Quantitative Method for the Gas Chromatographic Analysis of Short-Chain Monocarboxylic and Dicarboxylic Acids in Fermentation Media

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A method for the preparation and gas chromatographic analysis of the butyl esters of volatile (C_1-C_7) and nonvolatile (lactic, succinic, and fumaric) acids in microbial fermentation media is presented. Butyl esters were prepared from the dry salts of the acids. The esters were separated by temperature programming on a column of Chromosorb W coated with Dexsil 300 GC liquid phase and analyzed with a flame ionization detector. Apparent recoveries with butanol-HCl or butanol-H_2SO_4 as butylating agents were 80 to 90% for most acids. Chromatographic profiles of the butyl esters demonstrated that both volatile and nonvolatile acids can be detected and separated in 24 min on a single column. Standard calibration curves (peak area versus concentration) of the butyl esters were linear in the range of 5 to 40 μmol of acid per ml. The advantages of using an internal standard (heptanoic acid) for quantitating fatty acids in a mixture are given. Chromatograms of butylated fermentation media in which rumen anaerobic bacteria were grown illustrated that this method is useful for determining short-chain volatile and nonvolatile acids of taxonomic significance.

An important feature in the identification of anaerobic bacteria is an analysis of the acid fermentation products formed in culture media (9). These volatile (C_1-C_7) and nonvolatile (lactic, fumaric, and succinic) acids have been analyzed by various gas-liquid chromatographic methods. Problems arising from direct chromatography of the volatile components include free acids interacting with metal columns and adsorbing to polar supports and stationary phases, resulting in poor quantitation due to sample loss, peak tailing, and ghosting (10, 13). Moreover, many stationary phases have low thermal stabilities and tend to desorb ("bleed") from supports with repeated use.

In a report by Lambert and Moss (11) a procedure was described for the preparation and analysis of the butyl esters of short-chain volatile and nonvolatile acids on a Chromosorb-W support coated with a new, highly stable stationary phase, Dexsil 300 GC. This method is useful since it eliminates those problems associated with direct analysis of free acids, and both volatile and nonvolatile acids can be separated on a single column. The procedure described by Lambert and Moss, however, includes solvent extraction and evaporation steps which could lead to considerable loss of free acids from samples (5, 8). In this publication we report a modified and simplified Lambert and Moss method for butylation of the sodium salts of carboxylic acids which provides for chromatography of multiple samples of fermentation media. When acids were esterified from samples and compared with authentic butyl esters, the apparent recoveries were 80 to 90% for most acids. Furthermore, in these studies the addition of an internal standard (heptanoic acid) to samples facilitated quantitation of the volatile and nonvolatile acids.

MATERIALS AND METHODS

Gas chromatograph and auxiliary equipment. The gas chromatograph used was a Hewlett-Packard model 5754B equipped with a hydrogen flame ionization detector. Additional components used with this instrument were also Hewlett-Packard equipment and consisted of the following: (i) an autosampler (model 7670A) with operating controls and injection units set for single analysis per sample and minimum wash cycle; (ii) a strip-chart recorder (model 7123A) of 100 mV full-scale deflection set for a chart speed of 0.5 inch (1.3 cm)/min, and (iii) a digital integrator (model 3373B) set for minimum input sensitivity (with a resolution of 1 μV/s) and capable of printing retention time (midpoint of the peak area).

Column and chromatographic conditions. Fatty
acid butyl esters were separated on a coiled glass column (0.25 inch by 6 feet [0.64 by 182.9 cm]) packed with Chromosorb W (80 to 100 mesh, HP, DMCS, AW) coated with 10% Dexsil 300 GC (Analabs, North Haven, Conn.). Dexsil 300 GC is a recently developed (Olin Corp.) polyboronarosiloxane polymer stationary phase of moderate polarity which is stabilized against thermal degradation (maximum temperature limit, 450 to 500 °C). Physical and chemical properties of this material have been given by Finch (7). The coated packing was loaded into columns by gentle tapping with a vibrator. Silanized glass wool was used to plug the column ends. The column was conditioned prior to use by heating to 250 °C for 24 h and under a flow of nitrogen gas. Sample application was made by on-column injection.

The following additional chromatographic conditions were employed throughout this study: (i) gas flow rates of 84, 260, and 50 ml/min for hydrogen, air, and nitrogen, respectively; (ii) injection port temperature, 250 °C; (iii) detector temperature, 270 °C; and (iv) temperature program, initial column bath was set at 50 °C for 3 min followed by 10 °C/min temperature increase to 250 °C. The total time for chromatographic separation and integration of each sample was 24 min. A 5-min column bath cooling period followed after the last ester (dibutyl fumarate) was detected and integrated.

Sample preparation, butylation procedure, and recovery of esters. A standard mixture containing 40 μmol each of the following acids per ml was prepared in distilled water, and the pH was adjusted to 9 to 10 with 10 N NaOH: formic, acetic, propionic, isobutyric, butyric, isovaleric, valeric, caproic, heptanoic, lactic, fumaric, and succinic. Dilutions of the standard mixture were made to obtain additional samples containing 5, 10, 15, 20, and 30 μmol/ml. (All samples were made alkaline to convert the free acids to the ionized species and thus prevent their loss during subsequent lyophilization.) Samples (1 ml) of each fatty acid mixture (or individual fatty acids) were placed in culture tubes (13 by 100 mm) (Kimble Products), frozen in an alcohol-dry ice bath, and dried overnight on the shelf unit of a continuous freezer dryer (New Brunswick, model V-13). To the dry salts of the acids were added 0.5 ml of chloroform (or hexane) and 0.2 ml of 1-butanol saturated with anhydrous HCl. (Butanol was saturated with anhydrous HCl by bubbling until a pH of 1 or less was achieved. The butanol-HCl mixture will remain saturated in a stoppered bottle for about 1 month.) Butanol (0.2 ml)-H₂SO₄ (0.05 ml) and 0.2 ml of boron trifluoride-butanol (14% wt/vol; Applied Science) were also compared as butylating agents. After mixing on a Vortex spinner, the tubes were tightly capped (Teflon-lined, screw cap), and the mixture was heated at 90 °C in a temperature block (Lab-Line Instruments, Melrose Park, Ill.) for 2 h. Tubes were then cooled to room temperature and 0.2 ml of trifluoroacetic anhydride (TFA; Sigma Chemical Co.) was added to each; the solution was mixed and allowed to react for 1 h. The TFA was used to (i) react with hydroxyl acids (e.g., lactic) forming the trifluoroacetyl esters and (ii) react with any excess butanol in the reaction mixture. Samples were then washed twice with 1 ml aliquots of distilled water to remove excess TFA reagent, and the water layer was discarded. The chloroform layer (adjusted to 1 ml) which contained the butyl esters was placed into 1-ml vials with Teflon-lined aluminum seals (Wheaton Glass Co., Millville, N.J.). Two microliters of sample was injected and analyzed by gas chromatography.

Cultures of rumen anaerobic bacteria were centrifuged to remove cells, and the supernatant (1 ml) was treated in the same way as the standard acid mixture.

Authentic butyl esters of the various acids were made up as chroflow (5 to 40 μmol/ml concentration) and analyzed either singly or as a mixture.

Internal standardization and calculations. Calibration with an internal standard was performed by the addition of a known amount of heptanoic acid to standard mixtures of fatty acids or fermentation media samples before freeze drying and esterification. The formula used for calculating the quantity (micromoles per milliliter) of a sample unknown, containing an internal standard, is given by: (A₁ - CA₁(RRF)) / (A₀ - CA₀(RRF)), where A₀ and A₁ are areas of the unknown component and internal standard, respectively; C₁ is the concentration of internal standard in micromoles per milliliter; and RRF (relative response factor) is the ratio of A₁C₁-A₀C₀ where A₁ and C₁ are the area and concentration of prepared fatty acid butyl esters prepared from a standard calibration mixture containing 5 to 40 μmol each of C₁-C₇, lactic, fumaric, and succinic acids per ml.

The percent recoveries of prepared butyl esters were calculated by comparing peak areas (in microvolts per second integrator units) with those of authentic esters. Mean peak areas and standard deviation of samples run in triplicate were also determined for each butyl ester.

Chemicals and reagents. Authentic butyl esters of formic, acetic, propionic, isobutyric, butyric, isovaleric, caproic, heptanoic, lactic, fumaric, and succinic (dibutyl), and succinic (dibutyl) acids were obtained from Pfaltz and Bauer (Flushing, N.Y.), and butyl valerate was purchased from K & K Laboratories (Plainview, N.Y.). These butyl esters were 95 to 98% pure. Monocarboxylic (C₁–C₅), iso C₆ and C₇, and dicarboxylic (fumaric and succinic) acids were obtained as the free acid from Eastman Kodak Co. (Rochester, N.Y.) or Sigma Chemical Co. (St. Louis, Mo.). Chloroform and 1-butanol were of spectrograde quality (Mallinckrodt Chemical Works, St. Louis, Mo.). All chromatography gases were obtained from Matheson Gas Products (East Rutherford, N.J.). Other chemicals used were reagent grade.

 Cultures. Rumen anaerobic bacterial strains used in this study were obtained from M. P. Bryant (University of Illinois). Bacteroides ruminicola 23, Bacteroides melaninogenicus ATCC 25845, Butyri vibrio fibrisolvens D1, Megasphaera elsdenii B159, Ruminococcus albus 7, Eubacterium ruminantium GA-195, and Lactobacillus vitiulins T-185 were grown for 7 days at 37 °C in a rumen fluid-glucose...
medium (4) under a 10% CO₂-90% N₂ gas phase. Cultures were centrifuged to remove cells, and the fermentation medium was processed as described above for preparation and recovery of butyl esters. Uninoculated media samples served as controls.

RESULTS AND DISCUSSION

Effects of butylating agents on esterification of acids. Data on esterification and apparent recovery of various volatile and non-volatile acids using different butylating agents are shown in Table 1. With butanol-HCl, recovery of C₁-C₇ and lactic acids (relative to authentic butyl esters) varied from 80 to 95% (see also Table 2); recoveries for dibutyl succinate and dibutyl fumarate, however, were 78 and 68%, respectively. Using butanol-H₂SO₄ as the butylating agent, recoveries of acids were comparable to those with butanol-HCl, except that only 30% of the formic acid was recovered as the butyl ester. The low recovery of butyl formate with butanol-H₂SO₄ may be due to oxidation of the formic acid to CO₂ and water by the concentrated sulfuric acid. In contrast, esterification of similar acid mixtures with BF₃

| Esterified acid | Butylating agent (%) recovery† | Butanol-HCl | Butanol-H₂SO₄ | BF₃-Butanol |
|----------------|--------------------------------|-------------|-------------|-------------|
| Formic         |                                | 87          | 30          | 95          |
| Acetic         |                                | 86          | 86          | 85          |
| Propionic      |                                | 89          | 90          | 73          |
| Isobutyric     |                                | 81          | 81          | 54          |
| Butyric        |                                | 87          | 82          | 64          |
| Isovaleric     |                                | 85          | 82          | 44          |
| Valeric        |                                | 95          | 92          | 69          |
| Caproic        |                                | 80          | 79          | 54          |
| Heptanoic      |                                | 86          | 84          | 60          |
| Lactic         |                                | 94          | 92          | 81          |
| Succinic       |                                | 78          | 78          | 42          |
| Fumaric        |                                | 68          | 75          | 25          |
| Mean           |                                | 85          | 79          | 62          |

† Reaction mixtures contained 20 μmol of each acid per ml.

The freeze-dried salts of fatty acids were added 0.8 ml of chloroform and either 0.2 ml of butanol-HCl, 0.2 ml of butanol-0.05 ml of concentrated H₂SO₄ or 0.2 ml of BF₃-butanol; the pH of each reaction was 0, 1.1, and 2.0, respectively. Reaction mixtures were processed, washed, and extracted as described in Materials and Methods.

Relative to the response (peak area) with authentic fatty acid butyl esters each at 20 μmol/ml concentration. Percent recoveries are the mean of triplicate runs.

butanol resulted in butyl ester recoveries varying from 25 to 95%; poorest recoveries were obtained for succinic and fumaric acids.

Several aspects of the butylation reaction with butanol-HCl or butanol-H₂SO₄ have been considered. Fatty acids can be reacted and extracted in hexane as well as in chloroform with no appreciable difference in recovery or alteration of the chromatographic profile. Diethyl ether, however, is not a desirable reaction solvent because of its high volatility (boiling point, 35 C). Maximum butylation of fatty acid mixtures at concentrations of 5 to 40 μmol/ml each was achieved (particularly for formic and acetic acids) when the reaction was carried out for 2 h at 80 C. We have observed that recoveries of formic, acetic, and fumaric butyl esters were at least 5 to 10% lower when the butylation reaction was carried out for 30 to 60 min. Extending the heat step reaction beyond 3 h resulted in reduced recoveries (30 to 40% less) of the C₁-C₇ acids. It is necessary to allow the mixture to react with TFA to remove excess butanol, since butanol adsorbs tenaciously to the Dexsil column and interferes with elution of the butyl esters. TFA also reacts rapidly with the hydroxyl group of lactic acid forming the trifluoroacetyl ester of butyl lactate. In this respect, TFA esterification of lactic acid, presumably as the TFA and butyl derivative, was 10 to 15% (based on recovery) lower when the TFA was allowed to react for 15 to 30 min. However, authentic trifluoroacetyl butyl lactate (synthesized from TFA and butyl lactate, and structure confirmed by nuclear magnetic resonance spectroscopy and elemental analysis) co-chromatographs with butyl lactate under the conditions used in this study. In addition to having the same retention time, butyl lactate and trifluoroacetyl butyl lactate have similar response factors. These esters, therefore, were chromatographically indistinguishable. It is also likely that a mixture of butyl lactate and trifluoroacetyl butyl lactate is formed during the esterification reactions. For purposes of lactate quantitation, however, it is not necessary to know which derivative is formed, since the response factors for these esters are similar.

Although pyridine has been used as a catalyst to enhance trifluoroacetylation of hydroxyl groups on acids (2), our studies indicate that pyridine is not necessary for this reaction. In fact, addition of pyridine (10 to 50 μl) to the reaction mixture reduced recoveries of butyl lactate (15 to 70% less) and most other butylated short-chain acids (10 to 30% less), depending upon the butylating agent used.
It is common practice to extract the water-soluble, short-chain, volatile and nonvolatile acids into organic solvents (diethyl ether or chloroform) from acidified aqueous media. However, poor solvent extraction and recovery of volatile (e.g., formic and acetic) and nonvolatile (lactic and succinic) acids is a frequently reported observation (6, 8). Hankinson et al. (8) noted that only continuous shaking of aqueous samples with a mixture of diethyl ether and petroleum ether resulted in recovery of 57% of the formic acid and 80 to 100% of the other volatile acids present in milk. Doelle and Manderson (6) determined that it was necessary to extract these acids from acidified microbial fermentation media for 2 to 3 h with diethyl ether to recover 90 to 100% of the volatile acids. In this latter study, losses of 10 to 20% occurred due to evaporation of the mixture, whereas distillation resulted in losses of 80, 60, and 20% for acetic, propionic, and isobutyric acids, respectively. Our experience with rapid solvent extraction procedures of acids from aqueous media confirms observations of others (1); only 10 to 30% of the formic and acetic acids and 60 to 80% of the C3-C4 acids and lactic, succinic, and fumaric acids are recovered with diethyl ether or chloroform. Neither the use of hot solvents nor saturation of samples with salts significantly improves the extraction and recovery of most acids. If the extracted sample is evaporated to a smaller volume (e.g., 0.1 to 0.2 ml), we lose approximately 60% of the formic acid and 20 to 30% of the other volatile acids. Our method of lyophilizing media yields a greater recovery of fatty acids and, therefore, has an advantage over extractions and distillation techniques.

**Chromatographic profiles and calibration curves of acid butyl esters.** Typical gas chromatographic separations of a mixture of authentic butyl esters and those prepared from the salts of acids are shown in Fig. 1. The chromatographic profile of authentic and prepared esters of volatile (C1-C2) and nonvolatile (lactic, succinic, and fumaric) acids are superimposable, and individual acids were baseline separated on the Dexsil-Chromosorb W column (Fig. 1a and c). Only lactic and isovaleric did not separate as well, and thus integration and quantitation of the lactic and isovaleric acid peaks were affected. The butyl ester of 2-methyl butyric acid could not be separated from that of isovaleric acid. Retention times of the prepared esters are given in Table 2. A comparison of elution times between authentic and prepared butyl esters indicated they were highly reproducible and usually did not vary by more than 0.5 to 1.0% at the concentrations of acid tested. The profile of acids shown in Fig. 1 has been maintained for the last 6 months on the same preparation of column material (over 1,000 injections), demonstrating the remarkable stability of the Dexsil 300 GC stationary phase with repetitive column temperature programming. Baseline shifting or peak broadening, which may be indicative of

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**Fig. 1.** Gas chromatographic profiles of acid butyl esters. (a) Authentic butyl ester standard (2 µl of 10 µmol of each ester per ml); (b) control reaction mixture containing no added fatty acid; and (c) prepared butyl ester standard (2 µl of 10 µmol of each acid per ml). Acids were esterified with butanol-saturated HCl. Peak symbols: CF, chloroform; R, reagent; F, formic; A, acetic; P, propionic; iB, isobutyric; B, butyric; L, lactic; iV, isovaleric, V, valeric; C, caproic; H, heptanoic (internal standard); S, succinic; Fu, fumaric; U, Unknown components.
desorption or breakdown of the liquid phase, was not observed.

The reagent blank chromatogram in Fig. 1b shows that reagents or by-products of the butylation reaction contribute a minor peak (between 3 and 6 min) to the butyl formate peak; another peak elutes at about 9.5 min but does not interfere with the separation of any other butyl ester. Unknown peaks (U₁, U₂, and U₃) in the chromatogram of the prepared butyl esters of acids (Fig. 1c) were due to trace components contaminating the commercially available succinic and fumaric acids. Similarly, separation of the butyl esters of valeric and caproic acids individually produced minor peaks (trace amounts) which chromatographed under butyl propionate and butyl butyrate, respectively. All other purchased acids and authentic butyl esters were chromatographically pure and free of detectable contaminating components at the concentrations employed.

Standard curves relating peak areas and concentration of fatty acid are given in Fig. 2. Integrator response of acid butyl esters (with the exception of butyl lactate at 40 μmol/ml) was linear in the range of 5 to 40 μmol/ml (0.01 to 0.08 μmol of injected butyl ester). Similar calibration curves were obtained for authentic butyl esters at comparable concentrations. Calibration curves relating peak height to concentration tended to be nonlinear for most butyl ester mixtures containing more than 10 μmol of each acid per ml.

Use of an internal standard. Quantitation problems associated with the analysis of fatty acids in a sample arise from the fact that usually an external standard (mixture of fatty

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**TABLE 2. Retention times and percent recoveries of butylated acids relative to authentic esters**

| Butyl ester of acid | Retention time (min) | SD | 5 | 10 | 15 | 20 | 30 | 40 | Mean | SDc |
|---------------------|----------------------|----|---|---|---|----|----|----|------|----|
| Formic              | 5.47                 | 0.09| 95.2| 93.2| 94.4| 87.4| 81.7| 81.7| 88.9 | 6.2 |
| Acetic              | 7.94                 | 0.05| 86.7| 91.0| 91.3| 86.3| 88.0| 90.3| 88.9 | 2.0 |
| Propionic           | 10.34                | 0.06| 87.5| 87.2| 90.8| 88.3| 87.8| 93.0| 89.1 | 2.3 |
| Isovaleric          | 11.36                | 0.08| 82.4| 82.1| 83.3| 81.4| 81.0| 86.1| 82.7 | 1.8 |
| Butyric             | 12.37                | 0.10| 84.6| 85.2| 86.5| 85.5| 84.7| 89.9| 86.1 | 2.2 |
| Lactic              | 12.90                | 0.12| 72.2| 80.0| 94.2| 93.8| 87.2| 98.6| 87.7 | 10.0|
| Isovaleric          | 13.44                | 0.13| 86.1| 85.9| 86.6| 84.6| 84.7| 90.2| 86.4 | 2.1 |
| Valeric             | 14.43                | 0.14| 96.4| 96.5| 96.6| 95.4| 93.8| 95.7| 95.7 | 1.1 |
| Caproic             | 16.29                | 0.17| 79.2| 80.3| 81.8| 81.8| 80.3| 86.0| 81.6 | 2.4 |
| Heptanoic           | 18.04                | 0.19| 87.3| 86.8| 87.6| 86.3| 85.7| 91.1| 87.5 | 1.9 |
| Succinic            | 22.52                | 0.23| 78.7| 77.9| 78.8| 77.7| 77.7| 80.0| 78.5 | 0.90|
| Fumaric             | 23.08                | 0.23| 65.0| 64.8| 69.5| 68.0| 68.9| 71.2| 67.9 | 2.6 |

* Acid butyl esters were prepared with butanol-HCl.

* Average of triplicate runs at each concentration.

* SD, Standard deviation.

* Relative to the response (peak area) with authentic acid butyl esters. Average of triplicate sample runs at each concentration.
acids) and a sample are run as separate injections at different times. An internal standard compensates for variations between runs in column temperatures, gas flow rates, detector sensitivity, sample preparation, and injection of different amounts of sample, since both sample and the internal standard are co-chromatographed and thus are influenced by these factors to the same relative extent. Heptanoic acid (as the butyl ester) fulfills several criteria for use as an internal standard for determining those fermentation acids considered here; it is (i) chemically similar to the sample species but normally not present in such samples, (ii) available as a pure and stable compound which may be accurately added to the sample, (iii) nonreactive with other acid components in a sample, and (iv) resolved from other acid peaks but similar in its retention time. Table 3 gives a list of the calculated RRFs of various prepared acid butyl esters relative to that of the internal standard, butyl heptanoate (added to each concentration mixture as heptanoic acid). These values show that the RRFs vary with concentration by ± 0.4 to 9% for most esterified acids. Butyl lactate, however, varied as much as ± 30% (standard deviation, 0.122). The apparently low RRF for butyl lactate at sample concentrations of 5 and 10 μmol/ml is partly explained by the fact that the butyl lactate peak is not completely resolved from butyl isovalerate, and consequently there is interference with integration of this peak. In most fermentation media samples, isovaleric acid is a minor component and would not interfere with quantitation of butyl lactate. In our experience with microbial fermentation media, we have made use of an average RRF (see Table 3) and observed that most acids in a sample can be accurately quantitated to within 5 to 10% when they are present in concentrations ranging from 5 to 40 μmol/ml.

Application of the methods described in this paper to samples of microbial fermentation media are illustrated in Fig. 3 and Table 4. Chromatograms of fermentation products produced by rumen bacterial species in a rumen fluid-glucose medium are shown in Fig. 3, and quantitation of acids as the butyl esters using an internal standard is given in Table 4. Similar fermentation patterns (major and minor products) for these bacterial strains have been described by Bryant (3) and illustrated in the VPI Anaerobe Laboratory Manual (9). These data indicate that acids (C₁₋₃ lactate, and succinic) as the butyl esters can be detected and quantitated with little interference from other components of the medium. The methods described in this paper are suitable, therefore, for analysis of acid fermentation products produced by anaerobic bacteria for purposes of identification. Preparation and analysis of the butyl esters of monocarboxylic and dicarboxylic acids have also been useful to determine milk fatty acids (14) and to identify species of Pseudomonas (12) and Neisseria (2).

**Table 3. RRFs of prepared butyl esters of acids using heptanoic acid as internal standard**

| Butyl ester of acid | RRF* | Mean | SD*  |
|---------------------|------|------|------|
|                     | 5%   | 10%  | 15%  | 20%  | 30%  | 40%  |      |
| Formic              | 0.319| 0.326| 0.334| 0.359| 0.357| 0.348| 0.341|
| Acetic              | 0.416| 0.492| 0.500| 0.503| 0.548| 0.523| 0.497|
| Propionic           | 0.573| 0.607| 0.621| 0.634| 0.635| 0.642| 0.618|
| Isobutyric          | 0.687| 0.700| 0.708| 0.717| 0.719| 0.726| 0.710|
| Butyric             | 0.719| 0.738| 0.739| 0.750| 0.757| 0.759| 0.744|
| Lactic              | 0.216| 0.321| 0.423| 0.479| 0.472| 0.552| 0.411|
| Isovaleric          | 1.010| 0.981| 0.948| 0.947| 0.922| 0.929| 0.956|
| Valeric             | 0.881| 0.887| 0.886| 0.890| 0.894| 0.901| 0.892|
| Caproic             | 0.930| 0.947| 0.944| 0.950| 0.950| 0.957| 0.946|
| Succinic            | 0.957| 0.960| 0.954| 0.962| 0.951| 0.956| 0.957|
| Fumaric             | 0.849| 0.861| 0.895| 0.899| 0.905| 0.931| 0.890|

* Calculated from the peak areas of each fatty acid butyl ester relative to that of the internal standard (butyl ester of heptanoic acid). Heptanoic acid (10 μmol) was added to a mixture of fatty acids each containing 5 to 40 μmol. RRFs are the average of triplicate analyses for each acid.

* Micromoles of fatty acid per milliliter in mixture.

* SD, Standard deviation.
Fig. 3. Chromatographic profiles of esterified fermentation products in rumen fluid-glucose medium cultures of (a) uninoculated control, (b) Lactobacillus vitulinus T-185, (c) Bacteroides melaninogenicus ATCC 25845, and (d) Eubacterium ruminantium GA-195. Symbols are the same as given in the legend to Fig. 1.
**SHORT-CHAIN MONO- AND DICARBOXYLIC ACIDS**

**Table 4. Fermentation products formed by representative bacterial species**

| Species and strain                      | Fermentation productsa (µmol/ml of medium) | F | A | P | B | L | IV | V | C | S | Fu | Patterna |
|----------------------------------------|--------------------------------------------|---|---|---|---|---|----|---|---|---|----|-----------|
| Ruminococcus albus 7                   |                                            | 2.0| 14.3|   | 21.9|   | 1.2 | 3.3 | 16.0 |    | Af | BCfviv |
| Megasphaera elsdenii B159               |                                            | 9.7|    |    | 4.5 |    | 15.1 | 16.1 |       |    |    | FLBa     |
| Eubacterium ruminantium GA-195          |                                            | 22.4|    |    |    | 15.5 | 1.2 |       |    |    |    |           |
| Lactobacillus vitiilinus T-185          |                                            | 1.9|    |    |    | 32.7 |    |       |    |    |    | Laf       |
| Bacteroides ruminicola 23               |                                            | 3.8|    |    | 16.4| 9.3 | 3.8 |       |    |    |    | ASpf      |
| Bacteroides melaninogenicus ATCC 25845  |                                            | 10.7|    |    |    | 13.8 | 14.7|       |    |    |    | SA1fu     |
| Butyrivibrio fibrisolvens D1            |                                            | 11.2|    |    | 15.8| 6.1 |    |       |    |    |    | BF1       |

*a* Products formed were determined from duplicate cultures of each strain. Calculations are based on the internal standard, heptanoic acid, and are corrected for acids in uninoculated (control) media. Abbreviations: F, formic; A, acetic; P, propionic; B, butyric; L, lactic; IV, isovaleric; V, valeric; C, caproic; S, succinic; Fu, fumaric.

*b* Uppercase letters refer to acids formed in amounts of 10 µmoles/ml of medium or greater, whereas lowercase letters refer to amounts less than 10 µmol/ml. Products formed in less than 0.5 µmol/ml amounts are not given.

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