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Improvement of a Chemically Defined Medium for the Sustained Growth of *Lactobacillus plantarum*: Nutritional Requirements

Fabiana M. Saguir,[1] María C. Manca de Nadra[1,2]

[1]Facultad de Bioquímica, Química y Farmacia, Universidad Nacional de Tucumán, Ayacucho, 491 4000, Tucumán, Argentina
[2]Centro de Referencia Para Lactobacillus (Cerela) CONICET, Chacabuco, 145 4000, Tucumán, Argentina

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Abstract. The aims of this work were to improve a basal synthetic medium (BM) for the growth of *Lactobacillus plantarum* strains and to establish their amino-acid requirements. Amino-acid use was analyzed in the most nutritionally demanding bacterium. First, the improved BM (*L. plantarum* synthetic medium [LPSM]) was created by increasing some vitamins in the BM, especially p-aminobenzoic acid, vitamin B_{12}, and biotin; 5-fold phenylalanine, histidine, isoleucine, leucine, lysine, methionine, proline, serine, threonine, and tryptophan; and 10-, 60-, and 75-fold valine, arginine, and tyrosine, respectively. With these additions, the N8 and N4 strains of *L. plantarum* grew rapidly to reach final cell densities similar to those obtained in Mann–Rogosa–Sharpe medium. When cysteine, leucine, valine, isoleucine, threonine, and glutamic acid were individually removed from this medium, bacterial growth significantly decreased or ceased, indicating that these amino acids are essential for growth. The N4 strain also required lysine and tryptophan in addition to the six amino acids necessary for growth. *L. plantarum* N4 mainly consumed essential amino acids, such as valine, lysine, cysteine, and threonine as well as the stimulatory amino acid, arginine. Thus, the BM was improved mainly on the basis of annulling limitations with respect to amino acids. With this, improved medium cell densities in the order of 10^9 colony-forming units/mL have been achieved, indicating that LPSM medium could be used for conducting metabolic and genetic studies on *L. plantarum*. Their low levels in orange juice suggest that these amino acids may not satisfy the total nitrogen requirement for the development of *L. plantarum* in the natural environment.

Growth of lactic-acid bacteria (LAB) in some fermented beverages, such as wines and ciders, can be useful, but in soft drinks, fruits juice, and related products, LAB are considered spoilage micro-organisms. *Lactobacillus* and *Leuconostoc* spp. can multiply in apple juice, producing an undesirable buttermilk flavor caused by diacetyl production, a fermented flavor caused by organic-acid production, the swelling of packages caused by production of carbon dioxide [19]. The fact that these bacteria can multiply in fruit juices could be attributed to their metabolic diversity and tolerance to high acid environments. In a previous work, we isolated and identified *L. plantarum* strains from oranges peel [3]. The rate of growth of these bacteria depends on their ability to use the substrates available in the medium. LAB have numerous nutritional requirements for growth, including amino acids, vitamins, purines, and pyrimidines [1, 8, 18]. Kandler and Weiss [11] reported that among LAB, members of the *Lactobacillus* genus present complex nutritional requirements that can be satisfied only by culturing a medium containing energy sources, precursors for cell growth and division, and growth-stimulatory substances. However, few studies have been reported regarding amino-acid requirements for *L. plantarum* growth. The use of complex media did not allow these requirements to be met. Therefore, defined media must be used for nutritional investigations.

Correspondence to: María C. Manca de Nadra; *email:* mcmanca@fbqf.unt.edu.ar

Synthetic media have been mainly developed for *Lactobacillus* species rather than *L. plantarum* [5, 9, 10].

The aim of this work was to improve a synthetic medium for the sustained growth of *L. plantarum* strains. The single-omission technique was applied to each amino acid in the improved defined medium to pinpoint the amino-acid requirements. Amino-acid use was then analyzed in the most nutritionally demanding micro-organism.

Materials and Methods

Micro-organisms. *L. plantarum* N4 and N8 were isolated from oranges peel [3]. The strain was stored at –20°C in Mann–Rogosa–Sharpe (MRS) medium [7] supplemented with glycerol (30% v/v).

Media, growth conditions, and culture procedures. The synthetic medium, Tucumán, was used as basal medium (BM) [14]. Different synthetic media were prepared by modifying the vitamins and/or amino-acid concentrations for the BM as stated in the text. A semisynthetic medium, in which the amino-acid source, except for cysteine-HCl, was substituted by tryptone (4 g/L) was used for cell adaptation before inoculation into the synthetic media. Synthetic media were sterilized in an autoclave, with heating stopped immediately when the temperature reached 121°C. Cysteine-HCl, sterilized by filtration through a nylon membrane (0.22-μm pore size; Millipore), was added to the sterilized media. For the final culture in the experimental media, cells grown in MRS medium (pH 6.5) and incubated without agitation at 30°C, were harvested at the end of the exponential growth phase (8 hours) and precultured under the same conditions in the semisynthetic medium. Cells from the third subculture were harvested by centrifugation; washed twice with sterile distilled water to avoid carry-over of essential nutrients; and resuspended in sterile distilled water to optical density .560 nm = 0.90. Cell suspensions were used to inoculate the experimental media at a rate of 2%. All cultures were incubated statically at 30°C for 10 days.

Growth measurement. Bacterial growth was monitored by periodic spectrophotometric measurements at 560 nm using a Bausch and Lomb Spectronic-20 spectrophotometer during micro-organism growth. At the same time, the colony-forming units (CFU/mL) were determined.

Amino-acid requirements were estimated in the *L. plantarum* synthetic medium (LPSM) medium by omitting these amino acids one at a time. They were classified into three groups according to the extent of growth in each deficient medium. From 0% to 30%, the extent of amino-acid growth was considered as essential; from 30% to 70%, it was considered as stimulatory, and for >70%, it was considered as nonessential. Growth experiments were repeated at least three times.

Analytic methods. Amino acids were analyzed using reverse-phase high-performance liquid chromatography (RP-HPLC) using an ISCO liquid chromatograph (ISCO, Lincoln, NE). Samples were submitted to precolumn derivatization with *o*-phtaldialdehyde (OPA). The reagent solution for the derivatization consisted of 200 mg OPA in 9 mL methanol, 1 mL 0.4 M sodium borate (pH 10), and 160 μL 2-mercaptoethanol. Solvents used for separation were solvent A = methanol, 10 mM sodium phosphate buffer (pH 7.3), and tetrahydrofuran (19:80:1 ratio) and solvent B = methanol and 10 mM sodium phosphate buffer (pH 7.3) (80:20 ratio). Solvent gradient conditions were as follows: 6 minutes (0 B), 10 minutes (15% B), 4 minutes (30% B), 12 minutes (40% B), 16 minutes (80% B), and 5 minutes (0 B). All separations were performed on a Waters Nova-Pack C18 column (150 x 3.9 mm i.d., 60 Å, 4 μm) with a flow of 1.0 mL/min. The detection was by fluorescence using a model 121 fluorimeter (340-nm excitation filter and 425-nm emission filter). Samples were injected in triplicate onto the column after being filtered through a 0.22-μm filter. Before RP-HPLC analysis, all samples were diluted with 0.4 borate buffer (pH 10). Amino-acid standards were used to determine their concentrations. These solutions were prepared by dissolving each amino acid in 0.1 N HCl solution to reach a concentration of 2.5 μmol/mL. They were stored at –18°C. The precolumn derivatization and the column apparatus were at room temperature.

Statistical analysis. The experimental data were analyzed by one-way analysis of variance. Variable means showing statistical significance were compared using Tukey's test (Minitab student R12).

Results

Definition of an improved synthetic medium for *L. plantarum* growth. In BM, both micro-organisms grew to a small extent (5.0 x 10^7 and 7.1 x 10^7 CFU/mL for the N8 and N4 strains, respectively) and with growth rates of 0.13 and 0.19 h^{-1} for the N8 and N4 strains, respectively. Growth was strongly stimulated when increased four-fold riboflavin, 10-fold folic acid, and 1000-fold p-aminobenzoic acid; vitamin B_{12}; and biotin in the vitamin solution, and 5-fold phenylalanine, histidine, isoleucine, leucine, lysine, methionine, proline, serine, threonine, and tryptophan, as well as 10-, 60-, and 75-fold valine, arginine, and tyrosine, respectively, in the original amino-acid mixture. With these additions, N8 and N4 strains grew at growth rates of 0.44 and 0.56 h^{-1} to reach final cell densities of 1.0 x 10^9 and 3.3 x 10^9 CFU/mL, respectively (Fig. 1). Vitamins and amino-acid concentrations in the BM and in the improved BM, called LPSM medium, are listed in Table 1.

Amino-acid requirements. Bacterial growth results obtained for the individual removal of 19 amino acids from LPSM medium and the amino-acid requirements are listed in Table 2. No growth was observed when glutamic acid, isoleucine, threonine, or valine were eliminated from the medium, and the individual suppression of cysteine and leucine led to a decrease of approximately 85% in the extent of growth. The N4 strain also had an absolute requirement for lysine and tryptophan in addition to the six amino acids necessary for growth. Both strains grew in the medium without alanine, arginine, or phenylalanine, but when these were supplied, growth increased by 40%. Hence, these amino acids were classified as stimulatory for both bacteria. Lysine and tryptophan, essential amino acids for N4 strain, also showed a stimulatory effect for N8 strain development. Members of the serine family, except for cysteine and some amino acids from the aspartate family

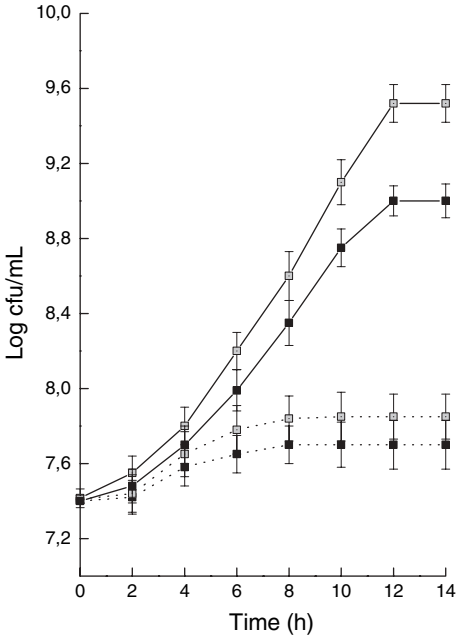

Fig. 1. *L. plantarum* N4 and N8 growth in synthetic media. N4 strain (open square), N8 strain (closed square), basal medium (dashed line), LPSM medium (line).

(aspartic acid, asparagine, or methionine), hystidine, and proline could be omitted without any effect or with minor inhibitory effect on bacterial growth of the analyzed strains. Therefore, these seven amino acids were classified as nonessential for growth. A similar result was observed for N8 strain in the absence of tyrosine.

Use of amino acids. Fig. 2 shows the essential and stimulatory amino acids use by *L. plantarum* N4 at the end of exponential growth phase in LPSM medium. Amino-acid use began immediately when growth began. The initial amino-acid levels decreased between 41 and 68% at the end of growth. In decreasing order of concentration the following five amino acids were mainly consumed: valine, lysine, arginine, cysteine and threonine. The aromatic amino acids, trytophan and phenylalanine showed the lowest consumption values.

Discussion

This study describes a chemically defined medium, LPSM, that supports excellent growth of *L. plantarum* strains. Moreover, the differences between the initial and final population levels were comparable with those obtained in complex MRS medium (data not shown). Increments of some vitamins and amino acids were responsible for the best bacterial growth obtained in the

Table 1. Vitamin and amino-acid concentrations in BM and LPSM medium

Constituent	Concentration (g/L)	
	BM[a]	LPSM
p-Aminobenzoic acid	0.00001	0.01
Vitamin B12	0.000001	0.001
Calcium pantothenate	0.001	0.001
D-biotin	0.00001	0.01
Folic acid	0.0001	0.001
Niacin	0.001	0.001
Piridoxal ethyl acetal HCl	0.0005	0.001
Riboflavin	0.0005	0.001
Thiamine HCl,	0.001	0.001
DL-Alanine	0.20	0.20
L-Arginine	0.005	0.30
L-Asparagine	0.20	0.20
L-Aspartic acid	-	0.20
L-Cysteine-HCl	0.20	0.20
L-Glutamic acid	0.15	0.15
L-Glycine	-	0.30
L-Histidine-HCl	0.05	0.20
L-Isoleucine	0.05	0.20
L-Leucine	0.06	0.30
L-Lysine-HCl	0.05	0.30
L-Methionine	0.05	0.20
L-Phenylalanine	0.04	0.20
L-Proline	0.04	0.30
L-Serine	0.10	0.30
L-Threonine	0.004	0.20
L-Tryptophan	0.05	0.20
L-Tyrosine	0.05	0.30
L-Valine	0.03	0.30

[a]BM contained in distilled water (l^{-1}): glucose 10 g; potassium acetate 10 g; potassium dihydrogen orthophosphate 2 g; sodium thioglycollate 0.5 g; magnesium sulphate $7H_2O$ 0.15 g; manganese sulphate $4H_2O$ 0.02 g; ferrous sulphate $7H_2O$, 0.01 g; Tween 80 1 mg; adenine 50 mg; cytidylic acid 50 mg; deoxyguanosine 50 mg; guanine HCl 50 mg.

LPSM medium. According to Hebert et al. [10], vitamin B12 and riboflavin are stimulatory for the growth of two strains of *L. delbrueckii* subsp. *Lactis*, whereas pyridoxal is stimulatory or essential depending the strain, and niacin and panthotenic acid are essential. Amoroso et al. [1] reported that vitamins were generally stimulatory for *Oenococcus oeni* strains. When adding all amino acids in concentrations 10-fold greater than those already in BM, a decrease in bacterial growth occurred (data not shown). This fact suggested that exogenous amino acids could have a negative impact on the pathways involved in the synthesis inside of the bacterial cell, acting by way of a feedback control mechanism. In concordance, Elli et al. [8] demonstrated a negative effect for 15 amino acids on *L. johnsonii* growth. As in previous works [2, 18] the nonessential amino acids, aspartic acid and glycine, were included in the BM. These amino acids were also included in the LPSM medium. This is

Table 2. Growth of *L. plantarum* N8 and N4 in LPSM medium deficient in one amino acid. Amino acid requirements

	L. plantarum strains			
	N8		N4	
Omitted amino acid	A[a]	Amino acid effect	A[a]	Amino acid effect
None	1.60 ± 0.08	-	2.10 ± 0.09	-
DL-Alanine	0.59 ± 0.03	S	0.78 ± 0.04	S
L-Arginine	0.64 ± 0.03	S	1.12 ± 0.05	S
L-Asparagine	1.61 ± 0.07	NE	2.03 ± 0.10	NE
L-Aspartic acid	1.60 ± 0.08	NE	2.09 ± 0.11	NE
L-Cysteine-HCl	0.24 ± 0.02	E	0.42 ± 0.02	E
L-Glutamic acid	NG	E	NG	E
L-Glycine	1.59 ± 0.07	NE	2.10 ± 0.10	NE
L-Histidine-HCl	1.53 ± 0.06	NE	1.72 ± 0.08	NE
L-Isoleucine	NG	E	NG	E
L-Leucine	0.16 ± 0.01	E	0.36 ± 0.02	E
L-Lysine-HCl	0.58 ± 0.03	S	0.28 ± 0.01	E
L-Methionine	1.60 ± 0.08	NE	1.64 ± 0.08	NE
L-Phenylalanine	0.61 ± 0.03	S	0.85 ± 0.04	S
L-Proline	1.62 ± 0.08	NE	2.09 ± 0.09	NE
L-Serine	1.61 ± 0.08	NE	2.09 ± 0.09	NE
L-Threonine	NG	E	NG	E
L-Tryptophan	1.05 ± 0.01	S	0.42 ± 0.02	E
L-Tyrosine	1.60 ± 0.08	NE	1.41 ± 0.07	NE
L-Valine	NG	E	NG	E

[a]A, difference in cell concentration (Log cfu/ml) between end of exponential growth phase and inocula. Values are the means of three independent experiments ± SD. Different times required to reach the end of exponential growth. Maximum time required was 24 hours.
NG, no growth[a]; E, essential; S, stimulatory; NE, nonessential.

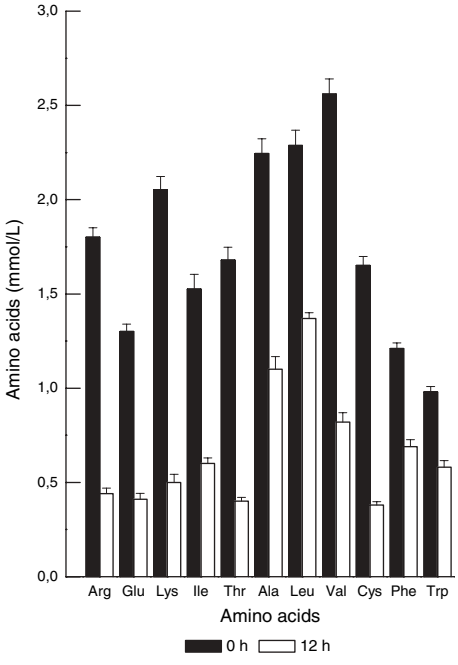

Fig. 2. Changes in concentrations of residual amino acids (indicated at the bottoms) in culture of *L. plantarum* N4 in LPSM medium at the end of exponential growth phase.

related to the fact that aspartic acid is a precursor of amino acids essential for the micro-organism's growth and glycine because it could be easily incorporated into cell material.

The absolute amino-acid requirements of the two strains tested on LPSM medium showed considerable similarities, although N4 strain was more demanding. Six common amino acids, mainly belonging to the aspartate and pyruvate families, were considered essential for growth. In contrast, Saguir and Manca de Nadra [17] demonstrated in *O. oeni* that amino-acid requirements differed by strain. Essential amino acids for N4 and N8 strains were not completely similar to those reported for strains of the same species isolated from green olive fermentations. Only glutamic acid, isoleucine, and leucine requirements were similar independently from the origin of the strain [16].

In our study, the absolute requirement for glutamic acid could be associated with the absence of functional glutamate dehydrogenase or with the presence of an inoperative TCA cycle unable to generate oxoglutarate, a precursor of glutamate. Morishita and Yajima [15] demonstrated in lactobacilli the lack of isocitrate dehydrogenase activity. Lapujade et al. [13] reported in *Lactococcus lactis* that the metabolic ''bottleneck'' responsible for the

limited growth rate in the absence of glutamate was located in the step leading to 2-oxoglutarate synthesis. *L. plantarum* strains assayed had absolute requirements for threonine, lysine, and isoleucine, all of which are part of the branched pathway forming the aspartic-acid family of amino acids [6]. It is possible to infer that genetic lesions affecting their biosynthetic pathways occurred below the divergence points, specifically forming each amino acid (for example, aspartic acid semialdehyde on the lysine formation and homoserine on the threonine and isoleucine synthesis). Cysteine is synthesized in two steps from serine by incorporation of sulfide or thiosulfate [12], and serine comes from 3-phosphoglycerate [18]. Glycine, also derived from serine and serine, were nonessential. Therefore, the auxotrophy for cysteine could be related to a deficient supply of sulfur under the assayed conditions.

Regarding amino-acid consumption, *L. plantarum* N4 used mainly the essential amino acids such as valine, lysine, cysteine, and threonine. This could be linked to a better incorporation of them in cell material. On the other hand, Elli et al. [8] emphasized the most important role of cysteine for *L. johnsonii* development, which was attributed to the disulphide bonds and sulphydrilic groups acting as one of the main redox potentials. It is interesting to note that arginine, a stimulatory amino acid for *L. plantarum* N4 growth, was also mainly used. This might be explained as an adaptation response to the conditions of orange juice medium, in which there is a majority of arginine. Furthermore, Arena et al. [4] demonstrated in *L. plantarum* N4 that growth was improved by arginine, which was degraded to citrulline, ornithine, and ammonium to produce additional energy. The consumption pattern of the essential amino acids and arginine explains the low bacterial counts in the BM, where they are in insufficient concentrations.

In conclusion, the BM was improved mainly on the basis of annulling limitations with respect to amino acids. With this medium, cell densities on the order of 10^9 CFU/mL have been achieved. Therefore, LPSM medium could be used for conducting metabolic and genetic studies on *L. plantarum*. The analyzed strains had absolute requirements for six common amino acids, with N4 strain being the most demanding. The low amino-acid levels in orange juice suggest that amino acids not may satisfy the total nitrogen requirement for *L. plantarum* development in the natural environment.

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