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Conversion of Unsaturated Food Fatty Acids into Hydroxy Fatty Acids by Lactic Acid Bacteria

KIM, MYUNG HEE[1], MEE SEUNG PARK[1], CHANG-HO CHUNG[2], CHEONG TAE KIM[3], YOUN SOON KIM[4], AND KYU HANG KYUNG[1]*

[1]*Department of Food Science, Sejong University, Seoul 143-747, Korea*
[2]*Audubon Sugar Institute, Louisiana State University Agricultural Center, Baton Rouge, LA 70803, U.S.A.*
[3]*Nongshim Food Co., Gyeonggido 435-030, Korea*
[4]*Department of Home Economy Education, Chosun University, Kwangju 501-759, Korea*

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Abstract The ability of 19 lactic acid bacteria to produce hydroxy fatty acids (HFAs) from unsaturated food fatty acids (USFAs) was tested. HFAs are related to human ailments, including steatorrhea. All the cultures produced HFAs from USFAs, unless their growth was inhibited by free USFAs. *Lactococcus lactis* subsp. *lactis* KFRI 131 converted oleic, linoleic, and linolenic acid into 10-hydroxyoctadecanoic acid (10-HODA), 10-hydroxyoctadecaenoic acid (10-HODEA), and 10-hydroxyoctadecadienoic acid (10-HODDEA), respectively. Both a USFA and a surfactant were needed for the bacterium to convert the fatty acid into the corresponding HFA. It was apparent that the production of 10-HODA was growth-related, while that of 10-HODDEA was not. It was unclear whether the production of 10-HODEA was growth-related.

Key words: Lactic acid bacteria, hydroxy fatty acid, unsaturated long-chain fatty acid, steatorrhea, *Lactococcus lactis* subsp. *lactis*

It has been previously reported that *Lactococcus lactis* subsp. *lactis* produces 10-hydroxyoctadecanoic acid (10-HODA) in an MRS broth [18]. 10-HODA is the major hydroxy fatty acid (HFA) in the fecal fat of humans with steatorrhea, diarrhea caused by malabsorption or maldigestion of fat [5, 21]. As such, it has been suggested that 10-HODA is produced from dietary fat by microorganisms in the gut, and that 10-HODA contributes to the diarrhea associated with steatorrhea. Kim and Spritz [11] concluded that the HFAs found in fecal fat are formed by the addition of water across the double bonds of dietary unsaturated fatty acids (USFAs), catalyzed by the enzymes of intestinal bacteria. They also reported that steatorrhea in dogs can be corrected by the administration of tetracycline, apparently by reducing bacterial growth in the intestine, and HFAs disappeared from the feces of the steatorrhea-cured dogs. The significance of hydrating USFAs into HFAs is still unknown. HFAs are common constituents of purgative oils. 10-HODA is chemically similar to ricinoleic acid (12-hydroxy-*cis*-9-octadecanoic acid), the major fatty acid in castor oil [20].

Other microorganisms reportedly to convert USFAs into HFAs are *Saccharomyces cerevisiae* [3], *Nocardia* sp. [12, 13, 16], *Pseudomonas* sp. [2, 22], and *Lactobacillus plantarum* [24].

Yamada *et al.* [24] reported that *Lact. plantarum* hydrates linoleic acid into 10-hydroxy-12-octadecaenoic acid (10-HODEA) and suggested that 10-HODEA resembles leukotoxin (9,10-epoxy-12-octa-decenoic acid), since 10-HODEA causes a decrease in muscular tension immediately after its administration, mimicking the physiological function of leukotoxin. HFAs have been found in higher concentrations in the low-density lipoproteins (LDL) of individuals with rheumatoid arthritis and atherosclerosis [6, 7]. Jira *et al.* [8] also suggested that the level of HFAs is probably an indicator of biological age.

Accordingly, we examined the hydration of USFAs, including oleic, linoleic, and linolenic acid, using dairy starter cultures into their corresponding HFAs, which are known to cause diarrhea and are related to other age-related ailments.

MATERIALS AND METHODS

Lactic Acid Bacteria and Culture Conditions

Lactococcus lactis subsp. *lactis* KFRI 131, *L. lactis* subsp. *lactis* KFRI 421, KFRI 422, *L. cremoris* KFRI

*Corresponding author
Phone: 82-2-3408-3225; Fax: 82-2-3408-3319;
E-mail: kyungkh@sejong.ac.kr

153, *L. diacetylactis* KFRI 185, *Lact. bulgaricus* KFRI 673, and *Lact. casei* KFRI 692, KFRI 709 were all purchased from the Korea Food Research Institute (Songnam, Kyonggi-do, Korea). *Lact. delbruckii* subsp. *bulgaricus* KCTC 1121, KCTC 3188, *Lact. casei* KCTC 2180, *Streptococcus thermophilus* KCTC 2185, *Lact. acidophilus* KCTC 3111, KCTC 3145, *L. lactis* subsp. *lactis* KCTC 3124, *Leuconostoc mesenteroides* subsp. *mesenteroides* KCTC 3505, and *Lact. reuteri* KCTC 3677 were purchased from the Korean Collection of Type Cultures (Taejon, Korea). *Bifidobacterium bifidum* LS and *Lact. casei* KY were donated by commercial dairy manufacturers.

All the dairy starter cultures were subcultured at 30°C in an MRS broth (Difco Laboratories, Detroit, MI, U.S.A.) in 16×150-mm glass culture tubes with caps [14, 19]. Then, 10 microliters of the culture grown overnight were inoculated into 10 ml of a TM broth [18] containing different fatty acids (0.01, 0.05, 0.1, 0.5, and 1.0%) with 0.05% different surfactants. The tubes were statically incubated at 37°C for 48 h.

Chemicals

Oleic acid, linoleic acid, linolenic acid, Tween 80, lecithin, and bile extract were purchased from Sigma Chemical Company (St. Louis, MO, U.S.A.). Tween 40 and cholic acid were from Yakuri Pure Chemical Company Ltd. (Osaka, Japan) and Acros Organics Company (New Jersey, U.S.A.), respectively. Glycerine monostearate, decaglycerine monolaurate, Span 20, Span 40, and Span 80 were gifts from Hyangwon Company Ltd. (Seoul, Korea).

Identification of Fatty Acids

The culture broths were extracted with 2 volumes of diethyl ether. The ether layer was washed twice with distilled water and de-watered using anhydrous sodium sulfate before the solvent layer was reduced to dryness *in vacuo*. The extracts were trimethylsilylated [1, 10, 23] before GC/MS analysis. The total ion chromatogram (TIC) and mass spectra of the trimethylsilylated samples were obtained using a Hewlett-Packard Model 6890 Plus capillary gas chromatograph equipped with a Platform II Mass selective detector (Micromass Ltd., Manchester, U.K.). A GC column (30 m capillary, J & W Scientific Inc., Folsom, U.S.A.) coated with DB-5 (0.25 μm thick) was coupled directly to the MSD capillary interface. After an initial hold of 10 min, the oven temperature was programmed to increase from 100°C to 260°C at 10°C/min and then maintain the final temperature for 15 min. The helium flow rate was 1 ml/min, with both the injector and detector temperatures at 270°C. Electron impact ionization (potential 70 eV) was used and the mass range scanned was 40-450 Daltons.

RESULTS

Effect of Surfactants on Formation of HFAs

Various surfactants were added to the TM broth [18] with or without oleic acid before the broth was inoculated with the test bacterium, *L. lactis* subsp. *lactis* KFRI 131, and incubated (Table 1). The bacterium produced 10-HODA without exception, when the TM broth contained both a surfactant and oleic acid. 10-HODA was not produced without oleic acid, except with Tween 80 and Span 80.

All of the dairy starter cultures tested produced 10-HODA from oleic acid within 3 days of incubation, except when there was no growth (Table 2).

Effects of pH on Formation of HFAs

When the initial pH of the growth medium was adjusted to between 5.0 and 8.8 (after autoclaving), *L. lactis* subsp. *lactis* KFRI 131 produced 10-HODA after 12 h in the broth at pH 7.5 and after 24 h at pH of 7.0, 8.3, and 8.8 (Table 3). The broth with an initial pH of 6.8 produced 10-HODA after 36 h, while there was no detectable amount of 10-HODA even after 72 h with an initial pH of 6.0 or lower. The rate of conversion of oleic acid into 10-HODA increased with the initial pH (data not shown). Very small quantities of 10-HODA were found by GC analysis in the broth with an initial pH of 5.1 and 6.0, where 10-HODA was not observed. At an initial pH of 8.8, the conversion rate was 65%.

Effect of Temperature on Formation of HFAs

The production of 10-HODA by *L. lactis* subsp. *lactis* KFRI 131 was more efficient as the cultivation temperature decreased to 25°C (Fig. 1). Temperatures ranging from 35°C to 45°C had no influence on the formation of the HFA by the bacterium. The conversion rate of oleic acid into 10-HODA at human body temperature after 48 h was only about 13.7%.

Table 1. Production of 10-HODA by *Lactococcus lactis* subsp. *lactis* KFRI 131 in TM broth containing oleic acid with different surfactants after 3 days of incubation at 37°C.

Surfactants (0.05%)	Without oleic acid	With oleic acid (0.5%)
TM broth control	-	-
Tween 80	+	+
Tween 40	-	+
Lecithin	-	+
Bile extract	-	+
Cholic acid	-	+
Glycerine monostearate	-	+
Span 20	-	+
Span 40	-	+
Span 80	+	+
Decaglycerine monolaurate	-	+

TM broth; TSB with 1.0% malt extract.

Table 2. Production of 10-HODA by dairy starters at 37°C in TM broth with 0.2% oleic acid.

Dairy starters	Surfactants							
	Bile extract	Cholic acid	Tween 40	Lecithin	GMS	Span 20	Span 40	DGML
Lactococcus lactis subsp. *lactis* KFRI131	1	1	1	1	1	1	1	1
Lactococcus cremoris KFRI 153	3	2	1	3	1	1	1	1
Lactococcus diacetylactis KFRI 185	2	1	1	3	1	1	1	1
Lactococcus lactis subsp. *lactis* KFRI 421	1	1	1	1	1	1	1	1
Lactococcus lactis subsp. *lactis* KFRI 422	1	1	1	1	1	1	1	1
Lactobacillus bulgaricus KFRI 673	1	1	1	1	1	2	1	1
Lactobacillus casei KFRI 692	1	1	1	1	1	2	1	1
Lactobacillus casei KFRI 709	1	1	1	1	1	2	1	1
Lactobacillus delbrueckii subsp. *bulgaricus* KCTC 1121	1	1	1	1	1	1	1	1
Lactobacillus casei KCTC 2180	2	1	1	2	1	1	1	1
Streptococcus thermophilus KCTC 2185	2	1	2	1	2	2	2	2
Lactobacillus acidophilus KCTC 3111	-[a]	-	-	-	-	-	-	-
Lactococcus lactis subsp. *lactis* KCTC 3124	1	1	1	1	1	1	1	1
Lactobacillus acidophilus KCTC 3145	1	1	1	1	1	2	2	1
Lactobacillus delbrueckii subsp. *bulgaricus* KCTC 3188	2	1	2	2	1	2	2	2
Leuconostoc mesenteroides subsp. *mesenteroides* KCTC 3505	-	3	2	2	2	3	3	2
Lactobacillus reuteri KCTC 3677	2	3	3	2	2	3	2	3
Bifidobacterium bifidum (LS)	2	1	2	2	2	1	2	1
Lactobacillus casei (KY)	2	1	2	2	2	1	2	1

Numbers are the initial day when 10-HODA appeared.
[a]-, no growth; GMS, glycerine monostearate; DGML, decaglycerine monolaurate.

Identification of HFAs Produced from Unsaturated Fatty Acids

The total ion chromatogram of the trimethylsilylated products showed a single additional peak in addition to the added USFA (data not shown). The mass spectra of the trimethylsilylated products matched the published ones (Wiley Library and Nist Library and Structures, Micromass, Ltd.) for oleic, linoleic, and linolenic acids and 10-HODA (Fig. 2).

The proportions of individual ion fragments for the ether-soluble portion of the culture medium were 73 (98), 129 (88), 215 (99), 331 (100), and 429 (26) for the HFA from oleic acid, 73 (98), 129 (99), 213 (56), 331 (100), and 427 (28) for the HFA from linoleic acid, and 73 (98), 129 (94), 211 (7), 331 (100), and 425 (16) for the HFA from linolenic acid. The mass spectra for the trimethylsilylated products of the HFAs did not show the expected molecular [M+] ions (M.W. 444, 442, and 440). However, they produced weak [M-15+] fragment ions, with *m/z* 429, 427, and 425. The fragment ion at *m/z* 331 served as the base peak in all three cases.

To interpret the mass spectra of the HFAs produced from the three USFAs, all three HFAs were hydroxylated at the 10th carbon. The saturated, and mono- and di-unsaturated trimethylsilylated products possessed the same marker fragment ions up to the site of unsaturation on the omega-side of the hydroxylated carbon. After this point, the marker fragment ions at *m/z* 213 and 427 of the mono-unsaturated product were 2 a.m.u. less than the expected fragment ion mass (215, 429) of the saturated HFAs. In turn, the fragment ion mass (211, 425) of the di-unsaturated product was 2 a.m.u. less than that of the mono-unsaturated HFAs (215, 429).

The HFA produced from oleic acid by *L. lactis* subsp. *lactis* KFRI 131 was 10-HODA, as we previously reported

Table 3. Change in pH and production of 10-HODA by *Lactococcus lactis* subsp. *lactis* KFRI 131 relative to initial pH at 37°C.

Incubation time (h)	Initial pH[a]						
	5.1	6.0	6.8	7.0	7.5	8.3	8.8
12	4.9	4.6	4.5	4.6	4.9	5.7	6.3
	(-)[b]	(-)	(-)	(-)	(+)[c]	(-)	(-)
24	4.8	4.5	4.4	4.5	4.8	5.6	6.3
	(-)	(-)	(-)	(+)	(+)	(+)	(+)
36	4.7	4.4	4.4	4.5	4.9	5.6	6.2
	(-)	(-)	(+)	(+)	(+)	(+)	(+)
48	4.6	4.4	4.3	4.5	4.9	5.6	6.0
	(-)	(-)	(+)	(+)	(+)	(+)	(+)
60	4.6	4.4	4.3	4.5	4.9	5.5	5.9
	(-)	(-)	(+)	(+)	(+)	(+)	(+)
72	4.6	4.4	4.4	4.5	4.8	5.5	5.9
	(-)	(-)	(+)	(+)	(+)	(+)	(+)

[a]pH after autoclaving.
[b](-); non-appearance of 10-HODA.
[c](+); appearance of 10-HODA.
[d]Culture medium was TM broth with 0.05% bile extract as surfactant.

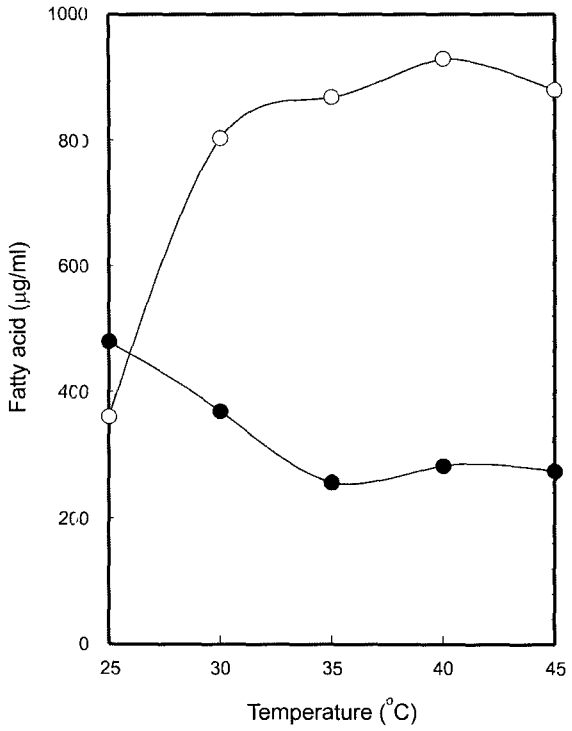

Fig. 1. Production of 10-HODA by *Lactococcus lactis* subsp. *lactis* KFRI 131 in TMB broth after 48 h with 0.2% oleic acid at different temperatures.
(●) 10-HODA, (○) oleic acid. TMB: TM broth with 0.05% bile extract.

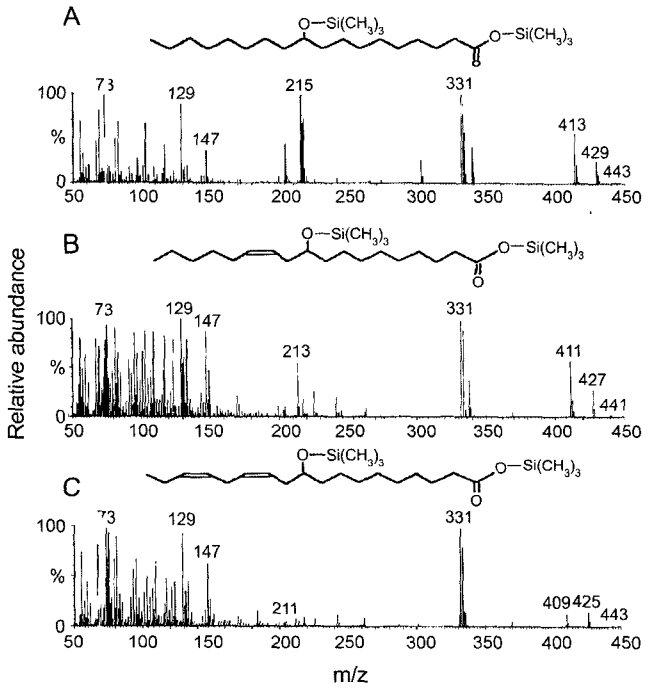

Fig. 2. Mass spectra of HFAs produced by *Lactococcus lactis* subsp. *lactis* KFRI 131 from oleic, linoleic, and linolenic acids.
A, 10-HODA; B, 10-HODEA; C, 10-HODDEA.

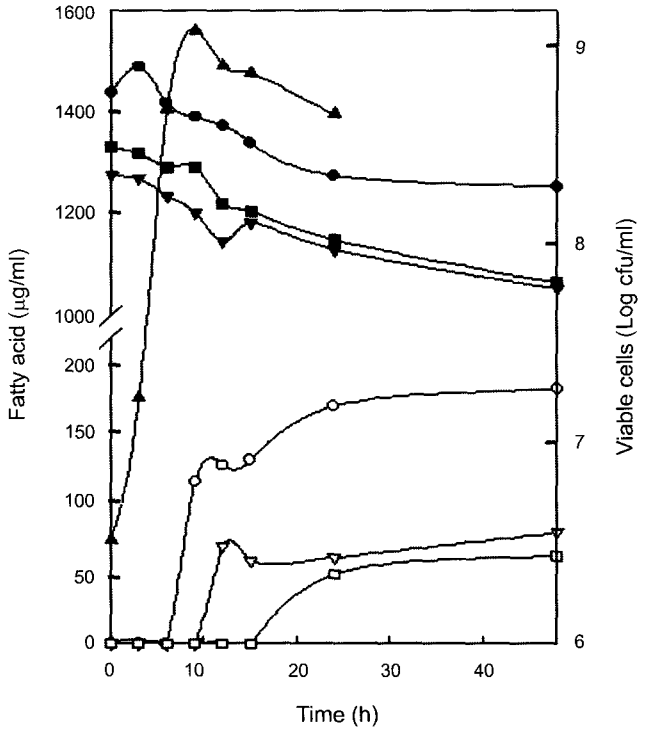

Fig. 3. Growth and production of HFAs by *Lactococcus lactis* subsp. *lactis* KFRI 131 with 0.2% individual fatty acid in TMB broth at 37°C.
(●) oleic acid, (○) 10-HODA, (▼) linoleic acid, (▽) 10-HODEA, (■) linolenic acid, (□) 10-HODDEA, (▲) viable cells (Log cfu/ml).

[18]. The mono- and di-unsaturated hydroxy fatty acids generated from linoleic and linolenic acid were 10-HODEA and 10-hydroxy-12,15-octadecadienoic acid (10-HODDEA), respectively (Fig. 2).

Bacterial Growth and HFA Production from Oleic, Linoleic, and Linolenic Acids

L. lactis subsp. *lactis* KFRI 131 was individually grown with 0.2% of each of the three USFAs (oleic, linoleic, and linolenic acids) using 0.05% bile extract as the surfactant, and the conversion of each USFA into the corresponding HFA was monitored (Fig. 3). The bacterium completed its growth within 10 h of incubation, and the production of 10-HODA from oleic acid paralleled its growth. 10-HODEA seemed to be produced either during the final stage of the exponential growth phase or immediately following the completion of bacterial growth, indicating that it began to appear much later than the other two HFAs.

DISCUSSION

L. lactis subsp. *lactis* KFRI 131 only produced an HFA in the presence of both an USFA and a surfactant, except with

Tween 80 or Span 80 (Table 1), both of which contain an oleic acid residue in their chemical structure. It has been previously deduced that the production of 10-HODA in an MRS broth by *L. lactis* subsp. *lactis* is due to the presence of Tween 80 in the medium [18]. However, *Lact. acidophilus* KCTC 3111 failed to grow with oleic acid in the medium, probably due to the inhibitory effect of the free fatty acid [8]. Therefore, the kind of surfactant did not seem to influence the production of HFAs.

The pH of the broth influenced the time of HFA formation. A weak alkaline pH allowed the formation of flocculent aggregates earlier than an acidic pH (Table 3). For example, 10-HODA was formed in the TM broth within 24 h at a pH of 7.0 or more, while it was not noticed when the broth had an initial pH of 6.0 or less, even after 72 h. Thus, the data suggest that the initial pH was more important for the production of 10-HODA than the pH during or after growth. The normal gut (fecal) pH of humans has been reported to be 6.2 [17] or 6.75 [15] and is relatively constant, varying only slightly with food intake. It is impossible to relate the two situations, since the tests converting USFAs into HFAs were carried out under unstable pH conditions.

Park *et al.* [18] previously identified 10-HODA produced in the form of flocculent aggregates by *L. lactis* subsp. *lactis* in an MRS broth. However, in the current study, no flocculent aggregates were observed with linoleic or linolenic acids. Oleic acid becomes saturated on the hydration of its double bond and its melting point increases, thereby separating the "saturated hydroxy fatty acid aggregates" from the aqueous layer. The aggregates were masses of 10-HODA filaments [18]. In contrast, the HFAs produced from linoleic and linolenic acids retained at least one double bond after the hydration of a double bond and remain in liquid form without forming saturated HFA aggregates.

The mono- and di-unsaturated HFAs generated from linoleic and linolenic acids were identified as 10-HODEA and 10-HODDEA, respectively (Fig. 3).

It has recently been found that the concentration of HFAs in LDL is related to human biological age, and that it is 20- to 50-fold higher in patients with rheumatoid arthritis and atherosclerosis [6-8], compared with healthy individuals of the same age. Yet, the relationship between the HFAs produced by intestinal bacteria and these age-related diseases are unclear.

The production of 10-HODA has been found to be growth-related, while that of 10-HODDEA is not. More study is still needed to determine whether the production of 10-HODEA is growth-related. Hudson *et al.* [4] reported that a ruminal strain of *Enterococcus faecalis* hydrated oleic acid into 10-HODA, yet it did not hydrate linoleic or linolenic acid. They suggested that the conversion into 10-HODA did not seem to be growth-related.

Currently, there is still no explanation for why microorganisms produce hydroxy fatty acids from unsaturated fatty acids.

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