Changes in Microbial Population During Fermentation of Feedlot Waste with Corn

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A new process for recycling feedlot waste involves the fermentation of liquid from this waste combined with corn. Changes in the flora of this silage-like fermentation were followed. The fermentation was dominated by lactobacilli and yeasts, which initially constitute 1% or less of the natural flora. The species of yeasts and lactics involved were characterized. The fermentation has two phases. A single heterolactic species multiplied rapidly for the first 24 h until it represented 95% of the lactobacilli and more than 90% of the total microflora. It displaced the betabacterium predominant among lactics of the original waste; the acid produced killed coliforms and other organisms in feedlot waste; and the acetic acid produced probably caused the death of the dominant native yeast Trichosporon cutaneum (de Beurm., Gougerot et Vaucher) Ota. The peak lactobacillus count remained constant (about 2 x 10^8 organisms/g [wet weight]) throughout the rest of the fermentation. Homolactics dominated the later phase and yeasts increased to 9.5 x 10^7 organisms/g (wet weight). At 6 days, a stable mixture of three lactobacilli was present, one streptobacterium, one thermobacterium, and one betabacterium. Similarly, yeasts stabilized as a mixture of two Candida sp. and one Pichia sp. The dominant species of lactics were characterized. Information on the sequence of microorganisms provides a basis for enhanced protein synthesis in the fermentation.

The use of animal waste, primarily as a nitrogen source, in the feeding or refeeding of livestock has been reviewed by Anthony (1) and Smith (11). About 70% of the nitrogen remains in the liquid portion when feedlot waste (FLW) is screened to remove fibrous material (11). Rhodes and Orton (R. A. Rhodes and W. L. Orton, Trans. Am. Soc. Agric. Eng., in press) devised a solid substrate fermentation that combined FLW liquid (FLWL) with corn. Changes in microflora during that fermentation and characterization of the dominant species involved are presented here.

MATERIALS AND METHODS

Raw FLW was mixed with water to give a homogeneous slurry containing about 15% solids. The slurry was separated into fibrous and liquid fractions by using a 30-mesh copper screen. Coarsely cracked corn and FLWL containing about 10% solids were mixed in a 2:1 ratio (wt/wt) so that the mixture had a 37 to 40% moisture content. Preparation of FLWL and large-scale fermentation procedures are detailed by Rhodes and Orton (in press).

Sampling procedure. Corn (350 g) and FLWL (175 g) were mixed in 2-liter Erlenmeyer flasks. The flasks were held at 9° from horizontal on a board rotating at 0.6 rpm. Incubation was at 28 C. Two 5-g samples were taken at 1, 6, 12, 24, 48, 72, and 144 h. One sample was triturated in 10 ml of distilled water for 10 min, and the pH was measured before drying at 100 C for 24 h to give the dry weight. The second sample was blended with 20 ml of cold (4 C) 0.1 M phosphate buffer (pH 7.0) for 30 s in a Waring blender, filtered through a loose glass-wool plug, and serially diluted (1:10) in 0.1% tryptone. Counts were made by spread plating 0.3 ml of selected dilutions in triplicate.

The following media were used for counts: Eugonagar for total count, L and LBS agars for lactobacilli, Streptosel for total streptococci, RF Streptococcal with triphenyl tetrazolium chloride for fecal streptococci, Staphylococcus 110 for staphylococci, eosin methylene blue for coliforms, and Mycofil with added dihydrostreptomycin sulfate (0.2 mg/ml) and penicillin G (330 U/ml) for yeasts and molds. All media were BBL products (BBL, Division of Bioquest, Cockeysville, Md.). Eosin methylene blue plates were incubated at 37 C for 18 to 24 h before counting; all other plates were counted after incubation at 28 C for 2 days.

Apparent coliform colonies of the 24-h sample were transferred from eosin methylene blue plates to lactose broth and were examined microscopically. Colonies of lactobacilli from LBS and yeasts from Mycofil were transferred, respectively, to Micro Assay Culture Agar (BBL) and YM agar (Difco Laboratories,
Detroit, Mich.). Isolates were incubated for 2 to 3 days at 28 C before storing at 4 C for subsequent examination. At each sample time, one to three plates of either the countable dilution or the next higher dilution were picked in entirety from LBS and from Mycofil (30 to 70 isolates per sample time).

**Characterization of lactobacilli.** Classification was based primarily on that of Sharpe et al. (9) supplemented with Bergey's Manual (2) and reports by Rogosa and co-workers (7, 8).

The primary screen for lactobacillus isolates included tests for acid and gas from glucose and arabinose, acid from raffinose and mannotol, nitrite from nitrate, catalase, oxidase, starch hydrolysis, and reaction in litmus milk (BBL). The basal medium for this screen contained neopeptone, 5 g; tryptone, 5 g; yeast extract, 5 g; 1-cystine, 0.5 g; Tween 80, 1 g; MgSO₄, 0.5 g; MnSO₄, 0.1 g; FeSO₄. 7H₂O, 0.05 g; and water, 1 liter. Carbohydrates at 1% final concentration and bromocresol purple, 0.0016%, were added for the fermentation tests; 1% glucose and 0.1% KNO₃ were added for the nitrite test; 1% soluble starch and 2% agar were included in the screening medium for the starch hydrolysis test. Except growth temperature studies, all tests to characterize lactobacilli were incubated at 30 C. Stock cultures were transferred to MRS medium (4) solidified with 1.5% agar and to MRS medium to establish ability to grow at 15 C; growth in three serial transfers was considered positive. In formulating the MRS medium, 0.5 g of neopeptone (Difco) and 0.5 g of tryptone (Difco) were substituted for each gram of Oxoid peptone, and Lab-Lemco (Oxoid) was replaced with beef extract (Difco). Cultures representing the groups differentiated in this preliminary screen were examined by Gram stain.

The three major subgroups of lactobacilli were delineated in greater detail. The method of Sharpe et al. (9) was followed for carbohydrate fermentations, with bromocresol purple as the indicator. Nutritional requirements were determined by the method of Rogosa et al. (6). Pancreatin-treated purified casein (Labco, "vitamin free") was replaced by tryptone in preparing the enzymatic casein digest following the method of Roberts and Snell (5); each charcoal absorption step was repeated three times with one and a half times the amount of Daclo G-60 indicated. Betabacteria were further differentiated by fermentation of cellubiose, xylose, and melezitose as well as by nutritional requirements for thiamine, riboflavin, pyridoxal, folic acid, nicotinic acid, and pantothenic acid. Streptobacteria were differentiated by the fermentation of melibiose, rhamnose, lactose, sorbitol, and salicin, by the percentage of acid formed in milk, and by the same nutritional requirements used for the betabacteria. Thermobacteria were differentiated by the fermentation of amygdalin, cellubiose, galactose, maltose, salicin, sorbitol, and trehalose.

Cultures were categorized by the preliminary screen and differential tests. Representatives of the larger groups were characterized in further detail by these tests: (i) Gram stain of an 18- to 24-h culture in MRS; (ii) nutritional requirements of the six vitamins used in the differential tests (repeated); (iii) fermentation of 18 carbohydrates in modified MRS medium (many repeated); (iv) pH of 7-day MRS culture; (v) production of ammonia from arginine according to the method of Briggs (3); (vi) growth in MRS plus 0.4% sodium dodecyl sulfate, as a replacement for Teepol; (vii) growth in MRS plus 6, 4, and 8% NaCl; and (viii) growth in MRS at 15, 37, and 45 C through three serial transfers. Fully characterized isolates were lyophilized and deposited in the ARS Culture Collection maintained at the Northern Laboratory.

**Characterization of yeasts.** Yeasts were grouped initially by colony type and by gas production in YM. Three cultures of each type from each sample time were characterized by Wickerham's methods (12). These included carbon utilization with glucose, acetate, lactate, and soluble starch as substrates and nitrogen utilization with (NH₄)₂SO₄, urea, and KNO₃ as substrates.

Acid tolerance of the yeasts was determined by adjusting the pH of YM broth with H₂SO₄ and acetic acid. Inhibition by acetic acid was also determined by back-titrating YM broth media of low pH with NaOH. Growth of three cultures of each yeast type was tested through three serial transfers; growth in the last transfer was measured after 1, 2, and 3 days with a Bausch and Lomb Spectronic 20 densitometer. Selected cultures of each yeast type were lyophilized and tentatively identified by the ARS Culture Collection taxonomist.

**RESULTS**

Growth patterns of the organisms are plotted in Fig. 1. Lactobacilli became the dominant group in 24 h, rising from about 1% to more than 90% of the total aerobic population; they remained dominant throughout the fermentation. Yeasts also increased sharply. Initially numbering 10⁴ organisms/g (wet weight), they attained a maximum count of 9.5 × 10⁶ organisms/g (wet weight) at 72 h. Coliforms, initially 10% of the aerobic population, died within 48 h. Colonies from the 24-h plating on eosin methylene blue did not produce gas when transferred.
into lactose broth. Fecal streptococci declined gradually throughout the fermentation, showing a thousand-fold reduction from the original 1.1 \times 10^6 organisms/g (wet weight). Staphylococci were not found.

The decline of pH from 5.5 to 4.2 reflected the growth of lactobacilli. Change in odor also accompanied the growth of lactics and yeasts. Within 6 to 12 h the acidic odor of a typical heterolactic fermentation replaced the fecal odor of FLWL. The odor of malic acid was detectable at 48 h; by the end of the fermentation, the odors of ethanol and ethyl acetate were apparent.

The lactobacilli were grouped according to the results of tests used in their characterization. Scattered isolates, less than five of any type, defied classification either as an established species or as a member of one of the groups listed.

The two largest groups of isolates were both betabacteria (Table 1). Group 1 isolates were similar to *Lactobacillus buchneri*. But unlike *L. buchneri*, group 1 isolates did not ferment melezitose but did ferment trehalose; they required folic acid; they did not produce ammonia from arginine and did not grow in 0.4% sodium dodecyl sulfate. Also, about one-fourth of the isolates fermented cellobiose and one-sixth of them did not ferment raffinose; a few strains required pyridoxal. On the basis of the primary screen, group 2 isolates were similar to *Lactobacillus fermenti*, with the fermentation of arabinose like that of a minority of *L. fermenti* strains. These isolates occurred as pinpoint colonies on LBS and grew only in the stab on Micro Assay Culture Agar slants. These stock cultures were dead about 3 weeks later.

Three types of streptobacteria were isolated (Table 2). The largest, group 3, was similar to *Lactobacillus plantarum*. Unlike this species, group 3 isolates were unable to grow in 0.4% sodium dodecyl sulfate and did not require pantothenic acid for growth; these isolates

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**Table 1. Characteristics of betabacteria from the fermentation of FLWL combined with corn**

| Tests on all isolates | Added tests on isolates typical of group |
|-----------------------|----------------------------------------|
| **Test**              | **Group (no. tested)**                  | **Test**                | **Group 1 (10 tested)** |
| Fermentation of       |                                        | Fermentation of         |                          |
| Glucose               | 179*                                   | Fructose                | 10                       |
| Gas from glucose      | 179                                    | Galactose               | 10                       |
| Arabinose             | 179                                    | Lactose                 | 6, (2)                   |
| Cellobiose            | 42                                     | Maltose                 | 10                       |
| Mannitol              | (163)                                  | Melibiose               | 10                       |
| Melezitose            | 0                                      | Rhamnose                | 0                        |
| Raffinose             | 115, (33)                              | Salicin                 | (2)                      |
| Xylose                | 48                                     | Sorbitol                | 0                        |
| **Nutritional requirements** |                                    | **Group 1 (10 tested)** |
| Nicotinic acid        | 51*                                   | NH₄ from arginine       | 0                        |
| Pantothenic acid      | 51*                                   | Growth in 0.4% SDS      | 0                        |
| Thiamine              | 179                                    | Growth at 37°C          | 10                       |
| Riboflavin            | 176                                    | Growth at 45°C          | 0                        |
| Pyridoxal             | 6                                      | pH at 7 days in MRS     | 4.68–4.70                |
| Folic acid            | 178                                    | Microscopic appearance  | G⁺ rods, 0.4×0.8–2.0 μm, s, p, sh ch |
| Nitrite from nitrate  | 0                                      |                          |                          |
| Catalase              | 0                                      |                          |                          |
| Oxidase               | 0                                      |                          |                          |
| Growth at 15°C        | 179                                    |                          |                          |
| Starch hydrolysis     | 0                                      |                          |                          |
| Litmus milk           | U, 167                                 |                          |                          |
|                       | a, 10                                  |                          |                          |
|                       | A, 1                                   |                          |                          |
|                       | AR, 1                                  |                          |                          |
|                       | ACR, 1                                 |                          |                          |

* Abbreviations used in Tables 1, 2, and 3: ( ), fermentation weakly positive or late; st, vitamin stimulatory for growth; SDS, sodium dodecyl sulfate. Litmus milk reactions: U, unchanged; a, weak acid; A, acid; R, reduction; C, coagulation. Microscopic appearance: s, single; p, pairs; sh ch, short chains.

* Number of isolates giving positive test.

* Fifty-one of 179 isolates tested all showed vitamin requirement.
Materials and Methods

Transient control vectors using LacZ as a reporter protein were constructed as in [27] (Table 1). The insertion of the Eco RI and Not I restriction sites was performed using the Promega PCR Cloning Kit (Promega, Madison, WI). The primers were designed to introduce unique restriction sites for the cloning of the synthetic LacZ fragment. The PCR was performed using the Promega PCR Cloning Kit (Promega, Madison, WI). The products were cloned into the pBluescript II SK(+) vector (Stratagene, La Jolla, CA) and sequenced by the dideoxy method using the Sequenase system (USB, Cleveland, OH). The plasmid DNA was prepared using the Qiagen PCR kit (Qiagen, Chatsworth, CA). The DNA was then purified using the Qiagen PCR kit (Qiagen, Chatsworth, CA).

Results

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Discussion

The results of the experiments are presented in Table 1. The LacZ expression was tested by Western blotting, and the results are shown in Figure 1. The data show that the LacZ expression is dependent on the presence of the synthetic LacZ fragment in the plasmid DNA.

Yeasts isolated during the fermentation are partially characterized in Table 5. Carbon sources used in utilization tests were those which occur in the fermentation mixture. Nitrogen sources tested were compounds that might be added to enhance single-cell protein production during fermentation. Trichosporon cutaneum was inhibited by acetic acid. When the medium was adjusted with H2SO4, it grew normally at pH 3.5 but was unable to grow at this pH when the medium was adjusted with acetic acid. Media adjusted to pH 3.5 with acetic acid and then back-titrated with NaOH to pH 4.0 afforded no growth. Comparable manipulation of the medium at pH 3.8 yielded normal growth. Apparently, acetic acid concentration was the cause for death of this organism during fermentation. The generic identification here used is a tentative one. The pattern of yeasts during the corn-FLWL fermentation is given in Table 6.
Table 3. Characteristics of thermobacteria from the fermentation of FLWL combined with corn

| Test                | Group 6 (21 tested) |
|---------------------|---------------------|
| Fermentation of     |                     |
| Glucose             | 21*                 |
| Gas from glucose    | 0                   |
| Amygdalin           | 1                   |
| Cellobiose          | 1                   |
| Galactose           | 1                   |
| Maltose             | 4                   |
| Trehalose           | 4                   |
| Arabinose, mannitol, raffinose, salicin, sorbitol | 0 |
| Nitrite from nitrate| 0                   |
| Catalase            | 0                   |
| Oxidase             | 0                   |
| Growth at 15 C      | 0                   |
| Starch hydrolysis   | 0                   |
| Litmus milk         | U, 21               |
| Microscopic appearance | G⁺ rod, 0.6 x 2-3 µm, s, p, sh ch, no granules |

* See Table 1, footnote a.
* Number of isolates giving positive test.

Table 4. Distribution of lactobacilli during the fermentation of FLWL combined with corn

| Type of lactobacillus | Time of fermentation (h) | 1   | 6   | 12  | 24  | 48  | 72  | 144 |
|-----------------------|--------------------------|-----|-----|-----|-----|-----|-----|-----|
| Betabacteria          |                          |     |     |     |     |     |     |     |
| Group 1               |                          | 5*  | 39  | 65  | 57  | 5   | 3   | 5   |
| Group 2               |                          | 43  | 7   | 0   | 1   | 1   | 0   | 1   |
| Others                |                          | 1   | 0   | 0   | 1   | 2   | 1   | 0   |
| Streptobacteria       |                          |     |     |     |     |     |     |     |
| Group 3               |                          | 3   | 2   | 2   | 12  | 12  | 13  |     |
| Group 4               |                          | 4   | 12  | 0   | 0   | 0   | 0   |     |
| Group 5               |                          | 0   | 0   | 0   | 3   | 2   | 0   |     |
| Others                |                          | 1   | 2   | 0   | 0   | 4   | 2   |     |
| Thermobacteria        |                          |     |     |     |     |     |     |     |
| Group 6               |                          | 0   | 0   | 0   | 5   | 7   | 7   |     |
| Others                |                          | 3   | 0   | 0   | 1   | 0   | 2   |     |
| Nonlactics            |                          | 1   | 0   | 0   | 7   | 7   | 2   |     |
| Dead*                |                          | 5   | 6   | 1   | 0   | 1   | 1   |     |

* Number of isolates from countable plate(s) of LBS agar; all colonies on plate(s) transferred.
* No growth on transfer from plate.

DISCUSSION

The corn-FLWL fermentation was a succession of dominant species which evolved into a stable mixed culture of lactobacilli and yeasts. At inception, the natural microflora contained about 10% coliforms, 1% lactobacilli, and 0.01% yeasts. L. fermenti-like group 2 betabacteria accounted for two-thirds of the initial lactics. The L. buchneri-like group 1 betabacteria, the L. plantarum-like group 3 streptobacteria, and L. casei var. alactosus-like group 4 each represented 5 to 10% of the naturally occurring lactics. During the lag phase, group 1 betabacteria became dominant, and group 4 streptobacteria increased to constitute about 18% of the lactic population; group 2 betabacteria decreased to 10% of the population, whereas numbers of group 3 streptobacteria remained constant. The yeasts T. cutaneum and Candida krusei (Castellani Berkhout) occurred in small numbers. The coliform count remained constant as the pH dropped from 5.5 to 5.2.

The logarithmic growth phase of the fermentation between 6 and 24 h was characterized by the growth of group 1 betabacteria, which represented 95% of the lactobacilli (2.6 x 10⁴ mg of protein). The fermentation of FLWL combined with corn

Table 5. Characteristics of yeast isolates from the fermentation of FLWL combined with corn

| Test                | Trichosporon cutaneum | Candida krusei | Candida valida | Pichia membranifaciens |
|---------------------|-----------------------|----------------|----------------|------------------------|
| Gas from glucose    | +                     | +              | -              | -                      |
| Carbon utilization  |                       |                |                |                        |
| Glucose             | +                     | +              | +              | +                      |
| Acetate             | +                     | +              | Wk             | Wk                     |
| Lactate             | +                     | +              | Wk             | Wk                     |
| Soluble starch      | -                     | -              | -              | -                      |
| Nitrogen utilization|                       |                |                |                        |
| (NH₄)₂SO₄           | +                     | +              | +              | +                      |
| Urea                | +                     | +              | +              | +                      |
| KNO₃                | -                     | -              | -              | -                      |
| Growth at           |                       |                |                |                        |
| 28 or 40 C          | +                     | +              | Wk             | Wk                     |
| 32-37 C             | +                     | +              | +              | +                      |
| Growth at           |                       |                |                |                        |
| pH 6.3              | +                     | +              | Wk             | Wk                     |
| pH 3.5 to 4.5       | +                     | +              | +              | +                      |
| (H₂SO₄)            |                       |                |                |                        |
| pH 4.0 to 5.5       | +                     | +              | +              | +                      |
| (HOAc)              |                       |                |                |                        |
| pH 3.8 (HOAc)       | Wk                    | +              | +              | +                      |
| pH 3.5 (HOAc)       | -                     | +              | +              | +                      |
| pH 4.0 (HOAc, 3.8; NaOH) | + | + | + | + |
| pH 4.0 (HOAc, 3.5; NaOH) | - | - | - | - |

* All isolates tested.
+ +, Normal growth; Wk, weak growth; -, no growth.
* Three isolates of each type from each sampling time tested.
* Three isolates of each type tested.
* Medium adjusted with either sulfuric or acetic acid as shown.
' Medium adjusted to the pH indicated in parentheses with acetic acid and then taken to pH 4.0 with NaOH.
organisms/g (wet weight) during this period. Neither group 2 betabacteria nor group 4 streptobacteria were isolated during the log phase, and only the former was occasionally isolated later in the fermentation. The heterolactic fermentation probably killed the \( T. \) cutaneum; \( C. \) krusei constituted 95% of the yeast population by 24 h. The coliform count, which remained constant for the first 12 h of the fermentation, then dropped to one-hundredth of the original number by 24 h. This decrease is probably understated because apparent coliform colonies on eosin methylene blue did not produce gas in lactose broth.

The 24- to 72-h period of the fermentation was one of transition for both lactic and yeast populations. By 48 h, the pH reached a minimum of 4.26, coliforms were no longer isolated, and the number of lactics was stabilized. Whereas total numbers of lactobacilli remained constant, heterolactics declined to 20% of the population. The persistent group 3 streptobacteria became the most numerous lactic, representing one-third of the total. Two newly emergent homolactics were isolated during this period: one, the \( L. \) casei var. casei-like group 5 streptobacteria representing 10% of the population; the other, the \( L. \) delbrueckii-like group 6 thermobacteria representing about 20% of all lactics. The logarithmic growth phase of the yeast population was complete by 72 h, when a total of \( 9.5 \times 10^7 \) organisms/g (wet weight) was present. At 48 h, \( T. \) cutaneum was not recovered. Two additional yeast species first appeared: \( Pichia \) membranaefaciens Hansen as 15% of the total yeasts and \( C. \) valida (Laberle) van Uden et Buckley, present in small numbers. The pH rose to 5.0 at 72 h, at which time about one-third of the yeast population was \( C. \) valida.

At 144 h, the pH had decreased again to 4.22, and both lactic and yeast populations were stable. The final lactobacillus population of 3.6 \( \times 10^9 \) organisms/g (wet weight) was composed of a 3:2:1 proportion of \( L. \) plantarum-like group 3 streptobacteria, \( L. \) delbrueckii-like group 6 thermobacteria, and \( L. \) buchneri-like group 1 betabacteria. The final yeast population of 2.6 \( \times 10^9 \) organisms/g (wet weight) was a 2:1:1 proportion of \( C. \) krusei, \( C. \) valida, and \( P. \) membranaefaciens.

Rhodes and Orton (in press) presented the general aspects of the fermentation. Their process conserved most of the nitrogen from FLWL while converting a waste to a feed of more desirable amino acid composition. Possible health and pollutant hazards inherent in the waste were reduced in the early heterolactic phase of this fermentation. Acid produced then caused the death of coliforms and most other organisms characteristic of FLW. The death of microorganisms like \( T. \) cutaneum and the change from fetid to silage odor probably resulted from acetic acid production. The subsequent fermentation phase, predominantly homolactic in character, provided a selective environment for yeast multiplication.

This change in microbial profile provides a basis for enhanced protein synthesis by the fermentation. A quantitative increase in yeast protein should be attainable by addition of inorganic nitrogen; a selective increase in specific amino acids may result from inoculation with yeast selected for protein composition. In either instance, a continuous but multistage fermentation system is suggested to obtain maximum benefit from both the early heterolactic fermentation and the later homolactic-yeast fermentation.

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**TABLE 6. Distribution of yeasts during the fermentation of FLWL combined with corn**

| Type of yeast               | Time of fermentation (h) |
|-----------------------------|--------------------------|
|                            | 1 | 6 | 12 | 24 | 48 | 72 | 144 |
| \( T. \) cutaneum            | 0*| 3 | 5 | 1  | 0  | 0  | 0  |
| \( C. \) krusei              | 0 | 1 | 5 | 42 | 26 | 13 | 22 |
| \( C. \) valida              | 0 | 0 | 0 | 2  | 14 | 12 | 12 |
| \( P. \) membranaefaciens    | 0 | 0 | 0 | 13 | 9  | 11 | 11 |

* Number of isolates from countable plate of Mycophil agar; all yeast colonies on plate were transferred.
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