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Effect of dominant specie of lactic acid bacteria from tomato on natural microflora development in tomato purée

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Abstract

The dominant lactic acid bacteria specie from tomatoes surface and its effect, as competitive microflora, on tomato purée during storage at 30 °C was investigated. Four genera were found *Leuconostoc mesenteroides* ssp. *mesenteroides* being the dominant group. *Leuc. mesenteroides* ssp. *mesenteroides* Tsc when inoculated on tomato purée, pH 4.1, grew approximately 2 log cycles in 48 h, inhibiting natural bacterial development and delaying the growth of yeasts. The faster organic acids production by inoculated microorganism contributed to the diminution in the natural microflora cells number. This microorganism could be considered to help to control the contaminates proliferation on tomato purée during storage at abusive temperature.

Keywords: Tomato; Lactic acid bacteria; *Leuconostoc mesenteroides*; Competitive bacteria; Metabolism

1. Introduction

Vegetables and vegetable-based food are normal parts of the human diet and are consumed daily in large quantities in most civilizations. The natural microflora of vegetables includes bacteria, yeasts, and molds representing many genera. The microflora can vary considerably depending on the type of vegetable, environmental considerations, seasonality, and harvesting conditions. In tomato nearly half of the total dry matter consists of the reducing sugars, glucose and fructose, about 10% is organic acid and about 1% is skin and seeds, with the remainder being alcohol, insoluble solids (cellulose, pectins, hemicellulose and proteins), minerals (mainly potassium), pigments, vitamins and lipids. Glutamic acid is the principal amino acid found in tomato (Hayes, Smith, & Morris, 1998). The organic acids content of tomato is responsible for a pH between 4.0 and 4.6 (Gutheil, Price, & Swanson, 1980). The low pH and the nature of the organic acid molecule per se select the growth of acid tolerant microorganisms, such as fungi (Splittstoesser, 1987) and lactic acid bacteria (LAB) (Brackett, 1988). Traditionally, fruits and vegetables have been regarded as microbiologically safer than other unprocessed foods such as meat, milk, eggs, poultry and seafood. Extensive handling and temperature abuse of vegetable provide opportunities for microbial contamination and growth of pathogens (Wiessinger, Chantarapanont, & Beuchat, 2000). For example, salmonellosis has been attributed to fresh vegetables and in particular to consumption of tomatoes (Hedberg, MacDonald, & Olsterholm, 1994; Wood, Hedberg, & White, 1991). Gombas (1989) reported that the use of a biological control system could be efficient for decreasing the microbiological spoilage of food.

The objective of this work was to determine the dominant specie of LAB from tomatoes surface and further to investigate the effect of its growth and metabolism on

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autochthonous microflora evolution on tomato purée during storage at temperature abusive.

2. Materials and methods

2.1. Microorganism

Leuconostoc mesenteroides ssp. *mesenteroides* Tsc was isolated from tomato. The strain was stored at −20 °C in MRS medium (De Man, Rogosa, & Sharpe, 1960) supplemented with glycerol (30%, v/v).

2.2. Samples

All tomatoes (Rio Grande type) were obtained from Farms of the Tucumán State, Argentina. The products were all of agreeable sensory quality.

2.3. Processing, enumeration and isolation of LAB from surface of tomatoes

For isolation of LAB, skins of tomatoes were washed three times with sterile distilled water. Each water washing was collected under sterile conditions. Successive decimal dilutions were carried out with sterile peptone water 0.1% (w/v). From each dilution 0.1 ml volumes were plated in duplicate on MRS agar (De Man et al., 1960) acidified to pH 5.0 and supplemented with 1.3 μg/ml of Pimaricin (Sigma) (MRS-P) to inhibit yeasts growth. Aerobic mesophilic bacteria were determined on plate count agar (PCA, Oxoid, Basingstoke, UK). Agar plates were incubated anaerobically, MRS-P plates, (BBL GasPak Anaerobic System) and aerobically (PCA) at 30 °C for 7 d before enumeration. The PCA agar plates were flooded after enumeration with 3% H_2O_2 in order to observe for presence of catalase positive colonies.

Colonies by random selection were picked up from MRS-P, purified by sub-culturing in MRS broth at 30 °C and further characterized. A small number of isolates were also obtained from PCA medium.

2.4. Phenotypical characterization of selected isolates

The isolates were characterized for Gram and catalase reaction, cell shape, cytochrome-oxidase activity, spore formation, production of ammonia from arginine (Devriese, Pot, & Collins, 1993) and fermentative catabolism of glucose. Gas and D- or L-lactic acid isomers production from glucose metabolism were determined in Gibson medium (Gibson & Abdel-Malek, 1945) and by using an enzymatic method, Boehringer Kit (Mannheim, Germany), respectively.

Ability of growth, under microaerophilic conditions in BBL GasPak jars in which the content of oxygen was reduced by use of a lighted candle, was determined on MRS agar plates incubated at 15, 30, 37 and 45 °C. Ability of growth at different NaCl concentrations (2, 4, 6 and 8% w/v) and pH values (4, 5, 5.5 and 6.5) were also investigated. Production of dextran from sucrose (5%) was determined on agar medium. Studies of fermentation of carbohydrates and related compounds were carried out in MRS broth without glucose and containing bromocresol purple (0.04 g/l) as a pH indicator, inverted Durham tube and supplemented with 1% of following carbohydrates: lactose, sucrose, xylose, arabinose, sorbitol, fructose, galactose, mannose, cellobiose, raffinose, melezitose and melobiose and by using API 50 CH galleries (BioMérieux, Marcy-l'Etoile, France).

2.5. Fluorescence in situ hybridisation (FISH)

This method uses fluorescent oligonucleotide probes, homologous to 16S rDNA of each species or genus. The 16S rRNA sequences used in this study were obtained from EMBL and GenBank databases by Blasco, Ferrer, and Pardo (2003). The eubacterial Eub 338 probe 5′ end-labelled with fluorescein by MWG biotech was used as positive control and Non 338 5′-fluorescein labeled, complementary to 338, as the negative control for non-specific binding (Amann et al., 1990). LU2 oligonucleotide probe 5′ end-labelled with fluorescein was specific for *Leuc.* FISH experiments were performed according to the method described by Blasco et al. (2003).

Fluorescence was detected with a Leica DMRB microscope fitted for epifluorescence microscopy with a 100-W mercury lamp high-pressure bulb and Leitz filter blocks A (UV light exciter BP 340–380 nm, beamsplitter RKP 400 nm, emitter LP 430 nm), I3 (blue light exciter BP 450–490 nm, beamsplitter RKP 510 nm, emitter LP 520 nm), and N2.1 (light exciter BP 515–560 nm, beamsplitter RKP 580 nm, emitter LP 580 nm). Color photomicrographs were obtained using Kodak Gold 800 ASA color print film. The exposure times used were 0.10–0.30 s for phase-contrast photomicrographs and 10–120 s for epifluorescence photomicrographs.

2.6. Preparation of tomato purée, growth conditions and culture procedures

Fresh tomatoes were washed with water, peeled, blanched in saturated steam for 2 min and immediately cooled in water at 20 °C. The tomatoes were then processed with a Brown Minipimer to provide purée. Tomatoes purée (100 g) with a pH 4.1 were aseptically dispensed into sterile glass flasks with caps. The time lapse between the preparations of tomatoes purées and microbial inoculation was approximately 15 min.

Cells of *Leuc. mesenteroides* ssp. *mesenteroides* Tsc grown in MRS broth were harvested at the end of exponential growth phase (8 h) by centrifugation, washed twice and resuspended in sterile distilled water to $OD_{560\,nm} = 2.0$. The cellular suspension was used to inoculate the tomato purée medium with ca. 10^6 cfu/g. The inoculum was thoroughly distributed in tomato purée by vigorously mixing with a

sterile stainless-steel spoon. The media were incubated statically at 30 °C for 30 d.

2.7. Microbial growth

Growth was determined by estimation of viable cells numbers (cfu/g). Three grams of a tomato purée sample was homogenized with 27 ml sterile distilled water. Aerobic mesophilic bacteria counts were performed by plating 0.1 ml aliquots of appropriate decimal dilutions on plate count agar (PCA). LAB counts were performed by platting 0.1 ml aliquots of appropriate decimal dilutions on MRS-P agar pH 6.5. Gram negative non-LAB were isolated by platting 0.1 ml aliquots of appropriate decimal dilutions on Mc Conkey agar pH 7.1 (Oxoid). Cultures were incubated at 30 °C during 72 h. Yeasts were enumerated by plating 0.1 ml aliquots of appropriate decimal dilutions on selective YGC medium (Merck) and incubating at 30 °C during 7 d.

2.8. HPLC analysis

HPLC analyses were carried out for the determination of glucose, fructose, organic acids and ethanol using an ISCO liquid chromatograph (ISCO, Lincoln, NE). All separations were performed on an ionic-exchange column (HPX.87H Bio-Rad 300 × 7.8) with a flow of 0.6 ml/min. A water refractive index detector (800 psi) was used. Prior to HPLC analysis, tomato juice, obtained by centrifugation of tomato purée at 8000*g* for 20 min, was clarified by centrifugation at 12000*g* for 15 min. The clarified juice was filtered through a 0.22 μm millipore filter before being injected in triplicate onto the column. Standards were used to determine the concentration of sugars, organic acids and ethanol.

3. Results

3.1. Isolation and identification of LAB

Determined on selective MRS-P agar plates, tomatoes surface contained levels of LAB ranging from log 3.5 to log 3.8 cfu/ml. A count approximately 1.0 log cycle higher was observed by use of PCA. Majority of colonies on the PCA plates were small, grey or white, catalase negative able to grow under aerobic conditions suggesting they were LAB. Lower numbers were found of small, positive catalase colonies as well as colonies belonging to Enterobacteriaceae and yeasts. The LAB constituted the predominant element of the microbial flora in tomatoes surface. A total of 35 isolates belonging to LAB group were selected from MRS-P agar medium. In addition five isolates were obtained from PCA plates. All LAB isolates were Gram positive, catalase negative, non-spore forming, non-motile cells. All isolates exhibited a fermentative catabolism from carbohydrates. The majority of isolates were obligatory heterofermentative (group I, II) while only a few isolates were homofermentative (group II–IV). Isolates were divided in four groups according to their morphological and physiological characteristics.

Group I: Twenty of heterofermentative isolates, identified as *Leuconostoc*, exhibited the following characteristics: production of gas and D-lactic acid isomer from glucose, no ammonia formation from arginine, growth at 15 °C but no growth at 45 °C and cocci arranged in chains. Eleven isolates belonging to this group were classified as *Leuc. mesenteroides* ssp. *mesenteroides* on the basis that they all produced slime layer when grown on agar containing sucrose, did not grow in 2% NaCl broth or in 10% ethanol, grew at 30 or 37 °C and by using of the API 50 CH kit.

Group II: Consisted of 10 isolates identified as *Lactobacillus*. Of these bacteria, three strains were assigned to *L. coryniformes* Lts1, Lts2 and Lts3 on the basis that they produced the D(-) lactate isomer from glucose by homo-lactic fermentation, grew at 15 °C but not at 45 °C, did not hydrolyse esculin and they fermented glucose, fructose, galactose, maltose, mannitol, melibiose, raffinose and rhamnose.

Group III: Consisted of six isolates identified as *Pediococcus* sp. They were differentiated from other LAB by their typical cell morphology, production of NH_3 from arginine, formation of D-L lactate isomers from glucose and ability to grow at 45 °C.

Group IV: Growth in 6.5% NaCl, at pH 9.6 and 45 °C made it possible to consider three strains as belonging to *Enterococcus* genus.

3.2. Identification of Leuc. mesenteroides by FISH technique

Whole-cell hybridisation results of *Leuc. mesenteroides* ssp. *mesenteroides* Tsc1, *L. coryniformes* Lts1 and *Klebsiella ornitolythica* Kts1, isolated from tomatoes surface during this study showed no autofluorescence and yielded strong hybridisation signals with the Eub338 oligonucleotide probe. The LU2 probe hybridised exclusively with the 16S rRNA of the strain of *Leuc. mesenteroides* ssp. *mesenteroides* Tsc, giving negative reaction with the other bacteria analyzed confirming the results obtained by phenotypic tests.

3.3. Growth of Leuc. mesenteroides ssp. mesenteroides Tsc in tomato purée medium

Fig. 1 shows the microbial population evolution in tomato purée medium stored at 30 °C for 26 d. Uninoculated tomato purée medium was used as control medium. In control medium, the total initial number of microorganisms found on PCA was 1.6×10^1 cfu/g and it was constituted 75% by bacteria belonging to LAB group and 25% by bacteria identified as *Staphylococcus* sp. on the basis that they were positive for gram staining, catalase activity and DNAse and coagulase tests. No Enterobacteriaceae and yeasts were isolated. Counts on MRS agar coincided with the counts on PCA plates for LAB. Genus of LAB identified were the same than those found on tomatoes

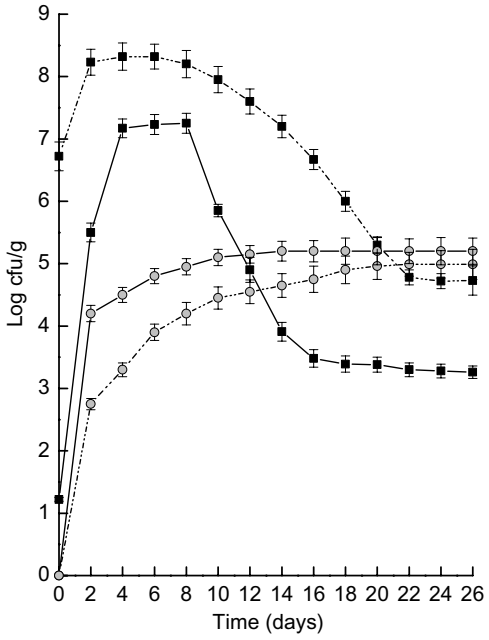

Fig. 1. Evolution of bacteria and yeasts in tomato purée media. Uninoculated medium (—) and inoculated medium with *Leuc. mesenteroides* ssp. *mesenteroides* Tsc (- - -) stored at 30 °C. Bacteria cfu/g (■), yeasts cfu/g (○).

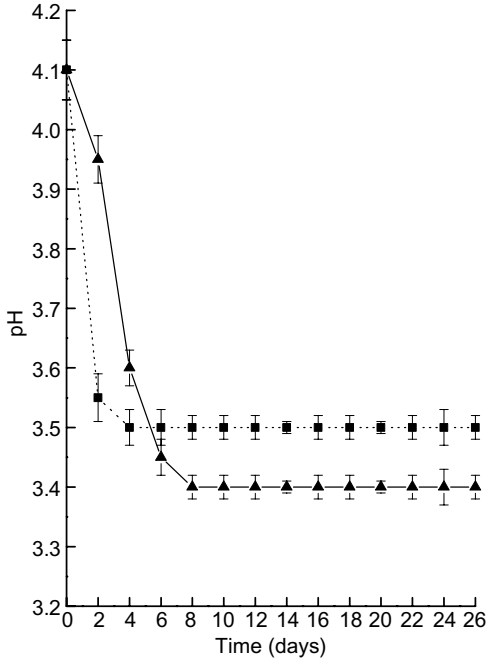

Fig. 2. Variation of pH in tomato purée media during storage time at 30 °C. Uninoculated medium (▲) and inoculated medium (■).

surface. During storage at 30 °C, initial counts increased significantly reaching a maximum value of 10^6 cfu/g at 4 d. After 8 d it began to decrease progressively to reach a final cell concentration of 1.8×10^3 cfu/g at 16 d. Yeasts count was positive (3×10^4 cfu/g) just at 2 d and most at 12 d. Yeasts did not decreased at anytime during the tomato purée storage period.

When the tomato purée medium was inoculated with the strain of *Leuc. mesenteroides* ssp. *mesenteroides* Tsc the initial bacterial population corresponded to 5.3×10^6 cfu/g. At 2 d incubation, the bacterial count became maximum increasing by about 2 log cycles, the inoculated organism being isolated. On the other hand, at 2 d storage, yeasts count was approximately 2 log cycle lower than that obtained in uninoculated medium. Yeasts continued growing to reach the same cell density as that observed in control medium. After 8 d incubation in inoculated tomato purée medium, the bacterial count also began to decrease but at a lower rate than that obtained in the uninoculated one.

Samples of uninoculated tomato purée medium were sensorially unacceptable at 8 d storage (odor and visual signs of alteration) whilst this effect was just observed in the inoculated medium at 19 d storage.

3.4. Variation of pH in tomato purée media

Fig. 2 shows that the initial pH changed from 4.1 to 3.9 in the natural medium during 2 d incubation and then it continued decreasing progressively until 3.4 for 8 d. By contrast in inoculated medium the pH rapidly decreased to 3.5 in 48 h incubation and then it remained almost constant.

3.5. Microbial metabolism in uninoculated tomato purée medium

The utilization of sugars, L-malic and citric acids and their metabolic products were measured during the natural microflora growth in control tomato purée during storage at 30 °C. Fig. 3 shows that the glucose and fructose consumption began immediately growth began and it was utilized faster during the first 2 d incubation. Maximum consumption corresponded to 48%, 12% and 40% of initial glucose and fructose respectively and it was reached at 4 d. At this time, significant amounts of D and L-lactic and acetic acids and ethanol were produced by the native microflora. Ethanol production continued until 6 d storage. The analytical balance of these end products from glucose + fructose consumed in mmol/mmol was: 0.30, 0.46, 0.49 and 0.45 for D-lactic acid, L-lactic acid, acetic acid and ethanol, respectively. Detected a level of total aroma compounds (diacetyl, acetoin and 2,3 butanediol) of 1.80, 4.0 and 7.2 μg/g at 2, 4 and 8 d incubation, respectively. Then they remained unchanged (data not shown).

Initial concentrations of citric and L-malic acids were 17.59 and 1.79 mmol/l, respectively. These results coincided with those reported for San Marzano tomato varieties from Italy (Loiudice et al., 1995). Forty-seven percentage and 87.5% of initial citric acid concentration was utilized at 2 and 4 d incubation, respectively (Fig. 4). Natural microflora consumed 88.8% of initial L-malic acid at 2 d incubation and almost 100% at 4 d (Fig. 4). Considering the low initial concentration of the dicarboxylic acid, only a very small amount of L-lactic acid could have been formed from its catabolism, this product being mainly formed from sugar metabolism.

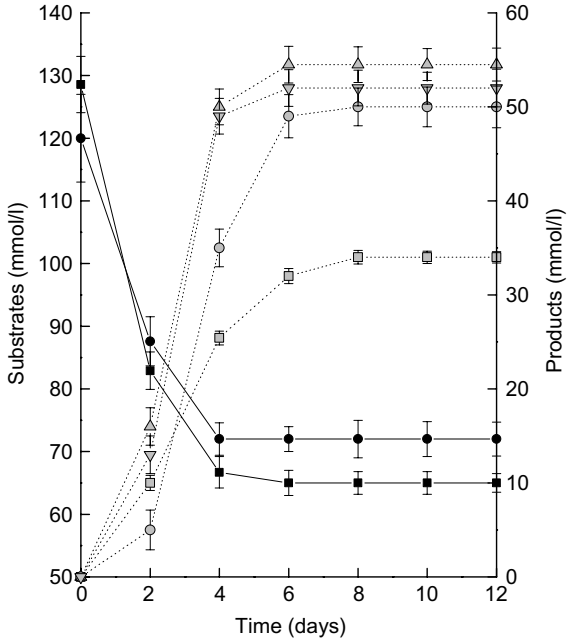

Fig. 3. Substrates consumption and metabolites production in uninoculated tomato purée medium during storage at 30 °C. Substrates: glucose (■), fructose (●). Products: D-lactic acid (□), L-lactic acid (▽), acetic acid (△), ethanol (○).

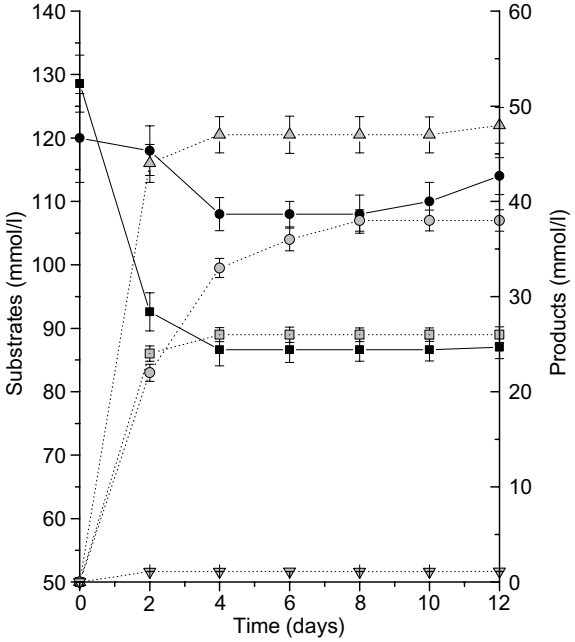

Fig. 5. Substrates consumption and metabolites production in inoculated tomato purée medium during storage at 30 °C. Substrates: glucose (■), fructose (●). Products: D-lactic acid (□), L-lactic acid (∇), acetic acid (△), ethanol (○).

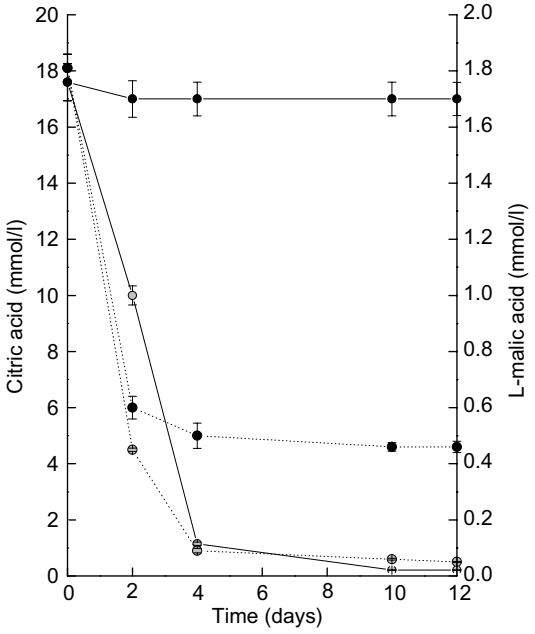

Fig. 4. Citric and L-malic acids consumption in tomato purée media during storage at 30 °C. Citric acid (—), L-malic acid (- - -). Uninoculated medium (○), inoculated medium (●).

3.6. Microbial metabolism in inoculated tomato purée medium

The time course of glucose and fructose utilization and L and D-lactic and acetic acids and ethanol production was studied in natural medium inoculated with *Leuc. mesenteroides* ssp. *mesenteroides* Tsc (Fig. 5). The utilization of glucose and fructose began immediately growth began and the major part of consumption being in the first 2 d. At this time 28% of initial glucose was utilized reaching a maximum value of 34.4% at 4 d. Only 10% of initial fructose was utilized. The carbohydrates utilization was accompanied by an increase in concentration of D-lactic and acetic acids and ethanol in culture medium, in a molar relation (mmol/mmol) of 0.5, 0.92 and 0.73, respectively. The rapid pH diminution was correlated with the organic acids formation (Fig. 3). The maximum aroma compounds production in this medium occurred at 2 d incubation and it was 37.5% lower than that obtained in uninoculated one. It was noticeable that citric acid was not utilized although 56% of initial L-malic acid was utilized (Fig. 4). L-lactic acid was accumulated into the medium throughout the period of L-malic acid degradation and accounted for approximately 100% L-malic acid consumed. The maximum production of L-lactic acid was significantly lower than that observed in natural medium.

4. Discussion

Numbers of LAB found on tomatoes surface (approximately $\log_{10}$ 4.5) was in accordance with the results reported by Brackett (1988) and Drosinos et al. (2000). Enterobacteriaceae and yeasts were also isolated from tomatoes surface in similar way as that reported by Drosinos, Tassou, Kakiomenou, and Nychas (2000).

Among isolates the predominance of *Leuc. mesenteroides* ssp. *mesenteroides*, might be related to better adaptation to

tomato conditions and be able to initiate growth more rapidly than other LAB. *Leuc.* spp. predominance, and more particularly *Leuc. mesenteroides* in unprocessed vegetables had been reported by Nguyen-the and Carlin (2000).

The lower initial microbial count on tomato purée medium, 10^1 cfu/g, than on tomatoes surface could be related with the removal of microorganisms by the washing, peeling and blanching procedures. In tomato purée medium the growth of *Leuc. mesenteroides* ssp. *mesenteroides* Tsc inhibited the development of indigenous bacterial microflora and reduced the growth rate of yeasts during storage at 30 °C. It is interesting to note that this fact would result in a decreased risk of human infection since *Enterococcus* and *Staphylococcus* strains, present in the natural microflora, were eliminated. Steinkraus (1983) demonstrated that *Leuc. mesenteroides* inhibited the aerobic mesophilic organisms growth that might destroy crispness in the cabbages or cucumbers. The prevention of natural microflora growth by *Leuc. mesenteroides* ssp. *mesenteroides* Tsc could be related with the inability to compete successfully with the inoculated strain or to the specific metabolic products, such as lactic and acetic acids, produced by the inoculated microorganism during storage. Vescoso, Orsi, Scolari, and Torriani (1995) reported that inhibitory effect of LAB on microflora associated with ready to use vegetables was due to bacteriocin production. In this study, *Leuc. mesenteroides* ssp. *mesenteroides* Tsc was not able to produce bacteriocin. Its inhibitory action was not destroyed by 1 mg/ml pepsin or trypsin.

Native microflora utilized glucose and fructose producing D, L-lactic and acetic acids and ethanol in a higher extent than inoculated microorganism. This was especially noticeable in the case of fructose consumption and L-lactic acid formation (80% and 98% higher in control medium than in inoculated medium, respectively). So the autochthonous bacterial microflora from tomato purée was not mainly composed by the predominant microorganism from tomatoes surface, even when heterofermentative microorganisms were present. This fact could be related with the low pH of internal tissue of tomato which would favor the growth of other LAB, such as Lactobacilli. On the other hand tomato composition could be an important factor in enabling the growth of LAB requiring growth factors. Babu, Mital, and Garg (1992) reported the stimulatory effect of tomato juice on the growth of *Lactobacillus* sp.

Metabolism of natural microflora was correlated with the changes in sensory properties of medium producing undesirable buttermilk and fermented flavor by the high diacetyl and organic acids concentration, respectively. Simsek, Con, and Tulumoglu (2004) reported in sourdough process the importance of role of the lactic and acetic acids on the aroma profile. The inhibition of natural microflora delayed these changes in the medium.

In tomato purée *Leuc. mesenteroides* ssp. *mesenteroides* Tsc preferred as main carbon and energy source glucose rather than fructose for growth. The high levels of residual sugars obtained at the end of *Leuc. mesenteroides* ssp. *mesenteroides* Tsc growth suggested that the fermentation stopped because of low pH rather than because of lack of carbohydrate substrate. A similar result was reported by Gardner, Savard, Obermeier, Caldwell, and Champagne (2001) in *Leuc. mesenteroides* growing in vegetable juice medium. The results of fermentation balances demonstrated that the D-lactic acid formed from sugars metabolism by inoculated microorganism was lower than expected theoretical value indicating that sugars metabolism could be involved in biosynthesis reactions. Saguir and Manca de Nadra (2002) reported in *Oenococcus oeni* that glucose metabolism was involved in the cysteine biosynthesis. In contrast, the amount of acetic acid + ethanol recovered from glucose + fructose was 65% higher than the expected theoretical value. So we can infer that acetic acid is also formed from other compounds present in the natural medium than carbohydrates.

The faster production of organic acids during the first 48 h incubation in inoculated medium with respect to control medium explained the rapid pH diminution. This may contribute to the inhibitory effect of inoculated microorganism growth on natural microflora. Citric and L-malic acids were consumed in control medium but in inoculated medium citric acid was not utilized. Starrenburg and Hugenholtz (1991) reported in *Lactococcus lactis* and *Leuconostoc* sp. that the citrate utilization is determined by the activity of the citrate permease which has an optimum pH between 5.5 and 6.0. In our study the pH of the natural medium, lower than 5.0, could explain the inability of this strain to utilize it.

In inoculated medium the changes in organoleptic properties were evident 11 d later than in uninoculated medium and this fact was related to the quantities of the metabolites produced (specially organic acids) in the different uninoculated/inoculated media during storage.

In conclusion, the dominant LAB isolated from the tomatoes surface, *Leuc. mesenteroides* ssp. *mesenteroides* Tsc, had an inhibitory effect on natural microbial population growth on tomato purée and it could be considered an important secondary barrier to help to control the proliferation of contaminants. At the same time, this microorganism could delay the changes in the sensory characteristics of tomato purée during storage at an abuse temperature.

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