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Effect of probiotics on blood fatty acid metabolism during the late middle stage of fattening period in Japanese Black cattle

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ABSTRACT. This study was conducted to investigate the effects of probiotics administration on fatty acid metabolism in Japanese Black cattle as per changes in blood fatty acid concentrations and blood biochemical tests. Eighteen clinically healthy Japanese Black female fattening cattle bred on the same fattening farm were randomly classified into the probiotics administration group (n=9) or the control group (n=9). In the probiotics administration group, 50 g of probiotics were started per animal per day at the age of 18 months, and the administration period was 2 months from the start date of the study. Blood was collected twice before starting the probiotics administration and at 2 months after starting the probiotics administration. In the probiotics administration group, palmitic, linoleic, arachidonic and α-linolenic acid tended to be higher at the end of the administration compared with those before probiotics administration. Additionally, as a result of multiple comparison test, monounsaturated fatty acids at Post was significantly higher, and the ω6 / ω3 ratio was significantly lower than in the control group. Vitamin A, E and albumin were significantly higher at the end of the administration than in the control group. In this study that administering probiotics to Japanese Black cattle in the late middle stage of fattening period did not have a significant effect on fatty acid metabolism during feed digestion and absorption, but suggested that may alter some blood fatty acids concentrations.

KEY WORDS: blood fatty acid, Japanese Black fattening cattle, probiotics

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Probiotics are widely used in livestock and are defined as "live microorganisms that when administered in adequate amounts confer a health benefit on the host" [9] and maintain the balance of the intestinal flora of domestic animals. Probiotics are used to effectively prevent pathogens that threaten both animals and consumers [13]. In ruminants, a stable rumen pH is thought to increase short-chain fatty acids production [1], enabling efficient rumen functioning and increasing microbial ecosystems, nutrient digestibility, and feed conversion ratios. Probiotics are expected to bring many beneficial clinical effects by improving growth ability [1] and redirecting the immune system to the anti-inflammatory phenotype [11].

On the other hand, lipids have various functions as biological membrane constituents, energy sources, and signal molecules. Among them, polyunsaturated fatty acids (PUFAs) containing double bonds in the molecule acquire physiological activity through an enzymatic oxidation reaction and play important roles as lipid mediators [5]. PUFAs are divided into three groups: ω3 fatty acids (FAs), ω6 FAs and some ω9 FAs, depending on the position of the double bond from the methyl end.

Since Bang *et al*. [3] reported the effects of fish oil lipids on living organisms in an epidemiological study of Inuit people, ω3 FAs, such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), have been shown to have a wide variety of physiological effects, including anti-inflammatory effects [6, 19].

In recent years, Japanese Black beef farms have become large-scale and intensive systems, and manage with the aim of marbled beef and increasing the weight by high-feeding of concentrated feed has become common from the early postnatal period. From such a background, we had previously investigated changes in blood FAs in Japanese Black cattle. We found that linoleic acid (LA), an ω6 FAs, increased, and EPA, an ω3 FAs, was significantly reduced after the early middle fattening period. Uyeno *et al*. suggest that administering probiotics may affect microbial ecosystems within the rumen because the probiotics alter the short-chain

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FAs levels [21].

However, no reports have been published on the effects of probiotics administration on long-chain FAs and very-long-chain FAs in the late middle stage of fattening period in Japanese Black fattening cattle.

This study was conducted to investigate the effects of probiotics administration on FAs metabolism in Japanese Black cattle as per changes in blood FAs concentrations and blood biochemical tests.

MATERIALS AND METHODS

Study period and animals

Eighteen clinically healthy Japanese Black female fattening cattle bred in the usual manner on the same fattening farm were used. The cattle were randomly classified into the probiotics administration group (probiotics group, n=9) or the control group (n=9) 9 months after the moving onto the farm. There was no difference the strain between the groups, and mean ages ± standard deviation (SD) were 18.0 ± 0.5 months in the probiotics group and 17.6 ± 0.5 months in the control group; these ages did not significantly differ. The blood biochemical test results contained no abnormal values at the start of the study.

Study outline

Table 1 shows the daily feed amounts fed during the study period and the components of the concentrate. The feeding environments were the same for both groups, rice straw and alfalfa hay cubes were used for roughage, and the feeding amount was measured using an automatic feeding device for concentrated feed. Both groups were given an additional 500 g/day of alfalfa hay cubes for 2 months from 1 week before the end of the administration period according to the farm feeding program. The prescribed amount of feed was fed continuously throughout the fattening period, and the cattle had free access to drinking water. BIO-THREE ACE (Toa Yakuhin Co., Ltd., Tokyo, Japan) was used as the probiotics. One gram of this probiotics contains 1×10^8 *Streptococcus faecalis T-110*, 1×10^6 *Bacillus mesentericus TO-A*, and 1×10^6 *Clostridium butyricum TO-A*. The cattle received 50 g of probiotics per animal per day in a top dress, and the administration period was 2 months from the start date of the study.

Blood was collected twice from the jugular vein using a vacuum blood collection tube for serum separation (Venoject II VP-H100K, Terumo, Tokyo, Japan) before starting the probiotics administration (Pre, pre-administration) and at 2 months (Post, at the end of administration). The control group received no probiotics and were studied simultaneously with the probiotics group. After blood collection, the samples were placed under shade at room temperature for 2–3 hr, then centrifuged at 3,000 rpm for 20 min to separate the serum and stored at −60°C until the day of measurement.

Measurement of FAs concentrations in total serum lipid

Sera were transported to a laboratory (Clinical Pathology Laboratory, Kagoshima Co., Ltd., Kagoshima, Japan) and measured. After dispensing, the samples were derivatized by adding a derivatizing reagent (Domestic Chemistry, Tokyo, Japan) and an internal standard solution (Domestic Chemistry), stirring and heating. Next, NaOH and n-hexane (Domestic Chemistry) were added, shaken, and centrifuged, and the upper layer was dispensed into sample tubes. The prepared samples were measured via gas chromatography (GC-2010, Shimadzu Corp., Kyoto, Japan) using a GC capillary column for cis-trans FAs separation (TC-70, GL Sciences, Tokyo, Japan). Concentrations of the 24-fraction FAs in total serum lipids were calculated using a data processing device (C-R7A, Shimadzu Corp.). The following FAs were analyzed: total FAs (TFAs); palmitic acid (PA) and stearic acid (SA) as saturated FAs (SFAs); oleic acid (OA) as a monounsaturated FAs (MUFAs); LA, dihomo-γ-LA (DGLA) and arachidonic acid (AA) as ω6 FAs; and alpha-linolenic acid (ALA), EPA, and DHA as ω3 FAs. We also calculated the EPA/AA and ω6/ω3 ratios.

Blood biochemical tests

Serum aspartate aminotransferase (AST), γ-glutamyl transferase (GGT), urea nitrogen (UN), total protein (TP), albumin (Alb),

Table 1. Feeding management status, viable agent administration period and blood sampling age

Age after birth (month)	17	18	19	20
Rice straw (saturated)	1 [1]	1	1	1
Concentrated feed [2]	10	10	10	10
Alfalfa hay cubes [3]				←–
Probiotics' administration period		←– – – – – – –→		
		↑		↑
Blood sampling [4]		Pre		Post

[1]Data are expressed daily feed amount (Kg/head). [2]Mixing ratio of raw materials; Cereal 67.0%, Bran 24.0%, Vegetable oil residue 7.0%, Others 2.0%, Total Digestible Nutrients; 74.0% or more, Crude protein; 13.0% or more. [3]An additional dose of 500 g/day was given for 2 months according to the farm feeding program. [4]Pre; immediately before the start of administration, Post; 2 months after the start of administration.

globulin (Glb), total cholesterol (T-Cho), calcium (Ca), inorganic phosphorus (iP), free fatty acid (FFA), magnesium (Mg) and β-Hydroxybutylic acid (BHB) were measured with an automatic analyzer (7180 type, Hitachi High-Tech Fielding, Tokyo, Japan).

Vitamin A and E

Serum vitamin A (VA) and vitamin E (VE) were measured via high-performance liquid chromatography (high-performance liquid chromatography LC-2000, JASCO Corp., Tokyo, Japan).

Statistical analysis

FAs concentrations and blood biochemical test values were shown as the mean ± SD. For comparison between groups of each data, the presence or absence of homoscedasticity was confirmed by Bartlett's test, and then the significance was confirmed by two-way ANOVA. In addition, the significance of the temporal transition of both groups was confirmed by one-way repeated measures ANOVA, and then multiple comparison test was performed by Bonferoni's method.

In addition, the correlation between FAs concentrations in all samples (n=36) and blood biochemical test values was determined by Pearson's correlation analysis. Risk rates below 5% were considered to be significantly different and EZR (http://www.jichi.ac.jp/saitama-sct/SaitamaHP.files/statmed.html) was used for all statistical analysis.

RESULTS

Blood FAs concentrations

Table 2 shows the FAs concentrations in total lipids of the cattle during the study period.

In this study, no significant difference was observed interaction effects in ANOVA. However, an independent multiple comparison test showed a significant difference about some items over time and in groups. That is, in the probiotics group, the Post values of ALA and EPA were increased compared with the Pre values, resulting in a significant increase in ω3FAs concentrations at Post (P<0.01). On the other hand, in the control group, the Post value of only EPA was increased compared with the Pre values.

OA and ALA at Post were significantly higher in the probiotics group compared with the control group. As a result, in the probiotics group, MUFAs and ω3 FAs at Post were significantly higher and the ω6 / ω3 ratio at Post was significantly lower than those in the control group. As a result of comparison of increase / decrease in FAs concentration of Pre and Post in both groups, LA, ALA, TFAs, and PUFAs in the probiotics group were significantly higher than those in the control group at Post (See Supplementary Fig. 1).

Blood biochemical test

Table 3 shows the blood biochemical test results.

Also in the Blood biochemical test, no significant difference was observed interaction effects in ANOVA. But compared with the Pre value, T-Cho tended to be higher at Post in the probiotics group. Result of an independent multiple comparison test, Mg and VA were significantly decreased at Post in both groups compared with the Pre value (P<0.01). However, VA in the probiotics group was higher than the control group at Post (P<0.05). Alb and Alb / Glb (A / G) in probiotics group were significantly higher than those in control group at Post. VE in the probiotics group was significantly higher than that in the control group after the end of the probiotics administration (P<0.05).

In the correlation between FAs and blood biochemical test values, T-Cho showed a significant positive correlation with PA, SA, LA, DGLA, AA, ALA and EPA (P<0.001). We also found that there was a significant positive correlation between FFA and OA, and between VE and PA, OA (P<0.001) (See Supplementary Fig. 2).

DISCUSSION

The probiotics used in this experiment was a mixture of *Bacillus mesentericus* TO-A, *Clostridium butyricum* TO-A, and *Enterococcus faecalis* T-110. This complexed probiotics is reported to have effects on livestock, such as reducing the incidence of diarrhea in sows and improving reproductive performance [10] and strengthening the immune systems of unvaccinated porcine epidemic diarrhea (PED)-infected sows [20]. Even in humans, this probiotics clinical uses are wide-ranging and include reducing postoperative infectious complications after a pancreaticoduodenectomy [16] and significantly reducing severe diarrhea during *Salmonella* or rotavirus infections [11]. Regarding the effects of other probiotic administration, Beauchemin *et al.* [4] reported that increased propionic acid concentration, decreased butyric acid concentration, decreased pH lowest point in the rumen and enhanced feed nitrogen flow to the duodenum was observed after *Enterococcus faecium* EF212 and Yeast (*Saccharomyces cerevisiae*) administration in the feedlot cattle. Colombo *et al*. [8] also reported improved the response to BRD treatment and improved immune capacity due to partially enhanced metabolism and dietary supplementation in steered cattle treated with a mixture of yeast-derived prebiotic and *Bacillus subtilis* probiotic. However, the exact mechanisms by which probiotics administration plays a beneficial role remain unclear, and the effects of probiotics administration on Japanese Black cattle in the late middle stage of fattening period on blood FAs and blood biochemical test remain unclear.

With the addition method used this time, probiotics did not significantly change fatty acid metabolism. However, in an independent multiple comparison test, in the probiotics group at the end of administration, some types of FAs were significantly increased compared to before administration. On the other hand, in the control group, only EPA increased at the Post. Interestingly,

Table 2. Blood fatty acids concentrations during the test period

		Group	Pre[2]	Post	*P*-value[1]		
					Group	Time	Group×Time
TFAs [3] (μg/ml)		Probiotics	2,166.4 ± 495.2	2,497.6 ± 429.0	0.302	0.236	0.246
		Control	2,184.8 ± 440.5	2,188.2 ± 262.2			
SFAs (μg/ml)		Probiotics	724.8 ± 156.4	803.0 ± 111.8	0.095	0.450	0.295
		Control	696.7 ± 137.1	683.9 ± 100.1			
	PA	Probiotics	280.3 ± 50.5	306.3 ± 45.6	0.079	0.522	0.313
		Control	268.0 ± 54.0	262.2 ± 33.5			
	SA	Probiotics	412.9 ± 103.7	461.2 ± 65.0	0.121	0.455	0.308
		Control	398.0 ± 80.8	390.5 ± 67.5			
MUFAs (μg/ml)		Probiotics	165.6 ± 18.6	190.1 ± 20.1*,##	0.003	0.075	0.330
		Control	146.9 ± 35.0	154.2 ± 26.9			
ω9FAs		Probiotics	150.8 ± 16.2	170.7 ± 17.2##	0.004	0.118	0.347
		Control	134.2 ± 32.0	139.3 ± 24.5			
	OA	Probiotics	141.5 ± 15.5	161.9 ± 15.9##	0.004	0.091	0.328
		Control	125.8 ± 30.6	131.4 ± 23.7			
PUFAs (μg/ml)		Probiotics	1,275.9 ± 327.1	1,504.6 ± 310.8	0.635	0.212	0.247
		Control	1,341.2 ± 273.1	1,350.0 ± 184.7			
ω6 FAs		Probiotics	1,230.8 ± 317.0	1,449.2 ± 301.6	0.664	0.223	0.246
		Control	1,297.7 ± 263.2	1,303.2 ± 178.9			
	LA	Probiotics	1,095.6 ± 289.3	1,299.3 ± 267.7	0.679	0.202	0.228
		Control	1,161.1 ± 229.7	1,166.8 ± 157.6			
	DGLA	Probiotics	46.9 ± 16.1	52.7 ± 13.6	0.703	0.585	0.525
		Control	48.1 ± 16.3	47.7 ± 12.4			
	AA	Probiotics	58.1 ± 16.7	65.9 ± 19.1	0.405	0.363	0.623
		Control	56.2 ± 16.7	58.6 ± 12.9			
ω3 FAs		Probiotics	44.2 ± 10.6	54.4 ± 9.6**,#	0.114	0.035	0.272
		Control	42.7 ± 9.8	46.0 ± 6.3			
	ALA	Probiotics	33.2 ± 8.0	42.6 ± 6.9**,#	0.110	0.011	0.236
		Control	32.2 ± 7.5	35.8 ± 5.9			
	EPA	Probiotics	2.7 ± 0.9	3.6 ± 1.1*	0.225	0.024	0.589
		Control	2.5 ± 0.8	3.1 ± 0.9*			
	DHA	Probiotics	1.1 ± 0.2	1.3 ± 0.3	0.834	0.034	0.754
		Control	1.1 ± 0.3	1.4 ± 0.4			
EPA/AA ratio [4]		Probiotics	0.0 ± 0.01	0.1 ± 0.01	0.277	0.003	0.829
		Control	0.0 ± 0.01	0.1 ± 0.01**			
ω6/ω3 ratio		Probiotics	27.8 ± 2.2##	26.6 ± 1.7#	0.001	0.007	0.406
		Control	30.6 ± 1.6	28.4 ± 1.6			

Data are shown as the mean ± SD. [1]*P*-value of main effect and interaction by two-way ANOVA, followed by Bonferoni's multiple comparison method was used to determine within-group differences. **P*<0.05, ***P*<0.01, compared to before administration (Pre); #*P*<0.05, ##*P*<0.01, compared to the control group at the same time. [2]Pre; immediately before the start of administration, Post; 2 months after the start of administration. [3]TFAs, total fatty acids; SFAs, saturated fatty acids; MUFAs, monounsaturated fatty acids; PUFAs, polyunsaturated fatty acids; PA, palmitic acid (16:0); SA, stearic acid (18:0); OA, oleic acid (18:1ω-9); LA, linoleic acid (18:2ω-6); ALA, alfa-linolenic acid (18:3ω-3); DGLA, dihomo-γ-linolenic acid (C20:3ω2-6); AA, arachidonic acid (20:4ω-6); EPA, eicosapentaenoic acid (20:5ω-3); DHA, docosahexaenoic acid (22:6ω-3). [4]EPA/AA ratio=eicosapentaenoic acid / arachidonic acid; ω6/ω3 ratio=ω6 FAs / ω3 FAs.

there was no difference in the change of SA between the both groups, whereas OA at Post in the probiotics group increased. Therefore, probiotics may have the effect of promoting the SA to OA reaction. In addition, the increase of ALA, EPA in both groups after the probiotics administration should be considered for the effect of adding alfalfa hay cubes. However, ALA at Post in the probiotics group showed significantly higher than those in control group, it suggesting that probiotics administration may have an ALA absorption promoting effect.

Results of blood biochemical tests, T-Cho tended to increase at the end of administration in the probiotics group compared to before administration. In addition, as a result of multiple comparison test, VE, Alb and A / G in the probiotics group were significantly higher than those in the control group at Post. These results suggest that the digestion and absorption of both lipids and proteins are affected by probiotics administration. Adachi *et al*. [2] showed increase of T-Cho and VE in the late fattening period on Japanese Black farms with excellent shipping results. In this study, the correlation between FAs and blood chemistry showed that T-Cho had the strongest positive correlation with LA, with significantly higher concentrations at the end of

Table 3. Blood biochemical test values during the test period

		Group	Pre[2]	Post	*P*-value[1]		
					Group	Time	Group×Time
Average age		Probiotics	18.0 ± 0.5	20.0 ± 0.5	0.014	0.000	1.000
		Control	17.6 ± 0.5	19.6 ± 0.5			
AST[3]	(IU/l)	Probiotics	78.7 ± 15.7	77.2 ± 16.4	0.175	0.759	0.641
		Control	86.9 ± 15.5	93.9 ± 46.4			
GGT	(IU/l)	Probiotics	28.4 ± 5.6	29.1 ± 7.4	0.186	0.544	0.752
		Control	24.7 ± 4.2	26.8 ± 9.0			
UN	(mg/dl)	Probiotics	17.0 ± 1.7	18.3 ± 2.7	0.171	0.045	0.558
		Control	15.4 ± 2.7	17.6 ± 2.6			
TP	(g/dl)	Probiotics	7.0 ± 0.2	6.9 ± 0.3	0.137	1.000	0.380
		Control	7.0 ± 0.3	7.1 ± 0.3			
Alb	(g/dl)	Probiotics	3.7 ± 0.2	3.8 ± 0.2$^{\#}$	0.004	0.861	0.504
		Control	3.6 ± 0.1	3.6 ± 0.2			
Alb/Glb		Probiotics	1.2 ± 0.1	1.2 ± 0.1$^{\#\#}$	0.000	0.957	0.142
		Control	1.1 ± 0.1	1.0 ± 0.1			
T-Cho	(mg/dl)	Probiotics	167.9 ± 39.3	197.7 ± 41.3	0.487	0.148	0.296
		Control	172.1 ± 32.4	177.0 ± 25.1			
Ca	(mg/dl)	Probiotics	9.4 ± 0.2	9.4 ± 0.3	0.948	0.651	0.948
		Control	9.4 ± 0.1	9.4 ± 0.3			
iP	(mg/dl)	Probiotics	7.2 ± 0.4	7.1 ± 0.4	0.555	0.039	0.178
		Control	7.4 ± 0.9	6.7 ± 0.7			
VA	(IU/dl)	Probiotics	75.8 ± 15.2	48.7 ± 7.3**$^{,\#}$	0.020	0.000	0.916
		Control	65.5 ± 14.1	39.1 ± 10.4**			
VE	(μg/dl)	Probiotics	393.6 ± 99.0	464.9 ± 104.3$^{\#}$	0.028	0.430	0.148
		Control	367.9 ± 81.1	346.5 ± 89.0			
FFA	(mEq/l)	Probiotics	160.8 ± 51.1	183.9 ± 33.1	0.262	0.554	0.499
		Control	152.6 ± 78.5	151.0 ± 42.7			
Mg	(mg/dl)	Probiotics	2.6 ± 0.2	2.4 ± 0.2**	0.007	0.001	0.664
		Control	2.4 ± 0.1	2.2 ± 0.2*			
BHB	(μmol/l)	Probiotics	307.7 ± 78.4$^{\#}$	303.4 ± 38.8	0.012	0.753	0.593
		Control	247.0 ± 34.4	263.2 ± 63.8			

Data are shown as the mean ± SD. [1]*P*-value of main effect and interaction by two-way ANOVA, followed by Bonferoni's multiple comparison method was used to determine within-group differences. **P*<0.05, ***P*<0.01, compared to before administration (Pre); #*P*<0.05, ##*P*<0.01, compared to the control group at the same time. [2]Pre; immediately before the start of administration, Post; 2 months after the start of administration. [3]AST, aspartate aminotransferase; GGT, γ-glutamyl transferase; UN, urea nitrogen; TP, total protein; Alb, albumin; T-Cho, total cholesterol; Ca, calcium; iP, inorganic phosphorus; VA, vitamin A; VE, vitamin E; FFA, free fatty acid; Mg, magnesium; BHB, β-Hydroxybutylic acid.

administration. T-Cho concentration has a positive genetic correlation with carcass weight and rib thickness, suggesting that it is an effective physiological indicator for improving these performances [12]. Therefore, administration of probiotics in the late middle stage of fattening period may have beneficial effects on improving shipping results.

The results of this study showed a positive correlation between FFA and OA. FFA is known to increase in dairy cows as a result of body fat mobilization in a negative energy balance state [22]. Nogalski *et al*. [15] reported that in the high condition loss group of high lactating cows, high mobilization of body fat reserves results in a significant increase of MUFAs (mainly OA) levels in milk. Therefore, the increase in OA observed at Post may need to be considered not only for synthesis, but also for preferentially mobilization from OA reserved in body fat, but this time we could not reveal the details.

Administering probiotics is thought to promote efficient FAs absorption by suppressing the decreased rumen pH by affecting the rumen bacterial communities [1, 24] and stabilizing the intestinal flora [7, 17]. Currently, each probiotic used as a feed additive in Japan has a strain-specific action and serves a different purpose [21]. It was impossible to clarify whether the effects of the probiotics used here on FAs were due to the action of a single strain or an interaction. However, in humans, the metabolic pathways associated with the intestinal flora of dietary PUFAs are being determined. Consequently, the FA molecular species produced in the intestinal tract depends on the metabolism of unsaturated FAs of lactic acid bacteria in the intestinal tract and may influence host health [14]. Furthermore, the culture supernatant of the *Bacillus mesentericus* TO-A in the probiotics used in this study promoted the growth of *Bifidobacterium* [18], and supplementation with *Bifidobacterium breve* NCIMB 702258 combined with ALA increased EPA concentrations in the liver and DHA concentrations in the brain in mice [23]. Therefore, it is possible that the

concentration of various FAs increased due to the effect of strain interaction between the enterobacteria and the metabolites of feed-derived PUFAs and the complexed probiotics used this time.

In conclusion, administration of probiotics by this method could not show a significant effect on FAs metabolism in relation to feed digestion and absorption. However, it has been suggested that administration of probiotics to Japanese Black cattle in the late middle stage of fattening period may alter some blood FAs concentrations.

CONFLICTS OF INTEREST. The authors declare no competing interests.

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