Augmentation of Vaccenate Production and Suppression of Vaccenate Biohydrogenation in Cultures of Mixed Ruminal Microbes

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ABSTRACT

To increase ruminal outflow of *trans*-vaccenic acid (*t*-VA), a new strain of *Butyrivibrio fibrisolvens* (MDT-10) was isolated that has a great ability to hydrogenate linoleic acid (LA) to *t*-VA. When strain MDT-10 was added to the batch cultures of mixed ruminal microbes (1% of the total number of viable ruminal bacteria), LA conversion to *t*-VA increased greatly; after 3 h, *t*-VA levels were >4-fold higher than the control. By 10 h, all of the *t*-VA was hydrogenated to stearic acid. However, when a new strain of *Bifidobacterium adolescentis* (HF-11), which has a high capacity for incorporation of *t*-VA, was added in conjunction with MDT-10 (1% of the total number of ruminal bacteria), *t*-VA levels after 10 h were 6 times higher than with MDT-10 alone. These results suggest that *t*-VA produced by MDT-10 was incorporated into HF-11 cells, resulting in protection of *t*-VA from *t*-VA-hydrogenating microbes. Similar results were obtained in a continuous culture of mixed ruminal microbes in which addition of HF-11 simultaneously with MDT-10 increased the amount of *t*-VA in the effluent 2.5-fold. Both MDT-10 and HF-11 appeared to grow readily in the presence of mixed ruminal microbes. Sixty-two percent of *t*-VA incorporated by HF-11 was present in the free form, whereas 19, 15, and 3%, respectively, were incorporated into monoacylglycerol, glycerophospholipid, and diacylglycerol fractions. Because these lipids can be digested in the small intestine, it is likely that most *t*-VA in HF-11 cells is absorbed. Thus, introduction of MDT-10 and HF-11 simultaneously to the rumen might increase the amount of *t*-VA absorbed and might consequently increase the conversion of *t*-VA to conjugated linoleic acid in tissue.

Key words: conjugated linoleic acid, *trans*-vaccenic acid, *Butyrivibrio fibrisolvens*, *Bifidobacterium adolescentis*

INTRODUCTION

Conjugated linoleic acid (CLA), particularly *cis* 9, *trans* 11 (*c9*, *t11*)-CLA, has been reported to exhibit

beneficial effects on health, including protection against carcinogenesis, atherosclerosis, and tumorigenesis and the improvement of hyperinsulinemia and immune functions (Pariza, 2004; Wahle et al., 2004). Furthermore, CLA, especially *t10*, *c12*-CLA, has reduced the ratio of body fat to muscle mass and has altered the ratio of low-density to high-density lipoprotein cholesterol (Pariza, 2004; Wang and Jones, 2004).

In contrast, it has been suggested that *t10*, *c12*-CLA has detrimental effects (Wahle et al., 2004), including procarcinogenic effects in animal models of colon and prostate cancer (Rajakangas et al., 2003). Thus, *c9*, *t11*-CLA appears to benefit health with less risk than *t10*, *c12*-CLA. In ruminants, *t10*, *c12*-CLA is a potent inhibitor of milk fat synthesis, whereas *c9*, *t11*-CLA has no such effect (Bauman and Griinari, 2003; Peterson et al., 2004).

Representative foods containing CLA include the meat and milk of ruminants, which primarily contain *c9*, *t11*-CLA. In ruminants, CLA is produced via 2 routes. One is microbial isomerization of linoleic acid (LA) to CLA in the rumen (Harfoot and Hazlewood, 1997). However, most of this CLA is hydrogenated to *trans*-vaccenic acid (*t*-VA) and then to stearic acid (SA). Therefore, only a small amount of nonbiohydrogenated CLA can be absorbed from the small intestine. The other route of CLA production is Δ^9 -desaturation of *t*-VA, absorbed from the small intestine, to CLA in the tissues of the host animals (Griinari et al., 2000). Recent studies indicate that a high percentage of milk CLA (78 to 93%) is derived from Δ^9 -desaturation of *t*-VA (Corl et al., 2001; Piperova et al., 2002), and meat CLA may also be produced from absorbed *t*-VA (Daniel et al., 2004). Thus, increasing *t*-VA production in the rumen may be a feasible method for increasing the CLA content of milk and meat. It has been reported that *t*-VA production in the rumen, as well as CLA content in milk and meat, was enhanced by feeding cows oils containing high levels of polyunsaturated fatty acids (PUFA), including soybean, corn, peanut, sunflower, linseed, and fish oils (Dhiman et al., 2000; Chouinard et al., 2001; Duckett et al., 2002). Because PUFA are known to have toxic effects on microbes (Harfoot and Hazlewood, 1997), this increase may be due to suppression of the

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growth or activity of some PUFA-hydrogenating microbes. The addition of ionophores, such as monensin (Fellner et al., 1997; Sauer et al., 1998), and copper (Morales et al., 2000) has also been reported to enhance the CLA content in milk, but these substances may be similarly toxic to PUFA-hydrogenating microbes. It has been suggested that decreasing pH increases postruminal flow of *t*-VA (Qiu et al., 2004), but low pH may also inhibit the growth of acid-sensitive microbes. Thus, great care is required for use of substances or treatments that suppress microbial growth, as they could depress overall ruminal metabolism, including fiber digestion (Hino and Asanuma, 2003).

Butyrivibrio fibrisolvens, a representative PUFA-hydrogenating ruminal bacterium, produces the highest levels of *t*-VA from LA among bacteria surveyed (Harfoot and Hazlewood, 1997; Jiang et al., 1998; Kim et al., 2000). Introduction of *Butyr. fibrisolvens*, especially strains that have a high capacity to convert LA to *t*-VA (Fukuda et al., 2005), into the rumen may increase *t*-VA production, and the prevention of *t*-VA hydrogenation to SA in the rumen may increase *t*-VA absorption. If *t*-VA is incorporated into microbial cells without hydrogenation, *t*-VA could be protected from *t*-VA-hydrogenating microbes (encapsulation of *t*-VA). However, the ability of ruminal microbes, including *Butyr. fibrisolvens*, to incorporate fatty acids (FA) is generally low (Hino et al., 1993a).

The principal objectives of this study were 1) to find *Butyr. fibrisolvens* strains with a high capacity to convert LA to *t*-VA, 2) to find gastrointestinal bacteria with a high capacity to incorporate *t*-VA, and 3) to examine whether the addition of a *t*-VA-producing strain and a *t*-VA-incorporating bacterium increases *t*-VA production and accumulation in cultures of mixed ruminal microbes.

MATERIALS AND METHODS

Sources of Bacteria and Culture Conditions

Butyrivibrio fibrisolvens TH1 was obtained as described previously (Fukuda et al., 2005). Strain MDT-10 was newly isolated from the rumen of a Japanese native goat by Hungate's role-tube method and was identified according to Bryant (1986). Similarly, *Bifidobacterium adolescentis* HF-11 (Scardovi, 1986), *Escherichia coli* HF-23 (Orskov, 1986), *Bifidobacterium breve* HF-31 (Scardovi, 1986), and *Lactobacillus fermentum* HF-3 (Kandler and Weiss, 1986) were isolated from human feces and identified in this study.

Each bacterium was routinely grown in 30- or 50-mL serum vials containing 15 or 30 mL of growth medium consisting of clarified ruminal fluid (Miyazaki et al., 1992) and a basal medium (1:3) containing (g/L) 0.45

K₂HPO₄, 0.45 KH₂PO₄, 0.9 (NH₄)₂SO₄, 0.9 NaCl, 0.12 CaCl₂·2H₂O, 0.19 MgSO₄·7H₂O, 1.0 trypticase-peptone (BBL, Becton Dickinson, Cockeysville, MD), 1.0 yeast extract (Difco Laboratories Inc., Detroit, MI), 3.0 glucose, and 0.6 cysteine·HCl (pH 7.0). Batch cultures were incubated in triplicate at 39°C, maintaining the pH between 6.5 and 7.0 (Miyazaki et al., 1992).

Both LA and *t*-VA were added in a mixture with BSA at 1:3 (Fukuda et al., 2005). Cell growth was estimated by measuring the optical density at 600 nm (OD₆₀₀) or cellular nitrogen (cell-N) as reported previously (Fukuda et al., 2005). Viable cells of *Butyr. fibrisolvens*, *Bifid. adolescentis*, and mixed ruminal bacteria were enumerated by the roll-tube method (Fukuda et al., 2005).

Isolation of Bacteria that Have a High Capacity to Incorporate *t*-VA, but Have No Capacity to Hydrogenate *t*-VA

Bacteria were isolated from the rumen of goats and from human feces by the role-tube method just described, and each bacterium was grown overnight in a medium containing *t*-VA (5 µmol/15 mL). After removal of *t*-VA adsorbed on the bacterial cell surface by washing with 0.1% (vol/vol) Triton X-100, total lipids in cells were extracted with chloroform:methanol (2:1), and the amount of *t*-VA was determined by GLC as described subsequently.

The ability to hydrogenate *t*-VA was determined by measuring LA isomerase (LA-I) and CLA reductase (CLA-R) activities as described subsequently. The rate of *t*-VA uptake was measured as follows. Each bacterial strain was grown to an OD₆₀₀ of 1.0, and after the addition of *t*-VA (5 µmol/15 mL of culture), the culture was incubated until an OD₆₀₀ of 1.5 was reached (late log stage). The rate of *t*-VA uptake was calculated for each culture (µmol/h) and then divided by the estimated cell-N (i.e., cell-N determined by the average of cell-N at an OD₆₀₀ of 1.0 and cell-N at an OD₆₀₀ of 1.5) to generate the rate of *t*-VA uptake per cell-N (µmol/h per mg of cell-N).

Culture Conditions of Mixed Ruminal Microbes

Mixed ruminal microbes were obtained from ruminal fluid by squeezing ruminal contents from a Japanese native goat with cheesecloth (Hino et al., 1993b). Growth medium and conditions for batch culture of mixed ruminal microbes were basically as described previously.

Apparatus and general procedures for continuous culture were as described previously (Hino and Hamano, 1993; Hino et al., 1993a). Briefly, the apparatus con-

sisted of 3 identical sets of equipment, including a reservoir bottle to supply saline solution, a peristaltic pump, an overflow-type fermenter (300 mL) in a water bath (39°C), and a bottle to collect the effluent in an ice bath (samples for analysis). Prior to and during culture incubation, CO₂ gas was purged through the entire system to avoid contact with O₂.

Mixed ruminal microbes were obtained by diluting ruminal fluid with saline solution (1:2), and the microbial suspension was introduced into fermenters under a stream of CO₂. Dilution rate was set at 0.1/h, and the pH was maintained at 6.8 to 6.9. A feed (3 g per fermenter) containing 50% cornstarch, 15% cellulose powder (Advantech, Tokyo, Japan), 10% fructo-oligosaccharide (Meiji Seika Kaisya, Tokyo, Japan), 20% casein, and 5% trypticase-peptone (BBL) was added to the fermenters every 6 h. Mixed with the feed, LA also was added every 6 h (70 µmol per fermenter). At the initiation of continuous culture, *Butyr. fibrisolvens* MDT-10 and *Bifid. adolescentis* HF-11 were added to fermenters at a concentration of 1% (1×10^9 cfu/mL) of the total viable counts of mixed ruminal bacteria (1×10^{11} cfu/mL). Effluents were collected every hour for analysis. Culture incubation was carried out for 24 h using one fermenter per test group, and the experiment was repeated 2 more times (n = 3) following a 3 × 3 Latin square design.

Identification of the Molecular Species of *t*-VA Incorporated in a *t*-VA-Incorporating, Non-*t*-VA-Hydrogenating Bacterium (*Bifid. adolescentis* HF-11)

Bifidobacterium adolescentis HF-11 cells grown with *t*-VA (5 µmol/15 mL of culture) to late log stage (5 h) were washed with 0.1% Triton X-100, and total lipids were extracted as described previously. The lipids were separated by thin-layer chromatography (TLC; Silica Gel B-10, Wako Pure Chemicals, Osaka, Japan; 10 × 20 cm) using a developing solvent of chloroform:acetone (96:4). To separate 1- and 2-monoacylglycerol (MG) more clearly, chloroform:methanol (95:5) was also used. As standard substances, triolein (triacylglycerol), oleic acid (free FA), diolein [mixture of 1, 3- and 1, 2-diacylglycerol (DG)], 1-monoolein, 2-monoolein, and phosphatidylcholine (phospholipids; PL), all purchased from Sigma (St. Louis, MO), were used. Subsequently, *t*-VA in each spot was quantified by GLC. Spots on TLC plates were detected by the conventional method (Fukuda et al., 2002), and PL was detected by spraying the Dittmer-Lester reagent (Dittmer and Lester, 1964).

Because *t*-VA was detected in the TLC spots corresponding to MG and DG, MG and DG were confirmed as follows. The bands on TLC plates corresponding to

MG and DG were scraped off the plates and extracted with chloroform:methanol (2:1). The extracts were divided into 2 equal parts; the first was for quantification of FA by GLC, and the other was for quantification of glycerol. Glycerol was quantified using a kit for the determination of blood triacylglycerol (Triglyceride E-Test Wako; Wako Pure Chemicals). Briefly, MG and DG were hydrolyzed by lipoprotein lipase, and liberated glycerol was measured by the enzymatic method reported by Spayd et al. (1978).

Quantification of FA and Organic Acids

Lipids were extracted by shaking cultures or effluents in continuous culture with isopropanol:isooctane:6 N H₂SO₄ (20:10:1) as reported previously (Fukuda et al., 2002). The lipids were then transmethylated with 5% HCl in methanol at 60°C for 20 min under a gas phase of N₂ (Fukuda et al., 2002). The methylated FA were analyzed by GLC and mass spectrometry as described previously (Fukuda et al., 2002). Organic acids produced by isolated bacteria and in the continuous culture of mixed ruminal microbes were analyzed by HPLC to identify the bacteria and to estimate the alterations in fermentation, respectively (Fukuda et al., 2002).

Assay of LA-I and CLA-R Activities

To measure LA-I and CLA-R activities in intact cells, cultures grown to the late log stage were immediately cooled in an ice bath and centrifuged (20,000 × *g*, 10 min, 4°C). The pellets were washed twice with anaerobic 50 mM KPi buffer (pH 7.0) and immediately subjected to enzyme assay (Fukuda et al., 2005). The LA-I and CLA-R activities were assayed by the method of Hunter et al. (1976) with some modifications as described previously (Fukuda et al., 2005).

Enzyme activity was expressed as specific activity (i.e., µmoles/min per mg of cell-N), which reflects the amount of enzyme per cell. Cellular nitrogen was determined by the Kjeldahl method followed by the quantitation of ammonia by the indophenol method (Fukuda et al., 2005).

Statistical Analyses

Differences among bacterial strains were analyzed by one-way ANOVA, and Tukey's test was used when the *F*-test was significant. In batch culture and continuous culture experiments, data were analyzed by 2-way ANOVA (bacterial addition and incubation time). Tukey's test was done when the interaction was significant. Differences with *P* values <0.05 were considered significant. Data are expressed as means ± SE. All sta-

Table 1. Capacity of isolated bacteria to incorporate *trans*-vaccenic acid (*t*-VA) and specific activities of linoleic acid isomerase (LA-I) and conjugated linoleic acid reductase (CLA-R)

Bacterium	<i>t</i> -VA incorporated ¹ ($\mu\text{mol}/\text{mg}$ of cell-N)	Specific activity ²	
		LA-I	CLA-R
<i>Bifidobacterium adolescentis</i> HF-11	0.76 ± 0.09^a	ND ^{3,b}	ND
<i>Escherichia coli</i> HF-23	0.18 ± 0.03^b	ND ^b	ND
<i>Bifidobacterium breve</i> HF-31	0.09 ± 0.01^c	0.07 ± 0.01^a	ND
<i>Lactobacillus fermentum</i> HF-3	0.07 ± 0.01^c	ND ^b	ND
<i>Butyrivibrio fibrisolvens</i> MDT-10	ND	0.52 ± 0.03	0.29 ± 0.02

^{a-c}Means with different superscript letters within a column differ significantly ($P < 0.01$).

¹Amount of *t*-VA in the cells grown to full growth (5 to 6 h; mean $\pm$ SE; $n = 3$); Cell-n = cellular nitrogen.

²Activities are expressed as $\mu\text{mol}/\text{min}$ per mg of cell-N (mean $\pm$ SE; $n = 3$).

³ND = Not detected ($P < 0.01$).

tistical analyses were performed with the SigmaStat Statistical Analysis System (Jandel Scientific, San Rafael, CA).

RESULTS AND DISCUSSION

Isolation of a *Butyr. fibrisolvens* Strain with a High Capacity to Convert LA to *t*-VA and Bacteria with a High Capacity to Incorporate *t*-VA

A newly isolated strain of *Butyr. fibrisolvens* (MDT-10) converted LA to *t*-VA approximately 30% faster ($P < 0.05$) than strain TH1, which had the highest capacity to produce *t*-VA reported so far (Fukuda et al., 2005; 1.6 vs. 1.2 $\mu\text{mol}/\text{h}$ per mg of cell-N). Activity of LA-I in MDT-10 was comparable with that in TH1 (0.53 vs. 0.45 $\mu\text{mol}/\text{min}$ per mg of cell-N; $P > 0.05$), whereas CLA-R activity in MDT-10 was nearly 40% higher than in TH1 (0.26 vs. 0.19 $\mu\text{mol}/\text{min}$ per mg of cell-N; $P < 0.01$). This result is consistent with the fact that MDT-10 rapidly converted LA to *t*-VA with little accumulation of CLA when the initial cell numbers were $>1 \times 10^{10}$ cfu/mL (data not shown). When the numbers were lower, CLA was accumulated once and then disappeared slowly. As explained previously (Fukuda et al., 2005), when cell numbers are low, more LA may be adsorbed to cells, and cell growth delays are possibly due to temporary cell damage. During this growth retardation, electron supply from fermentation may be decreased, which results in decreased electron donation to CLA-R.

Neither rumen nor stock ruminal bacteria, including *Butyr. fibrisolvens*, yielded bacteria that rapidly incorporated *t*-VA (data not shown). Even strain MDT-10, which efficiently converts LA to *t*-VA, incorporated only low levels of *t*-VA (Table 1). However, 4 bacteria that are able to incorporate *t*-VA were isolated from human feces. Of these, *Bifid. adolescentis* HF-11 demonstrated the highest concentration of incorporated *t*-VA ($P < 0.01$; Table 1).

The total amount of *t*-VA in cultures (cells plus culture supernatant) recovered after incubation was nearly equivalent to the amount of *t*-VA initially added to the medium. Because HF-11 does not synthesize *t*-VA de novo (see subsequent), these results indicate that HF-11 cannot hydrogenate *t*-VA. In addition, HF-11 demonstrated no LA-I or CLA-R activity (Table 1).

Strain HF-11 incorporated oleic acid (c9-C18:1) as readily as *t*-VA, whereas the rate of C18:0 (SA) uptake was one-third the rate of *t*-VA uptake ($P < 0.05$; Table 2). This is important in that HF-11 carries *t*-VA more readily than SA. The rate of C18:2 (LA and CLA) uptake was one-tenth the rate of *t*-VA uptake, and C18:3 (linolenic acid) was incorporated more slowly than C18:2 ($P < 0.05$; Table 2).

Molecular Species of *t*-VA Incorporated in *Bif. adolescentis* HF-11

When HF-11 was grown to saturation with *t*-VA (5 $\mu\text{mol}/30$ mL of medium), $>90\%$ of *t*-VA was recovered in the cells. The lipids in HF-11 contained mainly free FA, MG, DG, and PL (Figure 1). As shown in Table 3, *t*-VA was present in the free FA (62%), DG (1, 2-DG

Table 2. Capacity of *Bifidobacterium adolescentis* HF-11 to incorporate several fatty acids (FA)

FA	FA incorporated ($\mu\text{mol}/\text{h}$ per mg of cell-N ¹)
Stearic acid (C18:0)	0.04 ± 0.02^b
Oleic acid (<i>cis</i> 9-C18:1)	0.13 ± 0.03^a
Vaccenic acid (<i>trans</i> 11-C18:1)	0.13 ± 0.02^a
Linoleic acid (<i>cis</i> 9, <i>cis</i> 12-C18:2)	0.01 ± 0.01^c
Conjugated linoleic acid (<i>cis</i> 9, <i>trans</i> 11-C18:2)	0.01 ± 0.01^c
Linolenic acid (<i>cis</i> 9, <i>cis</i> 12, <i>cis</i> 15-C18:3)	ND ^{2,d}

^{a-d}Means ($\pm$ SE) with different superscript letters within a column differ significantly ($P < 0.05$; $n = 3$).

¹Cell-N = Cellular nitrogen.

²ND = Not detected ($P < 0.01$).

Figure 1. Thin-layer chromatograms of the lipids in *Bifidobacterium adolescentis* HF-11 grown in the presence (lane 2) or absence (lane 1) of *trans*-vaccenic acid (*t*-VA). Lane 3: Lipids in the cells grown with *t*-VA that were hydrolyzed with weak alkali [A: chloroform:acetone (96:4); B: chloroform:methanol (95:5)]. TG = triacylglycerol; DG = diacylglycerol; MG = monoacylglycerol; PL = phospholipid; Std = standard substances.

Table 3. Fatty acid (FA) composition of lipids in *Bifidobacterium adolescentis* HF-11 grown in the presence or absence of *trans*-vaccenic acid (*t*-VA; 5 μ mol/30 mL of medium)

Molecular species of lipids	<i>t</i> -VA ¹	Molar percentage of FA						Total FA (μ mol per vial)	<i>t</i> -VA in cells	
		C14:0	C16:0	C18:0	<i>cis</i> -9-C18:1	<i>t</i> -VA	Others		(μ mol per vial)	(%)
Free FA	–	4 $\pm$ 1	34 $\pm$ 3*	38 $\pm$ 4*	22 $\pm$ 2*	0 $\pm$ 0	1 $\pm$ 0	2.11 $\pm$ 0.11		
	+	0 $\pm$ 0	8 $\pm$ 1	3 $\pm$ 1	0 $\pm$ 0	89 $\pm$ 4*	0 $\pm$ 0	3.03 $\pm$ 0.18*	2.79	62
DG ²	–	17 $\pm$ 4*	48 $\pm$ 5*	35 $\pm$ 4*	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0.73 $\pm$ 0.05*		
	+	0 $\pm$ 0	8 $\pm$ 1	8 $\pm$ 1	0 $\pm$ 0	84 $\pm$ 3*	0 $\pm$ 0	0.23 $\pm$ 0.02	0.15	3
MG ³	–	15 $\pm$ 3*	42 $\pm$ 5*	43 $\pm$ 5*	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0.20 $\pm$ 0.02		
	+	0 $\pm$ 0	5 $\pm$ 1	6 $\pm$ 1	0 $\pm$ 0	89 $\pm$ 3*	0 $\pm$ 0	0.97 $\pm$ 0.10*	0.87	19
PL ⁴	–	13 $\pm$ 3*	58 $\pm$ 6*	6 $\pm$ 2	7 $\pm$ 2*	0 $\pm$ 0	16 $\pm$ 4	1.90 $\pm$ 0.13*		
	+	0 $\pm$ 0	7 $\pm$ 1	2 $\pm$ 1	0 $\pm$ 0	78 $\pm$ 4*	13 $\pm$ 2	0.88 $\pm$ 0.07	0.69	15

¹+ = Added; – = not added.

²DG = 1, 2-Diacylglycerol plus 1, 3-diacylglycerol.

³MG = 1-Monoacylglycerol plus 2-monoacylglycerol.

⁴PL = Phospholipid.

*Means with an asterisk in each block differ significantly ($P < 0.01$; $\pm$ SE; $n = 3$).

Table 4. Fatty acid (FA) composition of total lipids in *Butyrivibrio fibrisolvens* MDT-10 and *Bifidobacterium adolescentis* HF-11 cells grown in the presence of linoleic acid (LA)¹

Bacterial strain	Molar percentage						Other	Total FA (μmol per vial)
	C14:0	C16:0	C18:0	<i>cis</i> 9-C18:1	<i>trans</i> 11-C18:1	C18:2		
MDT-10	24 $\pm$ 2 ^a	38 $\pm$ 3 ^a	8 $\pm$ 1 ^a	0 $\pm$ 0	0 $\pm$ 0 ^a	0 $\pm$ 0	30 $\pm$ 3 ^a	4.9 $\pm$ 0.3
HF-11	0 $\pm$ 0 ^b	39 $\pm$ 3 ^a	40 $\pm$ 3 ^b	2 $\pm$ 1	0 $\pm$ 0 ^a	8 $\pm$ 1	11 $\pm$ 1 ^b	5.1 $\pm$ 0.3
MDT-10 and HF-11	10 $\pm$ 1 ^c	21 $\pm$ 2 ^b	4 $\pm$ 1 ^a	1 $\pm$ 1	51 $\pm$ 4 ^b	0 $\pm$ 0	13 $\pm$ 2 ^b	5.0 $\pm$ 0.2

^{a-c}Means with different superscript letters within a column differ significantly ($P < 0.01$).

¹Monocultures and cocultures were grown in the presence of LA (18 $\mu\text{mol}/30\text{ mL}$ of medium) for 16 h, and then bacterial cells were washed twice with 0.1% (vol/vol) Triton X-100 solution. Mean values of FA ($\pm\text{SE}$; $n = 3$) in the total lipids are shown.

and 1, 3-DG; 3%), MG (1-MG and 2-MG; 19%), and PL (15%) cell fractions with *t*-VA at molar percentages of 89, 78, 84, and 89%, respectively. When HF-11 was grown without *t*-VA, no *t*-VA was detected in either the cells (Table 3) or the culture supernatant (data not shown), indicating that HF-11 does not synthesize *t*-VA de novo. The sum of total FA in Table 3 (total FA in cells) was nearly equal between cells grown with *t*-VA (5.11 μmol per vial) and those without *t*-VA (4.94 μmol per vial), i.e., the increase in *t*-VA was nearly equivalent to the decreases in other FA, which demonstrates that de novo FA synthesis is suppressed by exogenous *t*-VA.

Diacylglycerol and MG, tentatively identified by TLC (Figure 1), were confirmed by the ratio of FA to glycerol (2:1 and 1:1, respectively) after hydrolysis by lipoprotein lipase. Weak alkali hydrolysis (0.1 N NaOH, 100°C, 1 h) suggested that glycerol-PL is the most abundant PL in HF-11 cells (Figure 1). Because these lipids are digested in the small intestine, much of the *t*-VA in HF-11 cells may be absorbed by the host animal.

Incorporation of *t*-VA Produced by *Butyr. fibrisolvens* MDT-10 into *Bifid. adolescentis* HF-11 Cells

Preliminary experiments showed that the specific growth rate of MDT-10 was similar to that of HF-11 (approximately 1.0/h) and that the ratio of concentrations of the 2 bacteria in cocultures, as estimated by microscopy, was unchanged until the stationary phase. These results suggest that there is no antagonism between MDT-10 and HF-11 growth.

When either MDT-10 or HF-11 was grown with LA in monoculture, no *t*-VA was detected in the washed cells (Table 4), demonstrating that MDT-10 does not incorporate *t*-VA and that HF-11 cannot hydrogenate LA to *t*-VA. However, when the 2 bacteria were cultured together in the presence of LA, *t*-VA made up 51% of total FA in the washed cells, and nearly 80% of added LA (18 $\mu\text{mol}/30\text{ mL}$ of medium) was recovered as *t*-VA in

cells. These results indicate that HF-11 can efficiently incorporate the *t*-VA produced by MDT-10. In the coculture, the percentages of other FA such as 16:0 and 18:0 were decreased, suggesting that de novo FA synthesis in HF-11 was suppressed by exogenously incorporated *t*-VA.

Effect of the Addition of *Butyr. fibrisolvens* MDT-10 and *Bifid. adolescentis* HF-11 on *t*-VA Production and Accumulation in a Batch Culture of Mixed Ruminal Microbes

Addition of MDT-10 (at 1% of the total number of viable ruminal bacteria) to batch cultures of mixed ruminal microbes (2×10^{11} cfu/mL) greatly enhanced the rate of LA conversion to *t*-VA, leading to concentrations >4-fold greater than the control at 3 h ($P < 0.01$; Figure 2, A and B). However, almost all of the *t*-VA was hydrogenated to SA by 10 h.

However, addition of HF-11 with MDT-10 (at an equal number) decreased the rate of *t*-VA hydrogenation, and *t*-VA accumulation after 10 h was >10-fold greater than in the absence of HF-11 ($P < 0.01$; Figure 2, B and C). When only HF-11 was added, the corresponding value was only 4-fold greater than the control at 10 h (12 vs. 41 μM ; $P < 0.05$).

Effect of the Addition of *Butyr. fibrisolvens* MDT-10 and *Bifid. adolescentis* HF-11 on *t*-VA Production and Accumulation in a Continuous Culture of Mixed Ruminal Microbes

The addition of MDT-10 to mixed ruminal microbes grown with LA in continuous culture at a level of 1% of total viable ruminal bacteria resulted in a 30% increase in the total amount of *t*-VA flowing from the fermenter in 24 h (55 vs. 42 μmol , $n = 3$, $P < 0.05$; Figure 3, A and B). The rate of LA conversion to *t*-VA, as well as the concentration of *t*-VA in the effluent, increased with an increasing number of doses of additional LA supplemented at 0, 6, 12, and 18 h (Figure

Figure 2. Effect of the addition of *Butyrivibrio fibrisolvens* MDT-10 and *Bifidobacterium adolescentis* HF-11 on *trans*-vaccenic acid (*t*-VA) production and accumulation in a batch culture of mixed ruminal microbes (total viable bacteria, 2×10^{11} cfu/mL). A) Neither of the bacteria was added (control); B) only *Butyr. fibrisolvens* (2×10^9 cfu/mL) was added; and C) both *Butyr. fibrisolvens* (2×10^9 cfu/mL) and *Bifid. adolescentis* (2×10^9 cfu/mL) were added. ○ = linoleic acid; ◇ = conjugated linoleic acid; ▲ = *t*-VA; □ = stearic acid. Bars indicate SE ($n = 3$).

3B). This may be due to an increase in the MDT-10 cell number with time, not due to an increase in LA-hydrogenating activity, because the enhancement of LA-I and CLA-R activities by LA attains the maximum within 2 to 3 h (Fukuda et al., 2005). It is likely that MDT-10 grew at a higher rate than dilution rate (0.1/h). The increase in *t*-VA concentration was greater than that in SA (Figure 3, A and B), suggesting that increased *t*-VA production caused by MDT-10 addition exceeds the capacity of mixed ruminal microbes to hydrogenate *t*-VA. Conjugated LA was not detected in the effluent, probably because the total cell number (or mass) of microbes was $>10^{10}$ /mL as mentioned previously.

Strain HF-11 added with MDT-10 to a fermenter increased 2-fold the total amount of *t*-VA in the effluent during 24 h (108 μ mol, $P < 0.05$; Figure 3C) compared with a fermenter that received only MDT-10 (55 μ mol; Figure 3B). The total amount of SA in the effluent greatly decreased (107 vs. 59 μ mol, $P < 0.05$; Figure 3, B and C) despite an increase in *t*-VA production. This suggests that biohydrogenation of *t*-VA was prevented by HF-11 incorporating *t*-VA into its cells and thus preventing the hydrogenation of *t*-VA by other microbes. Similar to the result obtained when MDT-10 was added (Figure 3B), *t*-VA in the effluent increased with an increasing number of additional doses of LA supplemented at 6-h intervals when both MDT-10 and HF-11 were added (Figure 3C). This trend (i.e., *t*-VA in the effluent increased with time) was more pronounced in the presence of both bacteria than with MDT-10

alone. Strain HF-11 may have also grown at a higher rate than the dilution rate (0.1/h) or *t*-VA-incorporating activity of HF-11 may have increased with time, or both.

Simultaneous addition of MDT-10 and HF-11 increased *t*-VA in the effluent 2.6-fold during 24 h relative to a culture that received neither of the bacteria (42 vs. 108 μ mol, $P < 0.05$; Figure 3, A and C). If MDT-10 and HF-11 are able to keep growing in the rumen, *t*-VA in the ruminal outflow might increase even when these bacteria are added at lower concentrations or less frequently.

In this experiment, continuous culture was performed for only 24 h, because short-term incubation better reflects the microbiota in the rumen. For example, the decline in the number of protozoa, as counted by microscopy, was not large (i.e., explicable by dilution rate). Because protozoa are extremely sensitive to unfavorable environmental conditions (Hino et al., 1993a), most microbes in the rumen might have been maintained. The fermentation products in continuous culture did not significantly change throughout the incubation period (acetate, propionate, butyrate, valerate, and lactate were 35 to 39, 17 to 20, 15 to 18, 2 to 3, and 4 to 6 mM, respectively), suggesting that addition of MDT-10 and HF-11 did not greatly affect the growth of ruminal microbes. Because ruminal contents flow out continuously in ruminants, an increased *t*-VA level in the rumen will result in more *t*-VA flowing out of the rumen as well. Therefore, the addition of both MDT-10 and HF-11 might increase the supply of *t*-VA to the host animal.

Figure 3. Effect of the addition of *Butyrivibrio fibrisolvens* MDT-10 and *Bifidobacterium adolescentis* HF-11 on the amount of *trans*-vaccenic acid (*t*-VA) in the effluent of continuous culture of mixed ruminal microbes (initial number of total viable bacteria, 1×10^{11} cfu/mL). A) Neither of the bacteria was added (control); B) only *Butyr. fibrisolvens* (1×10^9 cfu/mL) was added; C) both *Butyr. fibrisolvens* (1×10^9 cfu/mL) and *Bifid. adolescentis* (1×10^9 cfu/mL) were added. Linoleic acid (LA), carbohydrate, and protein were added every 6 h, and the amounts of LA (○), *t*-VA (▲), and stearic acid (□) in the effluent collected at 1-h intervals were measured. Values (means $\pm$ SE; $n = 3$) during 0 to 1, 2 to 3, and 5 to 6 h after the addition of LA are shown.

CONCLUSIONS

Addition of *Butyr. fibrisolvens* MDT-10 to the cultures of mixed ruminal microbes enhanced LA conversion to *t*-VA, and further, the addition of *Bifid. adolescentis* HF-11 protected *t*-VA against biohydrogenation. Introduction of the 2 bacteria to a continuous culture increased the amount of *t*-VA in the outflow. It is conceiv-

able that introduction of the 2 bacteria into the rumen increases *t*-VA flowing out of the rumen, which implies that *t*-VA absorption in the small intestine may be increased; consequently, the conversion of *t*-VA to CLA in tissues may be increased. This may be a beneficial means to increase the CLA content in milk and beef without suppressing overall fermentation in the rumen.

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