

Changes in Free Amino Acids During Fermentation of Wheat Doughs Started with Pure Culture of Lactic Acid Bacteria¹

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ABSTRACT

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Dynamics of free amino acid levels during fermentation of wheat bread doughs started with three strains of lactic acid bacteria species—*Lactobacillus brevis* (B33), *Lactobacillus plantarum* (B39), and *Enterococcus faecium* (B40)—were investigated by reversed-phase high-performance liquid chromatography of the dansyl amino acids. Twenty protein amino acids, γ -aminobutyric acid, and ornithine were quantified in doughs fermented 0, 4, and 24 hr with lactic acid bacteria. Changes were related to the nutritional requirements and proteolytic activities developed by these microorganisms. Nutritional requirements of lactobacilli and enterococcus for glycine were evidenced at the beginning of fermentation (0-4 hr), whereas asparagine was consumed later (4-24 hr). In addition, B33 metabolized glutamine and serine first, and aspartic acid, glutamic acid, γ -aminobutyric acid, tryptophan, and proline later; alanine was assimilated

during the entire fermentation (0-24 hr). B39 showed preferential uptake of aspartic and glutamic acids, glutamine, serine, alanine, ornithine, and threonine. Exoproteolytic activity of microorganisms was revealed by the rise in levels of some protein amino acids. During fermentation (0-24 hr), valine, leucine, and lysine gradually increased; however, in all doughs proline content increased only during the first 4 hr. Aspartic acid and histidine were accumulated only in doughs fermented with B40. Methionine and tryptophan accumulated in doughs fermented with B39 and B40. The degree and rate of proteolysis during fermentation were higher for bread doughs containing B40. For the strains of lactic acid bacteria considered, changes in γ -aminobutyric acid were connected to changes in glutamic acid. Higher amounts of ornithine in doughs fermented long times were presumably due to its release by microorganisms.

During dough fermentation and baking of wheat bread, many flavor compounds are developed (Maga 1974, Schieberle and Grosch 1987) whose qualitative and quantitative compositions strongly influence the acceptance of bread by the consumer. The use of bread starters, based on selected associated cultures of microorganisms with specific metabolic activities, is one recommended way to direct the course of fermentation and, consequently, to produce bread with controlled organoleptic characteristics and overall quality (Robinson et al 1958, Vadja et al 1984).

Among compounds produced by fermentation, amino acids play an important role in the metabolism of dough microorganisms (Baca and Golebiewski 1977, Spicher and Schröder 1979) and serve as an important source of bread flavor precursor material (Spicher and Nierle 1988).

During dough fermentation, amino acids and their derivatives can be released into the media by proteolysis (Spicher and Nierle 1984a,b), by metabolism of sugars (Okuhara and Harada 1971) and peptides (Nisbet and Payne 1979), by hydrogenation of keto acid derivatives (Cole et al 1962), and by enzymatic synthesis (MacWilliam and Clapperton 1969). They can also be supplied from the cell mass of the microorganisms (Thorn 1971, Rothenbuehler et al 1982).

Flour (Spicher and Nierle 1988), yeast (Dalaly and Al-Banna 1983), and particularly lactic acid bacteria (El-Soda et al 1982, De Giori et al 1985, Thomas and Mills 1981, Spicher and Nierle 1988) contain proteases and peptidases. Specific hydrolytic actions of these enzymes on dough proteins during fermentation release amino acids that can be metabolized by microorganisms (Marder et al 1977, Spicher and Schröder 1979). Specific requirements for amino acids are evidenced by fermentation microorganisms. Strains of *Saccharomyces inusitatus* grew only 4-12% when methionine was omitted in the growth medium (Ng 1976). The homofermentative sourdough bacteria have no requirement for alanine and serine, but they are unable to grow in the absence of glutamic acid and valine. Most of the heterofermentative sourdough bacteria show essential requirements for arginine, leucine, phenylalanine, tryptophan, tyrosine, and valine (Spicher and Schröder 1979).

Amino acid assimilation promotes the growth of microorganisms (Konova et al 1986), increases the fermentative activity

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and the alcohol tolerance of yeast (Rivareva et al 1983) and the lactic acid production of lactobacilli and streptococci (Desmazeaud and Hernier 1972). These factors have repercussions on the overall quality of bread.

The presence of specific amino acids in the medium stimulates or inhibits the synthesis of proteases in some species of streptococci (Westhoff and Cowman 1970) and promotes proteolytic activities of lactobacilli (El-Soda et al (1978). For example, phenylalanine inhibits the growth of *S. cerevisiae* (Marder et al 1977) and promotes the development of some strains of *Streptococcus* species (Law et al 1976).

On the other hand, the qualitative and quantitative compositions of amino acids of fermented doughs affect bread flavor mainly through the formation of Maillard compounds during baking. Leucine, proline, isoleucine, and serine reacting with sugars form typical flavors and aromas described as toasty and breadlike (Wiseblatt and Zoumut 1963, Kiely et al 1960). Excessive amounts of leucine in fermenting sponges lead to bread with unappetizing flavor; if valine is added, bread flavor is improved (Baker et al 1953). In addition, the condensation reaction of glycine with simple aldehydes forms volatile pyridines, associated with undesirable flavor (Suyama and Adachi 1980).

The study of the metabolism of amino acids during dough fermentation by pure strains of microorganisms and their mixtures has only been conducted in rye sourdoughs (Spicher and Stephan 1987). Lactic acid bacteria species from rye sourdough starters cause different increases in free amino acids, particularly basic sulfur-containing, aromatic, heteroaromatic, and hydroxy amino acids and cause slight variations in cyclic amino acids when compared with sourdoughs fermented spontaneously (Spicher and Nierle 1988). When yeast participates in the fermentation, the amino acid content undergoes a smaller increase due to consumption of alanine, glycine, and lysine (Spicher and Nierle 1984b). The trend, extent, and rate of the changes depend on the specific microorganisms present.

Despite the interest in the metabolism of amino acids during fermentation by selected microorganisms in connection with controlled flavor and performance of bread, the scarcity of data in wheat doughs is evident. This paper aims to contribute by a study of the dynamics of free amino acid levels during fermentation of bread doughs started by three strains of lactic acid bacteria species: *Lactobacillus brevis* (B33), *L. plantarum* (B39), and *Enterococcus faecium* (B40).

MATERIALS AND METHODS

Reagents and Chemicals

Dowex AG 1 × 2 (50–100 mesh, Cl⁻ form) anion exchange and Dowex 50W × 2 (50–100 mesh, H⁺ form) cation exchange resins were purchased from Fluka AG (Buchs, Switzerland). Amino acids, dansyl amino acids, dansyl chloride, and picric acid were furnished by Sigma (St. Louis, MO). Acetonitrile (high-performance liquid chromatography grade), acetone (ultraviolet grade), and ammonium hydroxide were obtained from Panreac (Barcelona, Spain). A standard mixture of 20 protein amino acids, ornithine, and γ -aminobutyric acid was prepared as described previously (Benedito de Barber et al 1989a).

Bread Dough Preparation

A commercial wheat bread flour was used, with an energy of deformation (W) = 126×10^3 ergs, curve configuration ratio (P/L) = 0.36, and 11% protein. Biomasses of pure strains of heterofermentative (*L. brevis*, B33), homofermentative (*L. plantarum*, B39), and *E. faecium* (B40) lactic acid bacteria previously isolated and identified from wheat sourdoughs (Barber and Báguena 1988) were prepared following the procedure described by Barber et al 1985. Unfermented bread doughs (UF) were prepared by mixing 300 g of flour, 150 ml of water, and 5.4 g of salt with 15 ml of physiological salt solution of each lactic acid bacterium (bacterial counts: $6-3 \times 10^{10}$ viable cells/ml) for 3 min in the mixer of a Brabender Farinograph (Duisburg, Germany) at 90 rpm (Martínez-Anaya et al 1989). Respective

UF doughs were fermented at 28°C and 80% rh for 4 or 24 hr in the fermentation cabinet of a Brabender Maturograph (Duisburg, Federal Republic of Germany). Doughs were frozen, freeze-dried, ground, and kept refrigerated at 4°C under nitrogen until analysis.

Amino Acid Extraction

Samples (10 g) of freeze-dried doughs were suspended in 20 ml of 0.85N NaCl and mixed with magnetic stirring for 3 hr at 4°C. The homogenate was centrifuged at $23,000 \times g$ for 20 min at 1–4°C, and the sediment was extracted again with 20 ml of solvent. The supernatant fractions were filtered through Whatman No. 1 paper and made up to 50 ml with Milli Q water (Millipore, Bedford, MA) (Benedito de Barber et al 1989b).

Amino Acid Purification

Aliquots (25 ml) of salt-soluble fractions were deproteinized by 1% picric acid (100 ml) precipitation (El Dash and Johnson 1970). Protein-free extracts were obtained by centrifugation at $18,000 \times g$ for 30 min at 1–4°C. Picric acid was removed from the protein-free extracts by anion exchange chromatography on Dowex AG 1 × 2. Carbohydrates, anions, and uncharged compounds were eliminated from extracts on a Dowex 50W × 2 cation exchange resin, previously equilibrated with 0.01N HCl, by elution with distilled water. Amino acids were eluted with 4M NH₄OH (Benedito de Barber et al 1989c). The characteristics of the ion exchange resins and the chromatographic conditions for amino acid purification are summarized in Table I. The ammonia eluates were evaporated to dryness in a vacuum heater (<40°C). Residues were redissolved in 50 ml of water and used for amino acid analysis.

Amino Acid Determination

Aliquots of purified dough extracts containing 75 μ g of amine nitrogen (Collar et al 1990) and aliquots of amino acid standard mixtures were derivatized with dansyl chloride (Navarro et al 1984, Benedito de Barber et al 1989a, Prieto et al 1990). Amino acids were quantified using the norvaline internal standard addition method (Benedito de Barber et al 1989a).

Chromatographic Conditions

A Waters Associates (Milford, MA) model equipped with two 510 pumps controlled by a 721 programmable system controller, a U6K universal liquid chromatograph injector, a column heater, a temperature control system, a 490 programmable multiwavelength detector, and a 730 data module reporting integrator was used.

An optical density scanner HS/3 column (3 μ m × 8.3 cm × 4.6 mm i.d.) and a precolumn (3.6 cm × 4.6 mm i.d.) packed with pellicular C-18 (Perkin-Elmer, Norwalk, CT) were used for analysis. The mobile phase was 12 mM K₂HPO₄ (pH = 7.00)/

TABLE I
Characteristics of the Exchange Resins
and Chromatographic Conditions for Amino Acid Purification

Characteristic	Resin	
	Ion Exchange Dowex AG 1 × 2	Cation Exchange Dowex 50W × 2
Mesh	50–100	50–100
Form	Cl ⁻	H ⁺
Stationary bed		
diameter × height, cm	2 × 6.3	2 × 36.5
volume, ml	20	73.2
Exchange capacity, meq/ml	0.7	0.7
Flow rate, ml/min	6	6
Sample volume, ml	125	140
Mobile phase volume, ml		
0.02N HCl	30	...
H ₂ O	...	200
4M NH ₄ OH	...	440

acetonitrile. A linear gradient from 10 to 43.6% acetonitrile during 42 min was applied at a flow rate of 1.5 ml/min. The column temperature was 30°C and the ultraviolet detection was set at 250 nm.

Data Analysis

Data were statistically analyzed in a Microvax computer (Digital, Northboro, MA) by applying the two-way analysis of variance (BMDP statistical package, 7D program). The Tukey test was performed to calculate significant interactions.

RESULTS AND DISCUSSION

Chromatographic Separation and Quantification

Coefficients of variation (CV) for relative retention times ranged from 0.11 and 1.26%, being less than 1% for most amino acids. Variability in response factors and in amino acid quantification was less than 7% except for threonine and tyrosine (CV ~ 10%).

Amino Acid Contents in Relation to Metabolic Activities of Lactic Acid Bacteria

The amounts of 22 free amino acids (20 protein amino acids, γ -aminobutyric acid, and ornithine) were determined in unfermented and fermented bread doughs started with B33, B39, and B40. No quantification of cystine could be observed in any of the tested doughs because it was present in trace amounts. Individual amino acids were grouped as dicarboxylic acids and amides, aliphatic, basic, sulfur-containing, aromatic, hydroxyl, cyclic, and γ -aminobutyric acid as reported in the literature (Morimoto 1966; Spicher and Nierle 1984a,b, 1988) for easier discussion of results. Contents of amino acid groups and total amino acids in each dough were determined by the sum of the individual amino acid contents.

Total Amino Acid Content

Total amino acid (TAA) content depends on both the strains of lactic acid bacteria and the period of fermentation (Fig. 1). Initial amounts of amino acids (per 100 g of dough, db) were not statistically different ($P < 0.05$). Mean values were 40.41 (B33), 37.20 (B39), and 34.36 mg (B40). At the beginning of fermentation (0–4 hr), a significant decrease ($P < 0.01$) was observed for doughs containing lactobacilli, being higher for B39 (20%) than for B33 (10%). At longer fermentation periods (4–24 hr), the amino acid content of B39-containing doughs increased by 65%, resulting in an overall rise over the 24 hr fermentation. An increasing trend of amino acid level was shown by doughs started with B40 during the entire fermentation. When a comparison between the amounts of amino acids measured by re-

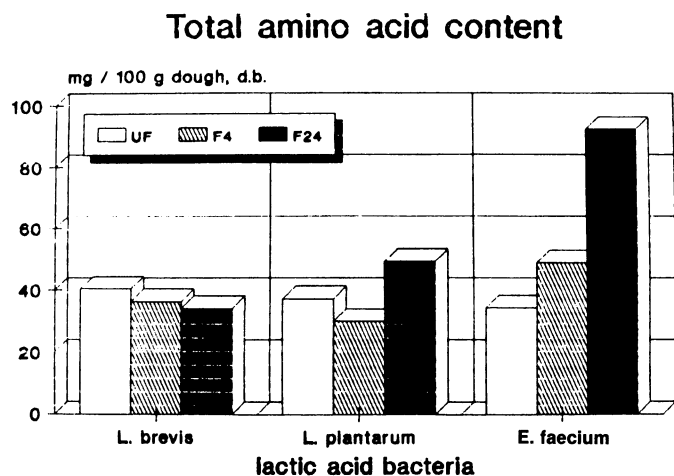


Fig. 1. Total amino acid content of unfermented (UF), and fermented bread doughs containing pure cultures of strains of *Lactobacillus brevis* (B33), *Lactobacillus plantarum* (B39), and *Enterococcus faecium* (B40). Fermentation time: 4 hr (F4) and 24 hr (F24).

versed-phase high-performance liquid chromatography and the nitrogen contents of α -amino of free amino acids (Collar et al 1990) was made, some differences in trends for early fermentation periods were observed. These differences can be attributed to different colorimetric responses for protein amino acids (α -amino) than for nonprotein amino acids (γ -aminobutyric acid and ornithine) in spectrophotometric determination. Production of amino acids during fermentation of rye sourdoughs started with *Lactobacillus* species has been described (Spicher and Nierle 1988) and attributed to a proteolytic action on higher molecular weight nitrogenous compounds. Enzymes from lactic acid bacteria are mainly responsible for the accumulation of amino acids. Hydrolytic action of endogenous flour enzymes and of indigenous microflora of flour must also be taken into consideration (Grant and Wang 1972, Spicher and Nierle 1988).

A significant consumption of amino acids by both lactobacilli was observed after 4 hr of fermentation and could be due to

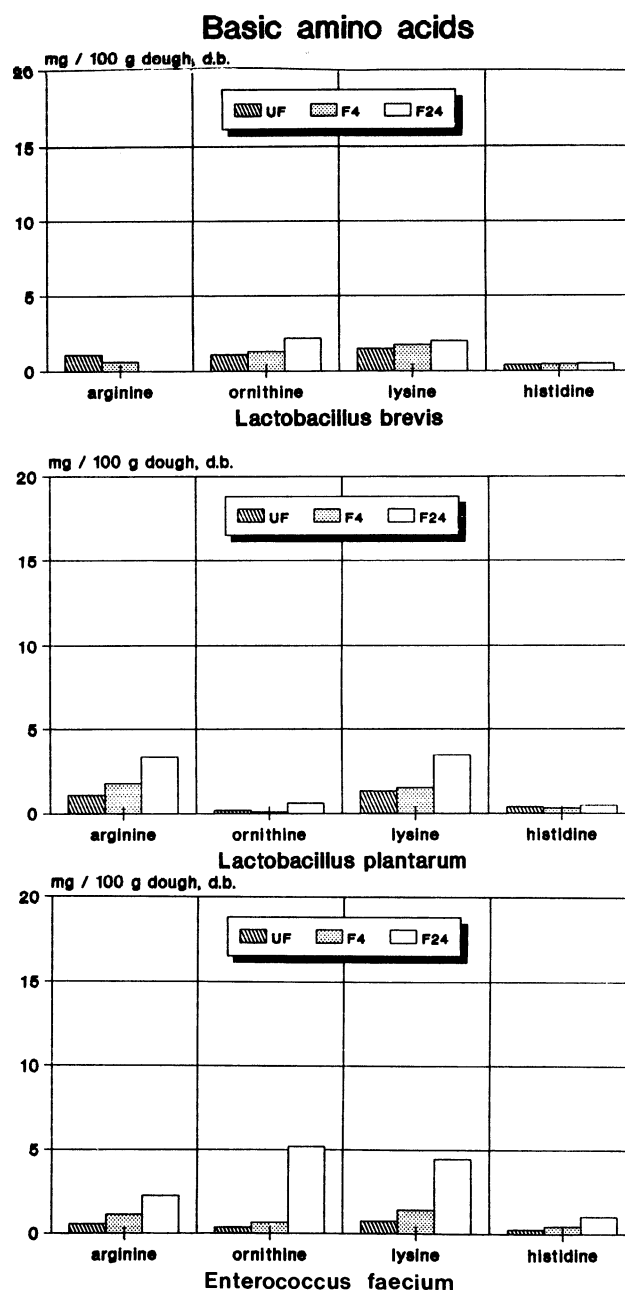


Fig. 2. Contents of basic amino acids in unfermented (UF) and fermented bread doughs started by strains of *Lactobacillus brevis* (B33), *Lactobacillus plantarum* (B39), and *Enterococcus faecium* (B40). Fermentation time: 4 hr (F4) and 24 hr (F24).

an early period of adaptation of microorganisms followed by a strong demand for assimilable nitrogen. After 4 hr of fermentation, lactobacilli were not yet able to hydrolyze peptide and protein chains at a noticeable rate because dough pH (5.3 in unfermented doughs and 3.8 after 4 hr of fermentation) (Martínez Anaya et al 1989) is lower than the optimum for proteolytic action (El-Soda et al 1982, De Giori et al 1985). At later times (4–24 hr) nonsignificant changes in TAA content for fermented doughs with B33 denoted the beginning of a slow change towards proteolytic activity. For B39 doughs, increased TAA content after 24 hr of fermentation is in good accordance with optimum aminopeptidase and dipeptidase activities developing at an early stationary phase (El-Soda et al 1983). The constant and highest rise in TAA levels for B40 fermented doughs reveals a faster and stronger proteolytic activity favored by the optimum initial pH (5.5) (De Giori 1985).

Amino Acid Groups

Dicarboxylic acids and amides and aliphatic and basic amino acids predominated in both unfermented and fermented bread doughs containing any of the strains of lactic acid bacteria tested (Figs. 2–4). Together, they accounted for approximately 80% of TAA and were responsible for 90–95% of the changes in TAA during fermentation. The minor amino acid groups were present only in small amounts (Fig. 5). Absolute overall values were higher for doughs fermented 24 hr, and a small decline in dicarboxylic acids and amides was observed for doughs started with lactobacilli (Figs. 2–4).

Different metabolic activities of lactic acid bacteria are evidenced by the variable extent and rate of the changes. Doughs started with B33 (Figs. 2–4) underwent a significant and gradual depletion (36%) in dicarboxylic acid and amide level during

Aliphatic amino acids

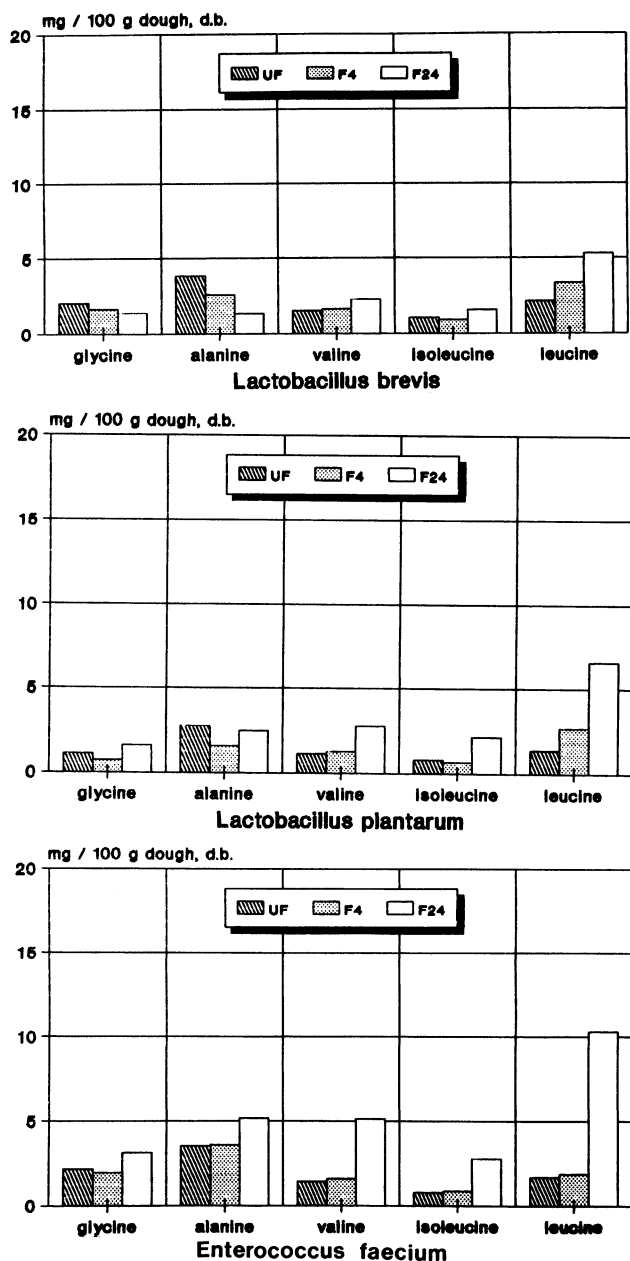


Fig. 3. Contents of aliphatic amino acids in unfermented (UF) and fermented bread doughs started by strains of *Lactobacillus brevis* (B33), *Lactobacillus plantarum* (B39), and *Enterococcus faecium* (B40). Fermentation time: 4 hr (F4) and 24 hr (F24).

Dicarboxylic acids & amides

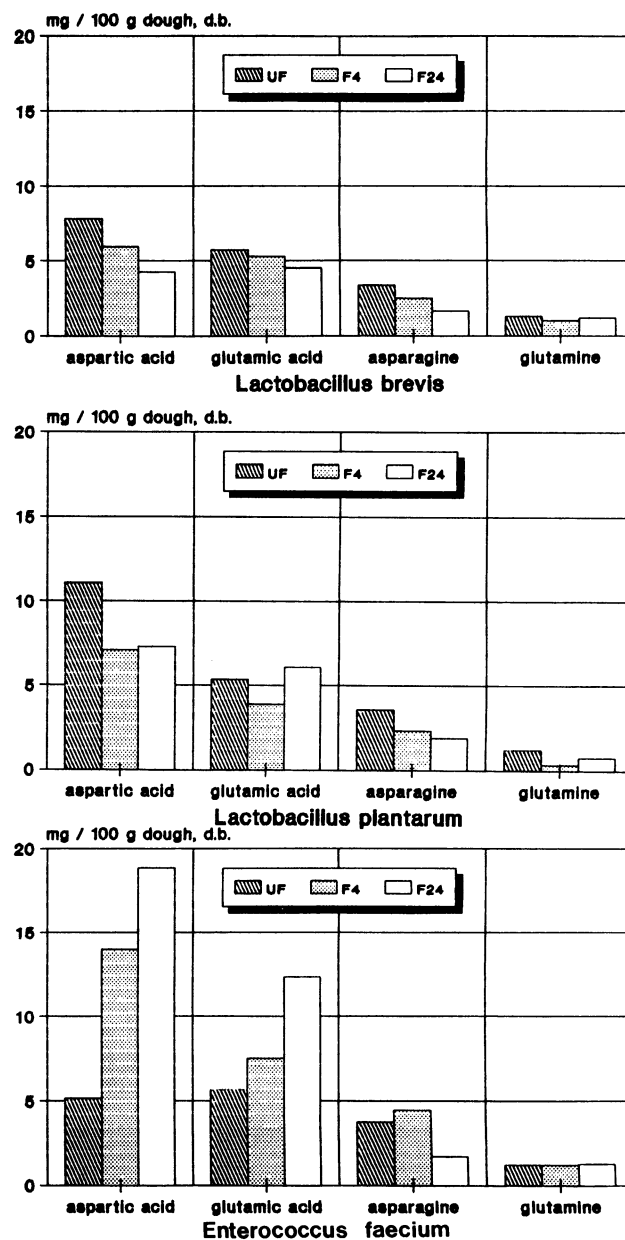


Fig. 4. Contents of dicarboxylic amino acids and amides in unfermented (UF) and fermented bread doughs started by strains of *Lactobacillus brevis* (B33), *Lactobacillus plantarum* (B39), and *Enterococcus faecium* (B40). Fermentation time: 4 hr (F4) and 24 hr (F24).

fermentation, whereas basic amino acids did not change. Aliphatic amino acid content decreased by 5% during the first 4 hr and later increased by 18%. As a result, an early decline and a later maintenance in TAA content was observed (Fig. 1). For doughs containing B39, the main changes occurred between 4 and 24 hr of fermentation, leading to increased aliphatic and basic amino acid levels by 125 and 116%, respectively (Figs. 2-4). Doughs prepared with B40 showed a continuous and significant increasing trend in the amount of dicarboxylic (115%), aliphatic (175%), and basic (600%) amino acids during the entire fermentation (Figs. 2-4). Similar trends in the changes in prominent amino acid groups were previously reported for fermentation of rye sourdough started with lactobacilli (Spicher and Nierle 1984a,b).

Individual Amino Acids

Determining the contents of individual amino acids in doughs (Figs. 2-5) provided information about fermentation dynamics regarding specific nutritional requirements of amino acids and exoproteolytic activities developed by the strains of lactic acid

bacteria tested. The net fall in glycine level in all fermented doughs (B33 20%, B39 35%, B40 10%) showed the strong demand for glycine by all microorganisms at the beginning of fermentation (0-4 hr). Asparagine was consumed later (4-24 hr), particularly by *E. faecium* (-60%). Contents of glutamic acid, asparagine, tryptophan, and proline, reported as essential amino acids for several strains of *L. brevis* (Spicher and Schröder 1979), were depleted by from 15% (glutamic acid) to 100% (tryptophan) as fermentation time increased from 4 to 24 hr. This heterobacterium also metabolizes glutamine (-22%), and serine (-70%) in the early stages (0-4 hr) and aspartic acid (-45%) and alanine (-66%) over the entire fermentation (0-24 hr). The net decrease in some individual amino acids suggests they are utilized as metabolites to a greater extent than they are being replaced by proteolytic activity. Doughs fermented with B39 showed an early uptake of aspartic (-35%) and glutamic acids (-25%), glutamine (-70%), serine (-65%), alanine (-40%), and threonine (-80%).

There was a particularly large increase (45%) in the content of γ -aminobutyric acid during the first 4 hr of fermentation as

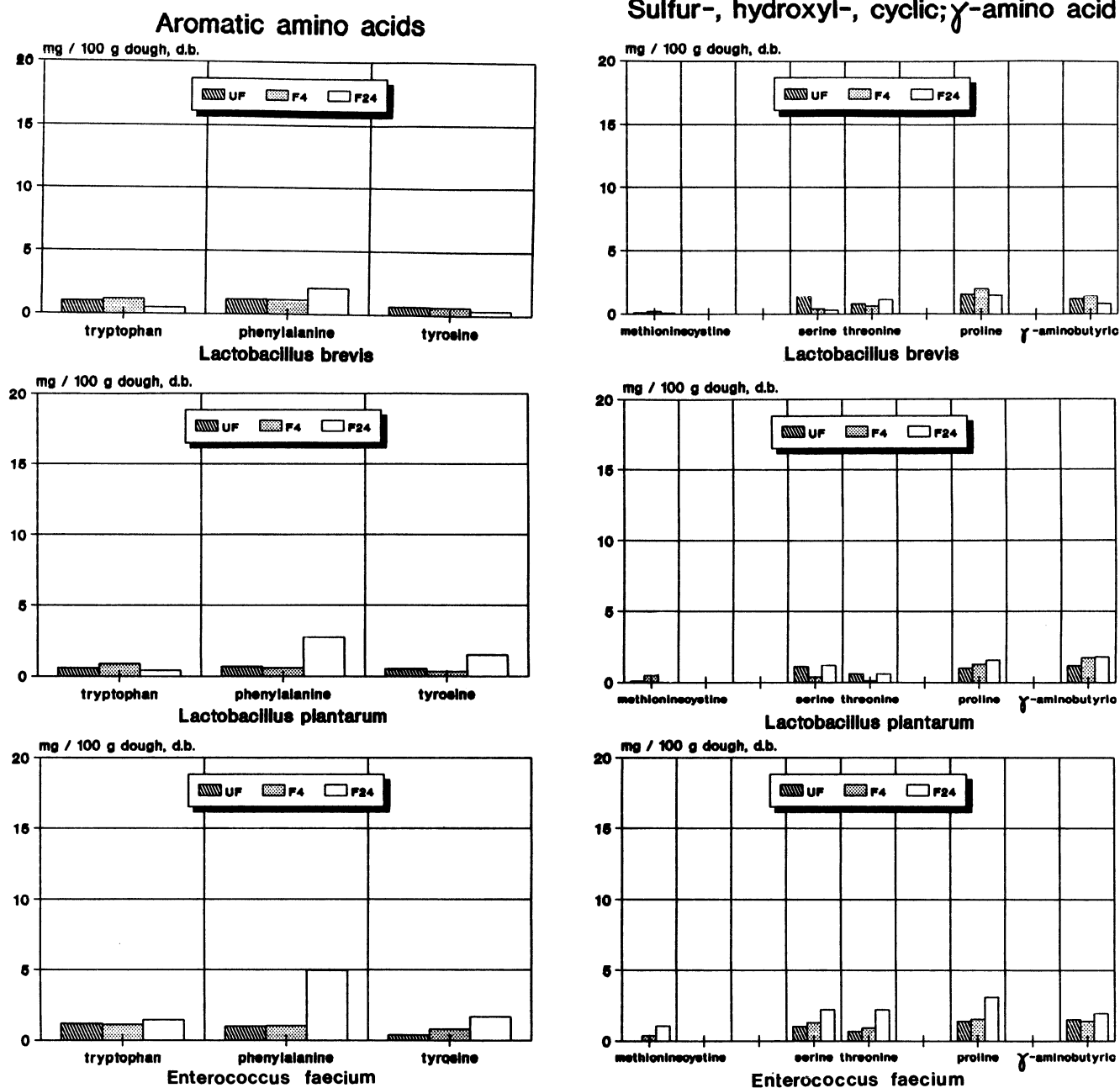


Fig. 5. Contents of aromatic, sulfur-, hydroxyl-, cyclic, and α -amino acids in unfermented (UF) and fermented bread doughs started by strains of *Lactobacillus brevis* (B33), *Lactobacillus plantarum* (B39), and *Enterococcus faecium* (B40). Fermentation time: 4 hr (F4) and 24 hr (F24).

a consequence of the effect of glutamate decarboxylase (Ponomareva et al 1964), and there was a simultaneous reduction in the glutamic acid content (-25%). Ornithine, which is involved in the biosynthetic pathway of arginine (Wiame 1971), deserves special mention; it was released to a variable extent, probably from a microbial mass (A. F. Mascarós, M. A. Brito, and C. Collar, unpublished) in fermented doughs (B33 100%, B39 250%, B40 1,500%). Only in doughs fermented with B39 was a considerable depletion (-50 %) in ornithine content observed in the first 4 hr of fermentation.

Exoproteolytic activity of microorganisms was disclosed by an increase in some protein amino acids (Figs. 2-4). After the active growth period, free amino acids can accumulate in fermented doughs because lactic acid bacteria no longer metabolize amino acids at a rapid rate. In this respect, over 24 hr of fermentation, valine, leucine, and lysine contents gradually increased, whereas the proline content in all doughs only rose after the first 4 hr (Figs. 2-4). In addition to aspartic and glutamic acids, serine, threonine, glycine, alanine, isoleucine, tryptophan, phenylalanine, histidine, and tyrosine were significantly generated in doughs started with B40.

Amounts of arginine, an essential amino acid for most homo- and heterofermentative lactic acid bacteria (Spicher and Schröder 1979), did not change significantly during fermentation in any dough. This evidence supports the view that the extent and the rate of assimilation and of enzymatic production are very similar.

The results reported here show that the lactic acid bacteria tested metabolized large amounts of free amino acids during bread dough fermentation. The nature, extent, and rate of the amino acid removal depend on both the species of microorganisms and the time of fermentation. Accumulation of free amino acids in fermented doughs occurs because of exoproteolytic actions. Degree and rate of proteolysis were higher and faster for doughs started with B40, producing significant levels of basic and hydrophobic amino acids. Since the importance of the amino acid composition of fermented doughs in controlling bread flavor has been recognized (Maga 1974, Spicher and Nierle 1988), expanded studies on the specific metabolism of amino acids by yeast and lactic acid bacteria and their associated cultures (now under investigation) will contribute to the selection of starters to produce bread with enhanced organoleptic characteristics and higher nutritive value.

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