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Fortification of Foods by Fermentation with Lysine-Excreting Mutants of Lactobacilli

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Most microorganisms regulate amino acid biosynthesis by mechanisms such as feedback inhibition and repression. Thus, they do not usually produce more amino acids than they need and in fact deplete those in the medium. However, some wild-type microorganisms and mutants have been found that do excrete specific amino acids. Some of these microorganisms are used to manufacture amino acids through fermentation procedures but none have been used directly to augment the amino acid content of food produced by fermentation. We used lysine analogues to select for spontaneous mutants of lactobacilli that over-produce and excrete lysine. Wild type *Lactobacillus acidophilus* and *L. bulgaricus* and lysine-excreting mutants were used to ferment soybean milk to yogurt. In all tests, mutants increased the lysine content of yogurt over that obtained with the wild-type. Mutants of *L. plantarum* were used to increase the lysine content of corn silage. The potential use of lysine-excreting mutants in producing fermented foods is suggested.

Many raw products can be fermented to provide different food, and the range of raw materials includes animal products such as milk, beef, and fish and such plant products as cereal grains, soybeans, coconuts, and peanuts (Hesseltine, 1965). Examples of fermented products are shown in Table I. Although fermentation may not improve the nutritive value of the raw material (van Veen and Steinkraus, 1970), it may enhance taste, sometimes making an inedible material palatable. In some parts of the world fermented foods are an important part of the diet and are classed as "traditional". The nutritive quality of some of these fermented foods could be improved by the use of mutant strains of the fermenting organisms that would excrete an essential amino acid into the food.

Lysine, one of the ten essential amino acids in animal nutrition, is often low in plant materials, and food has been fortified by adding lysine, either synthesized or purified from fermentation liquors (Altschul, 1974). Lysine intake may also be increased by eating varieties of plants that are richer in lysine (Brock et al., 1973). Mutant plants that contain up to twice the lysine in their protein than wild types have been described and used, e.g., high lysine maize, barley, and sorghum (Mertz, 1974).

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Table I. Examples of Fermented Foods and Starting Materials

Place	Product fermented	Product
United States	Milk	Cheese, yogurt
Orient	Grain	Soy sauce
Japan	Rice	Tamari sauce
Indonesia	Soybeans	Tempeh
Orient	Fish	Sauce
Hawaii	Taro	Poi
China	Eggs	Pidan
Java	Peanuts	Ontjon
Java	Coconuts	Bongkrek
Indonesia	Rice or cassava	Tape'
World-wide	Grain	Beer, bread

We suggest a third method to increase the lysine intake by selecting, from the normal food fermenting organisms, mutant strains that excrete this amino acid into the food and then use these strains to ferment food (Sands and Hankin, 1974). The key is finding organisms now used for fermentation that can be selected to excrete lysine.

To find a lysine excreting mutant for fermentation, several methods could be tried. Although many colonies from natural fermentations could be screened and tested for lysine excretion, our experiments with lactobacilli showed that none of the wild-type strains examined excreted lysine. However, most of the species tested on a lysine-free medium (Lysine Assay Medium, Difco, Detroit, Mich.) did produce sufficient lysine for their own growth.

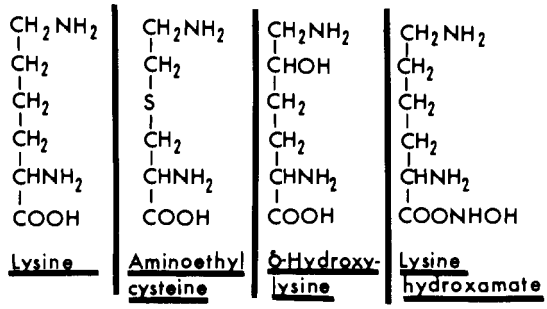

Figure 1. Structural similarity of lysine and lysine analogues.

A second method uses an enzyme, which would destroy any lysine in the medium. Leavitt and Ryan (1974) grew *Pichia* sp. (a yeast) on a medium that contained lysine decarboxylase, and they found mutant strains capable of excreting 2.3 to 5.0 ppm of lysine.

A more conventional approach is to use a lysine analogue (Brock et al., 1973). Growth of the organism is inhibited by the analogue unless the organism can produce and excrete large amounts of lysine. In that case the improved ratio of lysine to analogue presumably prevents inhibition by the analogue. Since selection may also occur for organisms able to inactivate or bind the analogue for organisms unable to transport the analogue, mutants must be tested later to see if they excrete lysine.

Both feedback inhibition and repression control lysine synthesis in bacteria (Stadtman, 1963) and in yeast (Tucci, 1969). To obtain bacterial mutants that overproduce lysine they must be both derepressed and not subject to feedback inhibition, or be "reverse" permease mutants (Halsall, 1975).

Organisms have been challenged with the analogue *S*-2-aminoethyl-L-cysteine and lysine excreters found (Halsall, 1975). None of the lysine excretors, however, are used in food fermentation. Since lactobacilli are used in many food and feed fermentations, we obtained wild-type strains currently being used as inocula in yogurt and experimental silage. Soymilk yogurt fermenters were *Lactobacillus bulgaricus* strain NRRL 724 and *L. acidophilus* strain NRRL B-1910 from C. W. Hesseltine (Wang et al., 1974). The *Lactobacillus plantarum* from silage was strain W 26 from M. K. Woolford, Grassland Research Institute, Hurley, Maidenhead, England.

The wild-type lactobacilli were tested against *S*-2-aminoethyl-L-cysteine (thialysine) and γ-hydroxylysine and an analogue not previously used, L-lysine hydroxamate (Figure 1) as follows (Sands and Hankin, 1974). A uniform suspension of the test lactobacillus was spread on the surface of medium that contained no lysine (lysine assay medium, Difco, Detroit, Mich.). An antibiotic-assay disk that contained the analogue was then placed on the surface. After incubation, a zone of inhibition was seen surrounding the disk, but a few colonies were growing within the zone of inhibition. Many colonies were tested for lysine excretion with *Leuconostoc mesenteroides* (Difco, 1972). Selected mutants were challenged with higher dosages of the analogues until a mutant was obtained that was insensitive to the analogues, even at 5%. *L. plantarum* was screened through four mutation cycles against *S*-2-aminoethyl-L-cysteine. The mutant finally selected excreted 72 ppm of lysine rather than the 1 ppm secreted by the wild-type cell. An example of how lysine excretion may be increased through mutation cycles against increasing concentrations of an analogue is shown in Table II. Similar results were obtained with the other lysine analogues: lysine hydroxamate and hydroxylysine.

Table II. Lysine Excretion by *Lactobacillus plantarum* Exposed to Stepwise Increases in Concentration of *S*-2-Aminoethyl-L-cysteine

Mutation cycle	Amino-ethyl-cysteine, μg/disk	Lysine produced, ppm/ml culture filtrate
Wild type		<1
1	20	9
3	1000	57
4	5000	72

Table III. Increase in Lysine Content of Chopped Maize after Fermentation with *Lactobacillus plantarum*

	No. of tests	Lysine, ppm
Wild-type inoculum	5	467 ± 64
Mutant inoculum	5	547 ± 72
Chopped corn, not inoculated	4	413 ± 28

Strains of *L. acidophilus* and *L. bulgaricus*, which had been used to ferment soybean milk (Wang et al., 1974) to yogurt, were selected for lysine excretion. We obtained strains able to excrete up to 100 ppm in lysine free broth compared with the less than 1 ppm of lysine secreted by wild type.

We tested our mutant selections in the two natural systems from which they originally came, yogurt and silage. Replicate samples of 50 g of chopped maize (corn) plants were placed in plastic bags, inoculated with 10^6 washed cells, and allowed to ferment under nitrogen at 30 °C for 3 days. The fermented corn was acid hydrolyzed with 6 N HCl for 6 h, neutralized, and assayed for lysine (Sands and Hankin, 1974). Inoculation of mutants of *L. plantarum* into chopped corn increased the lysine content about 17% (Table III).

Soy milk was prepared (Wang et al., 1974) from Rampage soybeans. The soy milk was inoculated with the mutant and wild-type strains of *L. bulgaricus* and *L. acidophilus* and incubated 1–2 days at 30 °C. The fermented material or yogurt was hydrolyzed and tested for lysine. Eight batches of soy milk yogurt made with *L. acidophilus* mutants showed increases in lysine content from 2 to 270%. It appears that the *L. acidophilus* strain we used is not uniform in its response and Dr. Hesseltine (USDA, Northern Regional Research Labs., personal communication) confirms this observation. However, we have noted that the level of inoculum can affect lysine excretion. High levels of excretion were routinely obtained except where little inoculum was used, and the bacteria did not ferment the soymilk rapidly. Four tests were made with *L. bulgaricus* mutants and a range in lysine content of 4 to 32% over that obtained with the wild type was found. This bacterium appears to be more uniform in its response than *L. acidophilus*. In all tests the mutants always produced more lysine than the wild type. Furthermore, the mutants appear to be stable. Some of our mutants have been kept in culture for over a year with bi-monthly transfer and, when retested, always excreted lysine. Interestingly, yogurt made with the wild type sometimes contained as much as 12% less lysine than the original soy milk.

Our experiments were designed to show that lysine-excreting strains of lactobacilli could be obtained from strains already used in natural fermentations, and we have shown that the mutant strains could be used with success. Obviously mutant strains should be obtained from bacterial cultures already being used. Both soy milk and cows' milk contain relatively abundant lysine, although we find it disconcerting that the wild-type strains sometimes

lowered the lysine content of the food. Fermentation of materials low in lysine would seem more advantageous. Completely new high lysine types of fermented foods might, of course, be made with lysine excreting mutants.

The lysine excreting strains were indistinguishable from the wild types in growth and rate of fermentation. The mutants, being spontaneous and easy to select and detect, can be obtained from current wild types to avoid possible taste differences from new strains.

To our knowledge high lysine producing mutants have not previously been used in natural food fermentations. We have obtained substantial increases in free lysine excretion with spontaneous mutants of lactobacilli. Mutants excreting even more lysine, or other amino acids, might be obtained either by the methods we describe, or other methods. Before any mutants are used, however, it is important to know if they are producing any unwanted metabolites. Furthermore, care must also be exercised to assure that there will not be a nutritional imbalance of some amino acids.

Our studies demonstrate the relatively simple procedures needed to allow selection of spontaneous mutants of high-lysine producing bacteria that could be used in natural fermentation of foods. Similar application of these techniques to find yeasts and molds used in fermentation that can excrete large amounts of essential amino acids should be possible.

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Hydrolysis of Milk Proteins by Bacteria Used in Cheese Making

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Literature pertaining to the role of lactic acid producing bacteria and *Brevibacterium linens* and *Propionibacterium shermanii* in the hydrolysis of proteins in cheese is reviewed. Particular emphasis is given to our work on the cellular location and proteolytic activity of selected strains of lactic streptococci. Cells of *Streptococcus lactis* C2 and *Streptococcus cremoris* ML1 harvested from Elliker's broth during late log, stationary, and early death phases of growth were ruptured in the Eaton press and fractionated into soluble, membrane, and ribosomal fractions by differential centrifugation. Enzyme activity on selected dipeptides and whole casein was determined on each cellular fraction. The soluble fraction contained the highest activity while the ribosomal fraction contained the lowest enzyme activity. *S. lactis* C2 had higher enzyme activity than *S. lactis* ML1. Activity on whole casein was reduced drastically in cells harvested during early death phase as compared to cells harvested during late log phase of growth while dipeptidase activity was relatively constant.

The manufacture of high quality cheese is primarily dependent on controlled microbial fermentation of milk constituents. Breakdown of lactose, fat, and protein is in one state or another necessary to obtain the desired curd type, texture, body, and flavor. Considerable attention has been directed to the importance of amino acids in cheese. Amino acids and peptides without doubt have an influence on cheese flavor (Storgårds and Lindquist, 1953; Harper, 1959). Proteins induce little, if any, flavor but are important for the body and texture.

The breakdown of proteins, primarily caseins, in cheese could be caused by rennet, milk proteinase, and microbial proteinases and peptidases. The milk proteinase seems to have only limited influence on cheese ripening (Zittle, 1965; Peterson, 1972). Only small amounts are found in milk and the presence of residual enzyme in pasteurized milk is questionable (Stadhouders, 1959; Kaminogawa et al., 1969; Chen and Ledford, 1971).

The effect of rennet on casein hydrolysis, besides the specific effect on κ-casein, has been a source of controversy. Stadhouders (1959) states that rennet is only of minor importance to casein degradation. On the other hand, Mabbitt et al. (1955) and Reiter et al. (1966) have characterized rennet as the most important source of proteolytic enzymes in cheese making. Others have claimed that rennet has minimum influence on cheese flavor because the products of casein hydrolysis by rennin are mainly protein fragments, and that only small amounts of amino acids are found (Sato and Ohmiya, 1966; Ohmiya and Sato, 1969a,b; Reiter and Sharpe, 1971). In a recent

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