Folia Microbiol. 28, 470—473 (1983)

Fatty Acids of *Lactobacillus bulgaricus* Determined by Gas Chromatography — Mass Spectrometry

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Received April 9, 1983

ABSTRACT. Fatty acids (in the form of pyrrolidinides) of *Lactobacillus bulgaricus* were identified by gas chromatography—mass spectrometry using two columns differing in the polarity of the stationary phase. In addition to the described fatty acids (primarily the most abundant vaccenic acid), we detected nine fatty acids that have not yet been described in the genus *Lactobacillus* (all of them minority compounds) and in four of them we determined the position of the double bond. The occurrence of vaccenic acid was related to other sources of positional isomers of octadecenoic acids (parsley seed — petroselinic acid; olive oil — oleic acid).

When studying metabolism of fatty acids a selective occurrence of position isomers of fatty acids can occasionally be observed (Holman 1978). The natural compounds of this type include isomers of *cis*-octadecenoic, of which oleic acid (9—18 : 1), petroselinic acid (6—18 : 1) and vaccenic acid (11—18 : 1) are best known. Plant oils are the main source of oleic acid, its content in olive oil being as much as 75 % (Gunstone 1967). Petroselinic acid is found primarily in seeds of plants of order *Umbelliferae*, family *Apiaceae* (Kleiman and Spencer 1982), for instance in parsley seeds it reaches 75 %, the oil content being 25 % of seed weight. Thus, the isolation of these two *cis*-octadecenoic acids in higher amounts is not too difficult.

Vaccenic acid in the trans form is contained in some animal fats (Gunstone 1967). In the *cis* form (asclepic acid) it is found only in two plants, of which only milkweed (*Asclepias syriaca*) grows rarely in southern parts of Czechoslovakia. The oil content is 19.4 % but, in addition to 15 % of vaccenic acid, the oil contains 15 % of oleic acid (Chisholm and Hopkins 1960). Similarly, most microorganisms growing anaerobically also produce vaccenic acid in a mixture with oleic acid. A mutant of *Escherichia coli* produces 48.9 % vaccenic acid bound in phospholipids (Mendoza *et al.* 1982). Species of the genus *Lactobacillus*, in which vaccenic acid had been dis-

covered (Hofmann *et al.* 1952) appear to be the most advantageous source of vaccenic acid that can be easily isolated after saponification. When studying these microorganisms as potential producers of vaccenic acid we continued our earlier studies concerning fatty acids found in natural sources (Řezanka *et al.* 1983).

Table I summarized the representation of position isomers of octadecenoic acids in various natural sources, from which the discrimination of their pyrrolidinides according to the double bond position can be seen. Vaccenic acid was found only in *L. bulgaricus* (55.2 %), petroselinic acid only in parsley seeds and oleic acid is a major compound in the olive oil; its content in parsley seeds and in *L. bulgaricus* is lower by an order of magnitude.

Table II presents the content (in %) of fatty acids in *L. bulgaricus*. Saturated fatty acids from C_5 to C_{18} were identified. Using the method of GC—MS of pyrrolidinide positions of double bonds in C_{16}—C_{20} acids could be determined. By using two columns with different stationary phases it was possible to discriminate between position isomers (9 and 11) of C_{16} and C_{18} acids and thus demonstrate oleic acid as a minority compound in *L. bulgaricus*. Fatty acids detected first in the genus *Lactobacillus* are altogether minority compounds (Table II, footnote *d*).

The content of total lipids in *L. bulgaricus* was 4.1 %.

EXPERIMENTAL

Microorganism and cultivation. Lactobacillus bulgaricus 370 (lyophilized stock culture from the Collection of pure dairy cultures Lactoflora, Prague, Czechoslovakia) was used throughout. The cultivation medium of Rogosa *et al.* (1951) was modified (without components containing fatty acids) as follows; composition in g: bacto-tryptone Difco 10, dextrose 20, potassium dihydrogen phosphate 6, triammonium citrate 2, sodium acetate 25, acetic acid 1, $MgSO_4.7H_2O$ 0.575, $MnSO_4.2H_2O$ 0.12, $FeSO_4.7H_2O$ 0.034, distilled water to 1 L, pH 5.8.

The lyophilized culture of *L. bulgaricus* 370 (1 ampoule) was precultivated in 5 mL of sterile fresh cow milk. After 2 d the culture was transferred to 250 mL of the cultivation medium in a round-bottom flask (500 mL) and used for 3 d to inoculate four Fernbach flasks each containing 500 mL of the nutrient medium. The last fermentation step was the fermentor with 20 L of the cultivation medium inoculated with 2 L of the 2-d old culture.

TABLE I. Content of octadecenoic acids in the biological material (relat. %)

Acid[a]	Parsley seed		Olive oil		*L. bulgaricus*	
	ref.[b]	detected	ref.[c]	detected	ref.[d]	detected
6—18 : 1	71.5	76.1	0	0	0	0
9—18 : 1	5.3	4.4	75	72.7	0	2.7
11—18 : 1	0	0	0	0	26.3—67.7	55.2

[a] Number before the dash indicates the double bond position, number after the dash indicates carbon atom numbers, number after the double fullstop indicates the number of double bonds.
[b] Kleiman and Spencer (1982).
[c] Gunstone (1967).
[d] Thorne and Kodicek (1962) (for various species of the genus *Lactobacillus*).

TABLE II. Content of fatty acids in *L. bulgaricus* determined by means of GC—MS (relative %) and splitting in MS[a]

Pyrrolidinide of an acid[b]	%	M[c]	Relative intensity		$12m_a$ interval
			lower	higher	
5 : 0[d]	0.1	155	—	—	—
7 : 0[d]	0.1	183	—	—	—
i-10 : 0[d]	0.1	225	196	182—210	—
N	0.1	—	—	—	—
ai-10 : 0[d]	0.1	225	182	196—168	—
12 : 0	0.7	253	—	—	—
i-12 : 0[d]	0.3	253	224	210—238	—
ai-12 : 0[d]	0.1	253	210	224—196	—
N	0.1	—	—	—	—
13 : 0	0.1	267	—	—	—
ai-13 : 0[d]	0.1	267	238	224—252	—
ai-13 : 0[d]	0.2	267	224	210—238	—
14 : 0	1.5	281	—	—	—
i-14 : 0	0.4	281	252	238—266	—
i-15 : 0	0.1	295	266	252—280	—
16 : 0	19.6	309	—	—	—
i-16 : 0	0.8	309	280	266—294	—
9—16 : 1[e]	5.4	307	—	—	196—208
11—16 : 1[e]	3.8	307	—	—	224—236
ai-17 : 0	0.1	323	280	266—294	—
10—17 : 1[e]	0.1	321	—	—	210—222
18 : 0	0.2	337	—	—	—
9—18 : 1[e]	2.7	335	—	—	196—208
11—18 : 1	55.2	335	—	—	224—236
N	0.3	—	—	—	—
c-11, 12—19 : 0	6.6	349	—	—	224—236
11—20 : 1[d]	0.1	363	—	—	224—236

[a] It serves to determine branching of fatty acids (iso-acids and anteiso acids) and double bond positions according to the rules formulated by Andersson (1978): 1) Splitting from the hydrocarbon end of fatty acid, ions with a different relative intensities (M—15, M—43 and M—29 in iso acids, M—29, M—57 and M—43 in anteiso acids) originate before and after the branching; 2) at the site of interruption of the sequence of $14m_a$ splitting ($12m_a$ is split) the double bond position can be localized.

[b] N — not identified; i — iso acid; ai — anteiso acid; number before the dash — double bond position; c and number before the dash — cyclopropane ring position.

[c] Molecular ion.

[d] It has not yet been described in the genus *Lactobacillus*.

[e] It has already been detected in the genus *Lactobacillus* but the double bond position has not been determined.

After a 3-d cultivation in the fermentor the biomass was centrifuged, washed with distilled water and lyophilized; 6.3 g of the product was obtained. All cultivations proceeded statiscally at 30 °C.

Isolation and preparation of pyrrolidinides of fatty acids. Oleic acid, in the form of saponified olive oil, is commercially available (Lachema Brno, Czechoslovakia). Petroselinic acid was isolated according to Skello and Spence (1952). Ten g of parsley seeds (Sempra, Czechoslovakia) were ground, saponified with 20 % NaOH in 50 % ethanol, acidified and after extraction with chloroform petroselinic acid (17.4 % per dry solid of seeds) was obtained. Vaccenic acid in a mixture with other fatty acids was isolated ac-

cording to Thorne and Kodicek (1962). The lyophilized mycelium was hydrolyzed with acid and alkali, acidified and extracted with diethyl ether. The solvent was evaporated and a mixture of acids was obtained. Ten mg portions of the mixture was esterified with a mixture of sodium methanolate in methanol and hydrochloric acid in methanol according to Jham *et al.* (1982). Pyrrolidinides were prepared from the formed methyl esters by boiling with pyrrolidine according to Anderson (1978) in a yield of 90 % (calculated per acids).

Gas chromatography—mass spectrometry. The analysis was performed in a LKB 9000 apparatus (LKB Produkter AB, Sweden) with glass columns 3 000×3 mm ID packed with Chromaton N-AW-DMCS (125—160 μm) (Lachema Brno, Czechoslovakia) with a 5 % covering with stationary phases OV-101 or OV-225 (Lachema Brno, Czechoslovakia). A temperature program of 140—245 °C at a rate of 5 K/min was used in the column with OV-225 and one of 140—295 °C at a rate of 5 K/min was used in case of the column packed with OV-101. Helium flowing at 25 mL/min served as carrier gas. Temperature of the glass injector of the mass spectrometer was 200 °C, of the separator 260 °C and of the ionic source 290 °C. Ionization energy was 11 aJ (70 eV) and voltage of the electrostatic field was 3.5 kV. Fatty acids were identified according to Anderson (1978).

The authors wish to thank Mr. J. Zajíček for the cultivation of *L. bulgaricus* in the 20-L fermentor.

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