Vitamin and amino acid requirements of *Lactobacillus plantarum* strains isolated from green olive fermentations

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J.L. RUIZ-BARBA AND R. JIMENEZ-DÍAZ. 1994. The requirement for essential amino acids and vitamins was determined in wild-type *Lactobacillus plantarum* strains isolated from green olive fermentation brines. All the strains were found to be auxotrophic with respect to the amino acids but some of them were prototrophic for pyridoxal, *p*-aminobenzoic acid and/or nicotinic acid. Their growth response to these nutrients was also studied and found to be quite heterogeneous. Nutritional requirement pattern as a criteria for selecting starter cultures is discussed.

INTRODUCTION

The spontaneous lactic acid fermentation of Spanish-style green olives relies upon the activity of the indigenous lactic acid bacterium *Lactobacillus plantarum* associated with the raw material (Andersson *et al.* 1988). Although *Lact. plantarum* is present in small numbers in the fresh product, the traditional process consisting of lye treatment, washing and brining of the fruits favours its rapid and predominant growth over other micro-organisms in the brines, e.g. Gram-negative bacteria, lactic acid cocci, yeasts and molds (González Cancho 1963; Borbolla y Alcalá *et al.* 1971; Fernández Díez 1983; Ruiz-Barba 1991). Thus, as *Lact. plantarum* ferments the sugars contained in the fruit into lactic acid, it becomes the predominant organism, aiding to the preservation of the olives. Although this natural fermentation usually leads to products of acceptable quality, occasional inconsistency in the final product or even spoilage may happen when *Lact. plantarum* grows poorly. The use of starter cultures of selected wild-type *Lact. plantarum* strains could provide more consistent fermentations and products of higher quality.

One of the traits cited as being desirable in starter cultures for application in vegetable fermentations is their ability to grow and predominate quickly (Daeschel *et al.* 1987). As the lactic acid fermentation of brined olives consists of solid reservoirs of nutrients (the olives) in a liquid environment (the brine), the growth rate of *Lact. plantarum* depends on the diffusion of nutrients from the fruits into the brine. This is particularly important, not only for fer-

mentable sugars but also for a number of amino acids and vitamins for which *Lact. plantarum* has been described as being auxotrophic (Rogosa *et al.* 1961; Koser 1968; Ledesma *et al.* 1977; Morishita *et al.* 1981; Kandler and Weiss 1982). In addition, the growth rate of *Lact. plantarum* is influenced by its response to limiting amounts of essential nutrients. As an approach to the selection of *Lact. plantarum* strains as inoculants for olive fermentations, the aims of this work were: (i) to determine the nutritional requirements, in amino acids and vitamins, of wild-type *Lact. plantarum* strains isolated from Spanish-style green olive fermentations; and (ii) to study their growth response to some of these requirements in synthetic media.

MATERIALS AND METHODS

Bacterial strains

The 24 *Lact. plantarum* strains used in this study belong to our stock collection. They were isolated from Spanish-style green olive fermentations and have been identified (Ruiz-Barba *et al.* 1991). Strains belonging to the series LPC and LPCO were isolated from fermenters located in Cabra (Córdoba, Spain) while those of the series LPS were from fermenters in our own processing pilot plant at Seville. All of them were isolated when the naturally occurring lactobacilli population was well established in the fermenting brines, that is during the so-called second phase of the green olive fermentative process (Fernández Díez 1983). As reference strains, *Lact. plantarum* ATCC 8014 and *Pediococcus acidilactici* ATCC 8042 were used. They were maintained as frozen stocks at -20°C in distilled water plus

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20% (v/v) glycerol and subcultured twice in MRS medium (Oxoid) before use.

Amino acid and vitamin requirement pattern

Screening for amino acid and vitamin requirements was performed with a replicator apparatus (Steers *et al.* 1959) in a defined medium described by Morishita *et al.* (1981). This medium contained the following ingredients per litre of deionized, glass-distilled water: D-glucose, 10 g l⁻¹; sodium acetate, 6 g l⁻¹; ammonium citrate, 1 g l⁻¹; potassium dihydrogen phosphate, 3 g l⁻¹; di-potassium hydrogen phosphate, 3 g l⁻¹; magnesium sulphate heptahydrate, 0.5 g l⁻¹; manganese sulphate heptahydrate, 50 mg l⁻¹; ferrous sulphate heptahydrate, 20 mg l⁻¹; Tween 80, 1 g l⁻¹; L-glutamic acid, 0.2 g l⁻¹; L-isoleucine, 0.1 g l⁻¹; L-leucine, 0.1 g l⁻¹; L-methionine, 0.1 g l⁻¹; L-phenylalanine, 0.1 g l⁻¹; L-tryptophan, 0.1 g l⁻¹; L-valine, 0.1 g l⁻¹; *p*-aminobenzoic acid, 200 µg l⁻¹; biotin, 10 µg l⁻¹; nicotinic acid, 1 mg l⁻¹; pantothenic acid, 1 mg l⁻¹; pyridoxal, 2 mg l⁻¹; and Agar Purified (Difco), 20 g l⁻¹.

The 24 *Lact. plantarum* strains were grown overnight at 30°C in Micro Inoculum Broth (Merck), subcultured in fresh Micro Inoculum Broth, and collected by centrifugation (5000 g, 10 min) when they were in log-phase of growth (*ca* 10⁸ cfu ml⁻¹). Pellets were washed twice in saline, and then inoculated at *ca* 2 × 10⁵ cfu ml⁻¹ in defined medium from which each amino acid or vitamin was omitted in turn. MRS agar (Oxoid) and the complete defined medium were used as controls, and *Lact. plantarum*

ATCC 8014 was used as a reference strain. Plates were incubated at 30°C for 2 d and then examined for the presence of bacterial growth.

Growth response studies

Four strains (LPS 1/112/1, LPS 2/112/1, LPS 1/43/1 and LPS 1/91/1) were selected for further studies on their growth response in the presence of increasing amounts of some essential amino acids and vitamins. These strains were chosen in order to eliminate as many variables as possible for they were isolated from the same place, during the same season, growing in the same type of fermenting olive brines and with identical amino acid and vitamin requirement profile (Table 1). Bacto Glutamic Acid Assay Medium, Bacto Methionine Assay Medium and Bacto Tryptophan Assay Medium (Difco) were supplemented with glutamic acid (range 0–40 µg ml⁻¹), methionine (0–6.0 µg ml⁻¹) and tryptophan (0–2.0 µg ml⁻¹), respectively. Vitamin Biotin Assay Broth, Vitamin Nicotinic Acid Assay Broth and Vitamin Pantothenic Acid Assay Broth (Merck) were supplemented with biotin ranging from 0 to 0.3 ng ml⁻¹, nicotinic acid (0–100 µg ml⁻¹) and pantothenic acid (0–10 ng ml⁻¹), respectively. Inocula of each *Lact. plantarum* strain were prepared as described above and added to 5 ml of broth at a final concentration of *ca* 5 × 10⁵ cfu ml⁻¹. Tubes were incubated at 30°C for 72 h and growth of *Lact. plantarum* strains in the presence of each amino acid or vitamin was determined in terms of acid production, titrating with 100 mmol l⁻¹ NaOH and

Table 1 Amino acid and vitamin requirements by wild-type *Lactobacillus plantarum* strains isolated from green olive fermentations

Nutrient omitted*	Growth of strains															
	ATCC 8014	LPC							LPCO		LPS		1/112/1	2/112/2	1/43/2	1/91/1
		2	5	9	12	14	16	26	6	10	10	15				
L-Isoleucine	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
L-Glutamic Acid	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
L-Leucine	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
L-Methionine	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
L-Phenylalanine	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
L-Tryptophan	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
L-Valine	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
<i>p</i> -Aminobenzoic Acid	—	—	—	—	—	—	—	—	+	+	+	—	—	—	—	—
D-Biotin	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Nicotinic acid	—	—	—	—	—	—	—	—	—	—	+	—	—	—	—	—
Pantothenic acid	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Pyridoxal	—	—	+	—	—	+	+	—	+	+	+	—	—	—	—	—

* The defined medium described by Morishita *et al.* (1981) was used in which each amino acid or vitamin was omitted in turn.

+, Growth; —, absence of growth.

expressing the results as mmol l^{-1} of lactic acid. *Lactobacillus plantarum* ATCC 8014 was used as reference micro-organism in the vitamin and tryptophan assays, and *Ped. acidilactici* ATCC 8042 was used in the glutamic acid and methionine tests.

RESULTS

Table 1 summarizes the vitamin and amino acid requirements of the 15 wild-type *Lact. plantarum* strains that were able to grow in both the complete defined medium and in MRS agar. The remaining nine strains were omitted as they failed to grow. All these 15 strains exhibited auxotrophy to all the amino acids tested. However, differences in the patterns of vitamin requirement were observed.

Thus, *Lact. plantarum* strain LPS10 was able to grow in the absence of nicotinic acid, *p*-aminobenzoic acid and pyridoxal, *Lact. plantarum* strains LPC06 and LPC010 grew in the absence of *p*-aminobenzoic acid and pyridoxal, and *Lact. plantarum* strains LPC5, LPC14 and LPC16 were able to grow in the absence of pyridoxal.

Diversity among different strains was also observed when their growth responses to some amino acids and vitamins were compared. In the case of the amino acids studied, Fig. 1 shows that most of the assayed wild-type *Lact. plantarum* strains reached their maximum growth, in terms of acid production, with significantly lesser amounts of amino acid than the control micro-organism. This effect is particularly clear for tryptophan and methionine with the strains 1/112/1, 2/112/1 and 1/43/1. All these strains produce their maximum of acid with several times less amino acid in the

Fig. 1 Growth response of four wild-type *Lactobacillus plantarum* strains isolated from green olive fermentations to increasing amounts of L-glutamic acid (a), L-tryptophan (b) and L-methionine (c). □, Strain 1/112/1; △, strain 2/112/1; ●, strain 1/43/1; ■, strain 1/91/1. *Pediococcus acidilactici* ATCC 8042 (○) in (a) and (c), and *Lactobacillus plantarum* ATCC 8014 (○) in (b) were used as reference strains

media than the required amount for the control strain to reach its own maximum. This is 10 times less tryptophan and three times less methionine.

In the presence of increasing amounts of biotin, pantothenic acid or nicotinic acid (Fig. 2), the growth response of the wild-type *Lact. plantarum* strain was very similar to the one shown by the control strain *Lact. plantarum* ATCC 8014, with the exception of strain 1/91/1, which reaches its maximum of acid production with six times less biotin.

DISCUSSION

The results show the complexity on amino acid and vitamin requirements displayed by wild-type *Lact. plantarum* strains isolated from green olive fermentations as it has been described before for strains of the same species iso-

lated from other sources (Morishita *et al.* 1981; Kandler and Weiss 1982). However, our results do not necessarily agree with the type of all of these requirements. On the other hand, the growth responses of our isolates to these nutrients were found to be heterogeneous.

Whereas most of the isolates were able to grow in the complete defined medium, nine of the strains did not. As this medium was supplemented with all the amino acids and vitamins described as essentials for *Lact. plantarum*, these nine strains must have some other nutritional requirements that will be further characterized. The study was therefore focused on the 15 strains that could grow in the complete defined medium. In this case, although their nutritional requirements in amino acids were similar to those previously reported for *Lact. plantarum* (Koser 1968; Ledesma *et al.* 1977; Morishita *et al.* 1981; Kandler and

Fig. 2 Growth response of four wild-type *Lactobacillus plantarum* strains isolated from green olive fermentations to increasing amounts of pantothenic acid (a), D-biotin (b) and nicotinic acid (c). □, Strain 1/112/1; △, strain 2/112/2; ●, strain 1/43/1; ■, strain 1/91/1. *Lactobacillus plantarum* ATCC 8014 (○) was used as a reference strain

Weiss 1982), some strains showed a pattern of vitamin requirement that differed from those published previously (Rogosa *et al.* 1961; Koser 1968; Ledesma *et al.* 1977; Morishita *et al.* 1981; Kandler and Weiss 1982). These differences could be explained in terms of an adaptative response of *Lact. plantarum* to the nutritional conditions present in a particular environment such as the fermentation brine. As lye treatment of olives before brining promotes alkaline hydrolysis of proteins contained in the fruits as well as making them more permeable (Fernández Díez 1983), it provides both peptides and free amino acids to the brines. However, this alkaline treatment destroys some of the vitamins contained in the fruits; other water-soluble and highly diffusible B complex vitamins like biotin, nicotinic acid, pyridoxal or *p*-aminobenzoic acid can be washed out of the starting brine during the removal of the lye and subsequent washes with water that are part of the traditional brining procedure. So the presence of these vitamins that are essential for *Lact. plantarum* would greatly depend on those that have been newly synthesized and excreted by the microflora which spontaneously grow in the brines (Ruiz-Barba 1991). Whereas essential amino acids would be present in amounts large enough to support the growth of *Lact. plantarum* in any olive fermentation brine, essential vitamins might not be present unless they are produced by other naturally occurring micro-organisms. This could explain why some of these strains were prototrophic for more vitamins than are described as essential for *Lact. plantarum*. These metabolic characteristics would provide some advantage to the strains that can grow in the brines in the absence of certain vitamins. Specific studies to find out which essential amino acids and vitamins and in what amounts are present during the different stages of the olive fermentative process are required and currently being carried out in our laboratory. However, we want to highlight the fact that the brines from which the different strains were isolated must have contained all the nutritional requirements that are needed, for all the lactobacilli were actively growing at the time of their isolation.

The strains used in this study were isolated from different fermenters at different locations and this could reflect the selection of particular combinations of *Lact. plantarum* strains with brines/olives of a characteristic nutritional content. In this sense, certain plasmid profiles have been found to be characteristic of certain olive processing places (Ruiz-Barba *et al.* 1991) and maintained in consecutive years in the *Lact. plantarum* population. Some of these plasmids are currently being studied for their possible involvement in the vitamin and amino acid synthesis in those strains found to be prototrophic.

Although other traits must be studied, i.e. ability to produce bacteriocins, tolerance to high levels of salt and acids in the media, lactic acid produced, etc., nutritional

requirement pattern and growth response to limiting amounts of these appear to be good criteria for the selection as well as offering many opportunities for optimization of *Lact. plantarum* inoculants for green olive fermentations. Selection of some of these strains as inoculants would have not only the advantages of a rapid growth and high levels of lactic acid produced quickly, but also would increase the nutritional value of the final product as those amino acids or vitamins would not be consumed by the predominant lactobacilli population at the same extent.

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