals and diets*
107 The animal study was conducted in the same way at the research institutes of
108 six prefectures (Toyama, Chiba, Aichi, Ishikawa, Ibaraki, and Kanagawa) in Japan. The
109 calves were cared for according to the Guide for the Care and Use of Agricultural
110 Animals in Agricultural Research of the National Institute of Livestock and Grassland
111 Science. We performed two experiments, one of which used 40 female calves (referred
112 to as experiment 1) and the other used seven male and seven female calves (experiment
113 2). Calves obtained from each institute were randomly assigned to one of three groups
114 in the order of birth: CE feeding group (n = 13), SB feeding group (n = 14), and control
115 group (n = 13) in experiment 1, CE feeding group (n = 7), and control group (n = 7) in

116 experiment 2. They remained at institutes where they were born through experiments.
117 All calves were given colostrum within 30 min of birth and housed individually in calf
118 hutches or pens bedded with sawdust. Ambient temperatures during experiments ranged
119 from 12 °C to 28 °C in experiment 1 and from 21 °C to 32 °C in experiment 2. The
120 calves were fed mother's milk for 1 – 3 d after birth and then abruptly switched to MR
121 without antibiotics (24% crude protein [CP] and 21% crude fat) dissolved in four times
122 (w/w) of warm water (experiment 1), or mother's milk without pasteurization
123 (experiment 2). Major ingredients of MR were skim milk powder, whey powder,
124 vegetable oil, hydrolyzed soy protein, and feed additives (vitamins ,minerals, and amino
125 acids). The liquid feed necessary for a daily body weight gain of 300 g (corresponding
126 to 370 – 500 g of MR powder and 3900 – 5200 g of mother milk [as fed]) based on birth
127 weight (NARO, 2006) was provided in two daily feedings at 07:30 and 17:00 using a
128 feeding bottle or a bucket with a nipple. The animals were weaned at 46 d. The CE
129 group and the SB group were fed CE (Nippon Paper Chemicals Co., Ltd, Tokyo, Japan)
130 at 5 g/day dissolved in the liquid feed. CE was mixed in starter at 10 g/day postweaning.
131 The SB group was fed 0.1 g (corresponding to 1.0×10^{8} colony forming units [CFU]) of
132 a commercial *Cl. butyricum* strain product (Miyagold Aqua Cello; Miyarisan Pharma
133 Co., Ltd, Tokyo, Japan) at the same time as CE feeding. Water was available at all times.
134 A commercial calf starter without antibiotics (New Make Star, consisting of 18% crude
135 protein and 2% crude fat, The National Federation of Dairy Co-operative Associations,
136 Tokyo, Japan) was offered at a daily maximum of 2400 g. The experimental period was
137 49 days (0 – 49 days of age). The animals' health and feed intake were monitored daily
138 and body weight was measured weekly before the morning feed. Fecal consistency was
139 scored and recorded daily according to the following definitions: 0 = firm; 1 = normal; 2

= soft but not runny; 3 = soft and runny; and 4 = watery (Cruywagen et al., 1996). Scores of greater than 2 were considered to indicate diarrhea.

2.2. Sampling, RNA extraction, and quantification of fecal microbes

An RNA-based, sequence-specific rRNA cleavage method (Uyeno et al., 2004) was applied to monitor the fecal bacterial community compositions of young calves associated with CE/SB feeding. Based on the findings of our previous study (Uyeno et al., 2010a), we analyzed the samples of both in the preweaning period and the postweaning period. Fecal samples were collected from calves by rectal stimulation on one day of each of weeks 4 (i.e., the preweaning period) and 7 (the postweaning period). Samples for microbial analysis (0.2 – 0.4 g) were suspended in 0.2 ml of PBS buffer plus 10 mmol/l ethylenediaminetetraacetic acid (EDTA), and were immediately cooled at 4°C and then processed within 1 h after collection. The prokaryotic cells in 1 ml of the suspensions were disrupted by glass bead beating (Uyeno et al., 2010a), and the total RNAs were extracted with 1 ml of phenol equilibrated with a buffer consisting of 10 mmol/l EDTA, 50 mmol/l sodium acetate (pH 5.1). Aliquots of 0.5 ml of the aqueous phase were obtained by centrifugation for 5 min at 12000 × *g* at 4°C, followed by purification using an RNeasy mini kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Solutions of the extracted RNA were stored at –80°C until use. For the detection and quantification of respective bacterial groups, we used eight scissor probes applied in previous studies under the same reaction conditions (Uyeno et al., 2008; Uyeno et al., 2010b). Probes and target groups were as follows: Bac303m (*Bacteroides* and *Prevotella*); Erec482m (*Clostridium coccoides–Eubacterium rectale* group); Atop291 (*Atopobium*); Bif164 (*Bifidobacterium*); Lab158m (*Lactobacillus* and *Enterococcus*); Fibr225 (*Fibrobacter*); Enter1251 (*Enterobacteriaceae*); 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).

2.3. Organic acid measurements

Samples were frozen at –20°C. The fecal samples (ca. 10 g) were weighed and dispersed in sterilized water (30 ml). The pH of the suspensions ranged from 6.3 to 6.8. Suspensions were centrifuged at 1000 × *g* for 5 min. The supernatants were used to analyze short-chain fatty acids (SCFA) and lactic acid with a high-pressure liquid chromatography system equipped with an electroconductivity detector (LC-20 model; Shimadzu Corp., Kyoto, Japan) as described previously (Miyamoto et al., 2005).

2.4. Statistical analyses

Measurements were analyzed using one-way ANOVA followed by Bonferroni test (experiment 1) or Student's *t* test (experiment 2). All analyses were performed using Stat View 5.0J (SAS Institute, Cary, NC). Differences were considered significant at $P < 0.05$. We applied the Tukey-Kramer test to verify whether there was any difference in observed data among six experimental sites. The difference did not reach statistical significance.

3. Results

3.1. Effects of CE or SB administration on animal performance

All calves were healthy and successfully completed the experiment. Total feed intake of the calves was 41.1 kg, 41.2 kg, 40.6 kg in control, CE, and SB group in experiment 1 (SEM, 0.92 kg; $P = 0.93$) and 43.6 kg, 44.1 kg in control, and CE group in

190 experiment 2 (SEM, 1.45 kg; $P = 0.87$), respectively. The daily weight gains were 0.57
191 kg, 0.58 kg, and 0.59 kg in experiment 1 (SEM, 0.02 kg; $P = 0.80$), and 0.57 kg and
192 0.54 kg in experiment 2 (SEM, 0.02 kg; $P = 0.58$). Total diarrhea days per head were
193 0.69, 0.61, and 0.71 in experiment 1 (SEM, 0.08; $P = 0.68$), and 0.63 and 0.89 in
194 experiment 2 (SEM, 0.25; $P = 0.61$). The addition of CE or SB did not improve fecal
195 scores, and did not show any advantages in performance measures.
196
197 *3.2. Bacterial composition and organic acid profiles of fecal and ruminal fluid samples*
198 Bacterial profiles of the calf feces are presented in Table 1. rRNAs of
199 *Bacteroides* and *Prevotella* as well as the *Cl. coccoides–Eu. rectale* groups constituted
200 the major fractions of microbiota for the seven weeks of the study, accounting for
201 approximately 39 – 48% and 10 – 29% of the total, respectively. *Atopobium* also
202 accounted for a proportion of the microbiota (4 – 8%). The *Lactobacillus–Enterococcus*
203 group and *Bifidobacterium* were shown to constitute approx. 1 – 3% each at four weeks
204 of age. The populations of *Lactobacillus–Enterococcus* and *Bifidobacterium* decreased
205 or remained almost unchanged as the animal aged, and these groups were present at low
206 proportions in the samples obtained at seven weeks of age. *Enterobacteriaceae*
207 accounted for approx. 1% throughout the experimental periods, but seemed relatively
208 high at week 4 in experiment 2. Fecal organic acid profiles were also determined in
209 these experiments (Table 2). The largest proportion in all sampling timings was acetic
210 acid (48.3 – 56.2 mmol/kg feces), followed by propionic acid and butyric acid. Lactic
211 acid and valeric acid were minor constituents of the total organic acids of calf feces. The
212 community composition and the organic acid profile were not different among the three
213 groups in experiment 1. In experiment 2, the fecal populations of *Cl. coccoides–Eu.*
214 *rectale* group were higher in CE group than control group both at 4 and 7 weeks of age

and fecal butyric acid concentration was higher (8.0 vs. 12.0 [mmol/kg feces]) at 4 weeks of age. Neither CE nor SB affected the populations of other groups, including "harmful" (*Enterobacteriaceae*) and "beneficial" (*Lactobacillus* and *Bifidobacterium*) bacteria. No Archaea species were detected in any fecal samples (data not shown).

4. Discussion

Oligosaccharides are a class of carbohydrates that are not absorbed or digested in the small intestine of animals and are readily fermented by specific microorganisms inhabiting the large intestine (Gibson, 1999; Gibson et al., 2004). Studies in various animal species have indicated that inclusion of oligosaccharides can alter populations of specific kinds of bacteria (Everard et al., 2011; Rastall et al., 2005) and then contribute to an increase in health-promoting bacteria, such as lactobacilli and bifidobacteria. Developed antimicrobial activities generated by the probiotics participate in defense of the host gastrointestinal system, as well as in the prevention and treatment of infectious bacterial and viral diarrhea (Gaggìa et al., 2010; Servin, 2004; Timmerman et al., 2005). These are a possible mode of action of well-known oligosaccharides like fructooligosaccharide (FOS) and mannan oligosaccharide (MOS). Various studies indicated that feeding FOS and MOS to calves showed certain health-promoting effects (Heinrichs et al., 2003; Quigley et al., 2002; Terre et al., 2007). In an *in vitro* study, cellobiose was shown to affect organic acid generation by mixed ruminal bacteria (Callaway and Martin, 1997; Lila et al., 2006). It was reported that CE feeding improved daily body weight gain in weanling pigs (Otsuka et al., 2004) accompanying substantial change in intestinal SCFA profiles. Therefore, we expected that CE would also act as a prebiotic and have benefits on health performance and GI ecology when

239 administered to preweaned calves, presumably in different modes of action due to its
240 bacterial specificity.

241 No effects were observed by CE or SB feeding in respect to health or growth in
242 either experiment 1 or 2. The low energy level setting of liquid feed may have been
243 responsible for the lower incidence of diarrhea in the present study, and the action of CE
244 may be unnecessary under such conditions. Such supplemental feed materials may not
245 always be required in healthy calves reared in suitable environments (Hill et al., 2005;
246 Quezada-Mendoza et al., 2011). In contrast, if variability among calves in early growth
247 rates and acceptance of dry feed exists, it seems more feasible to show beneficial effects
248 of probiotics to calves with lower performance. In addition, oligosaccharides may be
249 reserved for use in a certain period, e.g., to prevent the incidence of dyspeptic diarrhea
250 caused by the marked increase in solid feed intake before and after the day of weaning.
251 In relation to this, we performed performance measurements at eight weeks in
252 experiment 1, but no differences were observed among groups in the incidence of
253 diarrhea (data not shown). A recent report indicated that interindividual similarity in the
254 rectal flora of newborn calves decreases over time (Mayer et al., 2012). Therefore,
255 feeding oligosaccharides in the very early stages of the life may amend a variety of GI
256 microbiota among individuals to similar (and possibly desirable) microflora.

257 A previous in vivo study (Hasunuma et al., 2011) indicated that CE feeding in
258 calves improved DG and feed efficiency during the postweaning period, mainly due to
259 the enhancement of rumen SCFA production by affecting specific groups of rumen
260 microbes. The mechanisms may involve butyric acid produced by CE-utilizing bacteria
261 inhabiting the digestive tract and subsequent increases in plasma insulin concentration
262 by SCFA absorption. Butyric acid is also involved in the growth and differentiation of
263 intestinal cells, thereby improving digestion and absorption efficiency, which may

264 contribute to improved growth performance of the host animal (Neish, 2009). In this
265 study, we found in experiment 2 that CE also has the potential to change bacterial flora
266 in the large intestine, resulting in enhancement of butyric acid-producing bacteria
267 belonging to *Cl. coccoides–Eu. rectale*. That is, CE may have a specific nutritional
268 effect not only in the postweaning period but also in the preweaning period. A desirable
269 intestinal community composition in calves may contribute to the further improvement
270 of growth performance at an older age.

271 We employed a butyric acid-producing *Cl. butyricum* strain as a symbiotic
272 strain to CE in experiment 1, while no advantageous effects were observed at that time.
273 The effects of SB feeding were not assessed in experiment 2, but the addition of this
274 probiotic may contribute to further increase butyric acid concentration in lower GI than
275 only CE administration when applied to milk-fed calves. The amount of daily
276 supplementation of this strain may have originally been too low compared to the huge
277 numbers of bacteria inhabiting the large intestine. Therefore, the advantages of
278 introducing this strain may have been compromised. On the other hand, as it has been
279 recognized that the use of the strain with a similar level in diets (2.5×10^8 cfu/kg feed)
280 for weaning piglets and chickens can improve weight gain and feed efficiency (EFSA,
281 2011). Therefore, further trials are necessary to determine how the strain can exert the
282 nutritional effect on ruminants.

283 The type of liquid feed (MR or whole milk) employed in each experiment is
284 one of the reasons for the differences in results of intestinal ecology by CE feeding.
285 There have been no previous reports regarding evaluation of differences between whole
286 milk and MR in the effects of prebiotic supplementation on the health and growth of
287 calves, although both are commonly used as liquid feeds. Therefore, we performed an
288 indirect but comparable study to investigate the effects of CE in calves fed MR or whole

289 milk in the preweaning period on health and growth measures as well as fecal bacterial
290 profiles. The effects of CE were observed only in whole milk-fed calves, possibly
291 because of the intestinal community brought on by the whole milk ingestion and/or of
292 any milk-specific components. For example, the *Cl. coccoides–Eu. rectale* population at
293 seven weeks in the control group in experiment 2 was higher than at the same time point
294 in the control group in experiment 1 (20% vs. 13%), even though these data did not
295 permit a direct comparison. On referring to previous reports regarding prebiotic feeding
296 to calves, MR was mostly used as a liquid feed (Heinrichs et al., 2003; Quigley et al.,
297 2002; Terre et al., 2007). Health benefits of prebiotics have been indicated, in turn, there
298 was no evidence that lactobacilli and/or bifidobacteria were enriched by the
299 oligosaccharides in these experiments. Since they may have a different impact to the
300 intestinal community, the choice of liquid feeds and the combination with prebiotics
301 should be considered more carefully to successfully modulate the enteric flora of
302 preweaned calves.

303 This is also the first study to monitor the changes in composition of the fecal
304 bacterial community with a high-resolution molecular approach, to address the effects
305 of feeding a specific type of oligosaccharide on the intestinal ecology of calves. Our
306 results indicate that CE and SB supplementation has little effect on maintenance of the
307 levels of probiotic bacteria, regardless of the type of liquid feed. Prebiotics may not
308 necessarily bring health benefits to ruminants in the same manner as to monogastric
309 animals, in which continuous administration of prebiotics helps colonization of the large
310 intestine by beneficial microbes, as shown in previous studies (Mikkelsen et al., 2003;
311 Ouwehand et al., 2005; Paßlack et al., 2012). On the other hand, as another kind of
312 probiotic and prebiotic utilization, the promotion of bacterial groups specific to the

ruminant community (i.e., fibrolytic bacteria and lactic acid-utilizing bacteria) inhabiting the large intestine is a feasible manner of improving animal husbandry.

5. Conclusion

Cellooligosaccharides are utilized by specific microbes inhabiting the calf intestine, resulting in changes in the intestinal SCFA profile, while the effects of CE supplementation vary with experiment. On the other hand, CE and SB supplementation seemed to have no effect on maintenance of the levels of *Lactobacillus* and *Bifidobacterium* species in the large intestine of preweaning calves. Unlike monogastric animals, it was not easy to improve colonization by probiotic bacteria in ruminants by continuous administration of prebiotics. However, applying oligosaccharides to calves still seems advantageous with regard to two points. First, the use of prebiotic oligosaccharides may be useful as an alternative to antibiotics in calves suffering from dyspeptic scouring. Second, as another type of prebiotic utilization, it is possible to promote colonization by bacterial groups that are specific to the ruminant community, e.g., fibrolytic bacteria and lactic acid-utilizing bacteria.

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Conflict of interest statement

All the authors have no conflict of interest.

Table

Table 1. Fecal bacterial compositions of calves fed celloorigosaccharide (CE), CE plus *Cl. butyricum* (SB), or control and fed milk replacer (Experiment 1) and milk (Experiment 2)[1].

Item	Experiment 1				Experiment 2		
	Control	CE	SB	SEM	Control	CE	SEM
4 weeks							
Bacteroides/*Prevotella*	43.3	47.7	43.8	1.6	39.7	39.2	3.1
Cl. coccoides- Eu. rectale group	12.8	13.2	12.9	1.0	16.0[a]	27.5[b]	2.3
Atopobium	7.9	8.0	7.4	0.2	5.5	6.6	0.6
Enterobacteriaceae	1.3	1.2	1.6	0.3	0.8	1.0	0.2
Lactobacillus	0.7	0.8	0.7	0.5	2.3	1.6	0.4
Bifidobacterium	1.4	1.4	1.6	0.1	2.3	2.6	0.5
7 weeks							
Bacteroides/*Prevotella*	38.7	46.1	43.8	1.6	44.1	39.9	2.7
Cl. coccoides- Eu. rectale group	12.5	11.3	9.8	0.5	20.1[a]	29.3[b]	2.1
Atopobium	4.4	3.7	3.9	0.3	5.4	8.0	0.9
Enterobacteriaceae	0.9	1.1	0.9	0.1	0.8	0.9	0.2
Lactobacillus	0.8	1.1	0.7	0.1	1.7	1.7	0.3
Bifidobacterium	0.9	1.2	1.3	0.1	1.2	1.3	0.3

[1]Measurements are expressed as % of total 16S rRNA.

[a,b]Means in the same row with different superscripts are significantly different ($P < 0.05$).

Table 2. Fecal organic acid concentrations of calves fed cellooligosaccharide (CE), CE plus *Cl. butyricum* (SB), or control and fed milk replacer (Experiment 1) and milk (Experiment 2)[1].

Item	Experiment 1				Experiment 2		
	Control	CE	SB	SEM	Control	CE	SEM
4 weeks							
Lactic acid	0.4	0.3	0.3	0.0	0.1	0.3	0.1
Acetic acid	54.3	53.6	55.2	1.8	48.3	55.1	3.9
Propionic acid	9.3	6.0	5.9	1.0	10.0	12.2	1.8
Butyric acid	7.2	4.4	6.8	0.6	8.0[a]	12.0[b]	0.8
Valeric acid	1.1	1.2	2.2	0.5	0.9	1.6	0.3
7 weeks							
Lactic acid	0.2	0.2	0.3	0.1	0.8	0.4	0.2
Acetic acid	52.9	54.7	56.2	2.4	54.4	52.4	1.9
Propionic acid	6.7	9.8	9.1	1.3	16.9	14.1	1.2
Butyric acid	5.5	5.6	4.5	0.6	9.8	10.6	0.7
Valeric acid	0.8	1.5	2.6	0.4	0.7	0.6	0.1

[1]Measurements are expressed as mmol/kg feces.

[a,b]Means in the same row with different superscripts are significantly different ($P < 0.05$).