International Scholars Journals

Advances in Food Science and Technology ISSN: 6732-4215 Vol. 3 (8), pp. 332-338, August, 2015. Available online at www.internationalscholarsjournals.org © International Scholars Journals

Full Length Research Paper

Evaluation of Lactic acid bacteria isolated from fermented tomatoes to produce antimicrobial activities against several bacteria and fungi

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Accepted 20 July 2015

The aim of this study was to evaluate 40 lactic acid bacteria (LAB) and 20 *Bacillus* strains isolated from the fermented tomato (*Solanum lycopersicum*) for their capacity to produce antimicrobial activities against several bacteria and fungi. The strain designed LBc03 has been selected for advanced studies. The supernatant culture of this strain inhibits the growth of *Escherichia coli*, *Staphylococcus aureus* and *Aspergilus* sp. Based on the cultural, morphological, physiological and biochemical characteristics, LBc03 was identified as *Leuconeustoc* spp. Its antimicrobial compound was determined as a proteinaceous substance, but it is possible that the bacteriocin may also be bound to other molecules like a lipid or a carbohydrate moiety. Metabolite extracts from selected LAB were more effective in preserving tomato paste and sauce stored at 4°C against spoilage bacteria like *E. coli* and the application of bio-preservative should be encouraged in food processing industries.

Key words: Biopreservation, tomato (*Solanum lycopersicum*), bacteriocins, lactic acid bacteria.

INTRODUCTION

Microbial spoilage of fruits and vegetable is known as rot, which manifests as loss of texture (soft rot), changes in color (black or grey) and often off odor (Trias et al., 2008). Also, the high water content in tomatoes makes it very susceptible to spoilage bacteria and fungi during storage, harvesting and transportation (Spadaro and Gullino, 2004). Fresh food like fruits and vegetables, are normal part of the human diet and are consumed in large quantities in most countries. These products are rich in carbohydrates and poor in proteins with pH value from slightly acidic to 7.0 and provide a suitable niche to several bacteria, yeasts and moulds (Wiessinger et al., 2000; Trias et al., 2008 ; Ogunbanwo et al., 2014). Tomato (*Solanum lycopersicum*) is one of the highly nutritious food ingredient used in the preparation of food all over the world (Ogunniyi and Oladejo, 2011; Ogunbanwo et al., 2014). Its utilization as an ingredient in vegetable salads, other dishes and its processing into different products like puree, ketchups and juice is well documented. Nutritionally, it contains a large amount of water, niacin, calcium and vitamins especially A, C, E which are important in the metabolic activities of man and protects the body against diseases (Taylor, 1987). Lycopene (acarotene) an essential component of tomato contributes in the prevention of cardiovascular disease and cancer of the prostrate (Clinton, 1998; Bernard et al., 1999). The characteristic flavor of

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Table 1. S01, S02, S03, and S04 tomatoes *Solanum lycopersicum* samples.

Samples	Origin	Tomato Variety	Characteristics	Additives added	Temperature of storage (°C)	Storage time
S01^{C1}	Mascara, Algeria	*Solanum lycopersicum*	Fresh-cut tomato	Absence	25	24 h
S02^{C2}	Mascara, Algeria	*Solanum lycopersicum*	Tomato paste was boiled for 90°C for 02 h	Absence	25	24 h
S03	Mascara, Algeria	*Solanum lycopersicum*	Fresh-cut tomato	Absence	-20	06 months
S04	Mascara, Algeria	*Solanum lycopersicum*	Tomato paste was boiled for 90°C for 02 h	05% NaCl and oil	+4	06 months

C1and C2 are control samples.

of tomato is produced by the complex interaction of the volatiles and non-volatile components (Petro-Turza, 1987; Buttery, 1993). The nutritional value of tomato products is a topic attracting much attention, particularly regarding the effects resulting from food processing and storage treatments (Capanoglu et al., 2010). Among the common post-harvest fungal pathogens of tomatoes are *Pencillium expansum*, *Monilinia laxa* and *Rhizopus stolinifer* (Ogawa et al., 1995; Pla et al., 2005). Many LAB strains are able to produce protein compounds with efficient antimicrobial effect, which are known as bacteriocins (Davidson and Harrison, 2002). In recent times, the understanding of the preservation mechanisms of LAB is being exploited for industrial production of foods (Trias et al., 2008) because of their natural acceptance as Generally Recognized as Safe (GRAS) for human consumption and exhibit antimicrobial property (Aguirre and Collins, 1993). There is a complimentary effect by the production of acid and antimicrobial compounds that increases inhibition of both pathogen and spoilage bacteria (Edwards et al., 1983; Artés et al., 1999). Although many efforts have been made to develop bio-protective lactic acid bacteria strains, the application of these strains in fresh fruits and vegetables have not been developed yet (Toivonen and DeEll, 2002).

This work is designed to investigate the effectiveness of lactic acid bacteria and *Bacillus* metabolites in preserving tomato paste and sauce against *S. aureus, E. coli*, *Clostridium* sp., *Aspergillus* sp., and *Penicillium* sp.

MATERIALS AND METHODS

Samples collection

Fresh tomato samples *Solanum lycopersicum* (Table 1) were purchased from markets in Mascara, Algeria. The samples were collected in separate sterile polythene bags and immediately transported to the laboratory for analysis.

Determination of the physical-chemical characteristics of the tomato samples (S01, S02, S03, and S04)

Moisture content

The moisture content of the samples (S01, S02, S03, and S04) was determined before storage time by weighing into moisture cans then weighed and then placed in an oven at 80°C for 24 h to dry to a constant weight. Then, brought out and allowed to cool in desiccators and then reweighed (A.O.A.C., 1995).

$$\text{Thus, moisture content} = \frac{(\text{initial weight} - \text{final weight})}{\text{initial weight}} \times 100$$

Dry matter content

Five grams of each sample were obtained and placed into pre-weighed crucibles and dried in at 100°C for 12 h. The dried samples were weighed after cooling in the desiccators (A.O.A.C., 1984).

Determination of lactic acid produced by lab isolates

This was achieved based on the methods described (Ogunbanwo et al., 2008; Bamidele et al., 2011). For these measurements lactic acid was determined by transferring 25 ml of the tomato juice (S01, S02, S03, and S04) into conical flasks and 3 drops of phenolphthalein were added as indicator. From a burette, 0.1 M NaOH was slowly added to the samples until a pink color appeared. Each ml of 0.1 M NaOH is equivalent to 90.08 mg of lactic acid.

Determination of the pH

pH was determined after and before fermentation of the tomato samples (S01, S02, S03, and S04) by the used of pH meter (Inolab MLM) (Lehninger, 1981).

Detection of pathogens, contaminants, LAB, and bacillus

The main objective of this experiment was to detect microbial contaminants in tomato samples (S01, S02, S03, and S04) in addition to other related hygiene tests. Total aerobic bacterial count, total coliform

count, Total anaerobic bacteria count, *Salmonella*, *S. aureus*, *Clostrudium*, lactique acid bacteria, and *Bacillus* were detected (Delarras et al., 2006).

Isolation and selection of LAB strains

LAB strains were isolated from the tomato samples (S01, S02, S03, and S04). The samples were plated directly on MRS as detailed by De Man, Rogosa and Sharpe (Merck, Germany) and M17 agar (Merck, Germany) at 30°C for 2-3 days (pH6.5) under aerobiosis and anaerobiosis conditions. They were routinely propagated and stored at – 20°C supplemented with glycerol (20%, v/v, final concentration). Working cultures were sub-cultured twice (1 inoculum, 24 h, 30°C) prior to use (Djadouni and Kihel, 2012; Ogunbanwo et al., 2014).

Identification of the LAB isolates

The pure isolate selected as a potential bacteriocin - producer was identified on the basis of its cultural, morphological, physiological and biochemical characteristics. The selected LAB isolates were characterized by Gram stain, absence of spores and catalase test. Gram+, catalase and spores negative strains were maintained frozen until needed for the antimicrobial activity testing. Confirmation of the identification was based on the use of Bergey's manual of systems bacteriology (Sneath et al., 1986).

Isolation and Identification of bacillus strains

Samples of tomato were weighed as 1 g portions and thoroughly homogenized insterile distilled water; serial dilutions were plated on LB agar (Luria–Bertani media, Merck, Germany) plates were incubated at 30°C for 2-3 days (Kalil et al., 2009). The pure isolate was examined macroscopically and microscopically and identified with reference to Holt et al., (1994). Isolates were identified by colonial appearance, gram positive, and presence of spores, the presence or absence of β-haemolysis, lecithinase activity, motility, penicillin susceptibility and biochemistry (Abriouel et al., 2010).

The indicator strains

The indicator strains (*S. aureus, E. coli*, *Clostriduim* sp., *Aspergillus* sp.*,* and *Penicillium* sp.) used in this work was provided by the Laboratory of Bacteriology, Microbiology Department at the Faculty of Sciences, Es-Senia, Oran University, Algeria. For the antimicrobial assay, the pathogenic cultures were grown in the nutrient agar media (NA) at pH, 7.4 (Ogunbanwo et al., 2014).

For antifungal activities determination, *Aspergillus* sp. and *Penicillium* sp. were grown in potato dextrose agar (PDA, Merck, Germany) for 7 days at 30°C. Spores were collected in sterile distilled water and then concentrated to 10^4 spores ml^{-1} (Smaoui et al., 2010).

Preparation of cell-free filtrate

MRS broth (1000 µl) were inoculated separately with isolates (LAB or *Bacillus*) previously characterized and incubated at 30؛C for 72 h. After incubation, a cell free supernatant was obtained by centrifuging (Spectrafuge 24D, Labnet, USA) the bacterial culture at 10.000 rpm for 45 min, followed by filtration of the supernatant through 0.2 mm pore size filter paper thus obtaining cell free filtrate (Khalil et al., 2009; Djadouni and Kihel, 2013).

Antagonistic activity of LAB and bacillus metabolites against spoilage microorganisms

Sixty isolates (40 LAB and 20 *Bacillus*) were grown in MRS broth for 72 h at 30°C and the broth cultures were centrifuged at 10.000 rpm for 30 min and the supernatant containing the metabolites were obtained and 100 µL of the supernatant was transferred into wells (6 mm diameter) bored in Muller Hinton and potato dextrose agar previously seeded with the spoilage bacteria cells and fungi spores. The culture plates were incubated at 30 °C for 48 h and 7 days respectively and observed for zones of inhibition (Ogunbanwo et al., 2014).

Characterization of the antimicrobial substance

The isolated crude antimicrobial substance was characterized with respect to the effect of proteolytic enzymes on the bacteriocin activity. Selected enzymes were tested on the cell free supernatant (Bizani and Brandelli, 2002). Proteolytic enzymes including trypsin, pepsin, and papain were dissolved in 40 mM Tris-HCl (pH, 8.2), 0.002 M HCl (pH, 7), and 0.05 M sodium phosphate (pH, 7.0) respectively to a final concentration of 0.1 mg ml^{-1}. Other enzymes such as lipase and α-amylase were dissolved in 0.1 M potassium phosphate (pH, 6.0), and 0.1 M potassium phosphate (pH, 7.0) respectively to a final concentration of 0.1 mg/ml. Equal aliquots of both filter sterilized of each test strain and each enzyme solution were mixed, incubated at 30°C for each enzyme for 2 h and heated in boiling water for 5 min to inactivate the enzymes. These sample mixtures and the controls (without enzyme treatment) were inoculated with the indicator strains as previously mentioned and tested for antimicrobial activity by the optical density method (ODM) (Smaoui et al., 2010; Djadouni and Kihel, 2013).

Shelf life study of tomato paste and sauce

The tomato paste and sauce were boiled for 10 min and dispensed in 20 g amount separately into three pre-sterilized containers. Twenty milliliters of the crude bacteriocin-like substance of *Leuconostoc* spp. were added (v/v) differently to the tomato paste and sauce inoculated with *E. coli* 10^7 CFU g^{-1} (2 ml) and stored at 4°C for 72 h. Microbial load of each treatment was monitored by determining the colony forming unit (CFU ml^{-1}) of *E. coli* on the hektoen medium (Safdar et al., 2010; Cottaz et al., 2008).

Statistical analysis

Data were expressed as mean ± standard deviation. Statistical significance was determined using one-way analysis of variance on the replicates, where a p-value of ≤ 0.05 was considered significant.

RESULTS AND DISCUSSION

The results of the physical and chemical analyses of tomato samples are shown in Table 2. The percentage of moisture contents decreased in S02 and S04 samples to 60.30 and 62.50%; this may be due to the boiling at 90°C for 2 h and the production process steps of tomato paste, that is made by cooking tomatoes for several hours to reduce moisture, straining them to remove the seeds and skin (Adam, 1998; Ife Fitz and Bas uipers 00 rgera et al., 2007; Souci et al., 2008). Dry matter content was stable

Table 2. Physical-chemical characteristics of tomatoes samples (S01, S02, S03, and S04).

Samples	Moisture content %	Dry matter content (g)	lactic acid content (°D)	pH	Firmness, taste and color
S01	94.20 ± 0.1	5.9 ± 0.1	0.99 ± 0.02	04.20±0.03	No change
S02	60.30 ± 0.1	5.7 ± 0.2	0.75 ± 0.02	04.43±0.03	Change in color
S03	94.20 ± 0.1	5.8 ± 0.1	0.89 ± 0.02	04.28±0.02	No change
S04	62.50 ± 0.1	5.9 ± 0.1	01.58 ± 0.02	03.70±0.01	Change in color, taste, and production of CO_2

stable along the different time and temperature of storage in S01, S02, S03 and S04 from 5.9 g to 5.7 g, but the pH values decreased to 03.70 in sample S04 that was stored at 4°C for six months, and lactic acid concentration increased to 01.58 D° during this storage period with an important change in the color, taste, and CO_2 production; this changes may be due to the thermal treatment, fermentation process and organic acids (citric acid, ascorbic acid, and lactic acid) produced by LAB during storage in anaerobic conditions in presence of oil and NaCl (Jean-Louis, 2007).

The fresh-cut tomato S03 was not affected during cold storage and shelf-life. On the other hand, S01 and S02 did not show significant differences among them. Color of fresh-cut tomatoes was significantly affected by the antimicrobial treatments and the storage time (Ayala-Zavala et al., 2008). Previous experiments demonstrated that firmness losses of fresh-cut tomato slices were probably linked to the ripening stage of whole fruit at processing and storage temperature. In addition, processing operations may have triggered important losses in membrane integrity due to mechanical stressing of plant tissues. In this regard, diminishing membrane integrity could cause a loss of texture related enzymes and their substrates, leading to a rise in Xuids and solute exchanges as well as an increase in the enzymatic activity (Ayala-Zavala et al., 2008). Results obtained in this study show that transformation process, temperature and storage period can affect the tomato components and quality (Goodman et al., 2002; Chong et al., 2009; Ife Fitz and Bas Kuipers, 2003). During transport and storage, intact fruits and vegetables are prone to deleterious changes induced by respiratory, metabolic and enzymatic activities, as well as by desiccation, pests, microbial spoilage, and temperature-induced injury. Many of these changes adversely affect the antioxidant status of the tomato products (Lindley, 1998). Capanoglu et al. (2010), worked closely with a Turkish tomato paste factory and obtained samples from each step during the paste production process, from the tomatoes arriving at the factory gate to the final canned product. Both targeted (for carotenoids) and untargeted (LC-MS of semi-polar, hydrophilic extracts) metabolic approaches were used to follow biochemical changes after each step.

Results show that a multitude of modifications took place, involving both increases and decreases in individual components. Those steps causing the greatest changes were identified and predictions were made as to how these steps could be tackled in a modified processing strategy to improve the antioxidant capacity of the end product. Gahler et al. (2003) investigated home preparation methods such as peeling, tomato soup preparation, etc., and three different steps of tomato juice production including sieving, homogenization, sterilization, filling, and pasteurization. Results suggested that homogenization increased the hydrophilic antioxidant capacity of the different tomato products. However, the exact mechanism still remains unclear. Similarly, in industrial processing,

Capanoglu et al. (010) also showed the “breaker” or homogenization step most significantly altered the biochemical composition of tomato paste. Abushita et al. (2000) also analyzed samples taken from three steps of paste processing (raw tomato, crushed sieved puree, and pasteurized paste) which were obtained from a canning factory in Hungary. The results show that the contents of ascorbic acid and tocopherols decreased during processing while carotenoids either remained unchanged or were found to increase.

Absence of *Salmonella*, *S. aureus*, and *Clostridium* in the tomatoes samples (Table 3) showed that thermal treatment and cold storage of tomato inhibit the growth and developments of pathogens and spoilage micro-organisms. However, thermal treatments applied during industrial preparation of tomato products may involve various chemical reactions leading to the degradation of these antioxidants. Besides, addition of vegetable oil for the preparation of tomato sauce may lead to lipid oxidation contributing to the micro-constituent instability (Chanforan et al., 2010a and b). The presence of some aerobic mesophilic microorganisms, coliforms, and anaerobic bacteria compared to control samples was related to the averments conditions and preparations methods of tomatoes. Ayala-Zavala et al. (2008) suggest that mechanical damage during minimal processing enhance contamination by epithelial microflora, and promote leaking of nutrients, which are rich substrates to microorganisms, supporting the fast microbial development in contrast with intact tissue.

The highest count of LAB and *Bacillus* in S04 sample was explained by the storage of tomato paste for 06 month at 4°C in the presence of oil and NaCl, which favor the fermentation and organics acids production that

Table 3. Detection of pathogens, contaminants, LAB, and *Bacillus* in the tomato samples (S01, S02, S03, and S04).

Samples	Total aerobic bacterial count (CFU ml^{-1})	total coliform count (CFU ml^{-1})	Total anaerobic bacteria count (CFU ml^{-1})	*Salmonella* (CFU ml^{-1})	*Clostrudium* (CFU ml^{-1})	*S. aureus* (CFU ml^{-1})	LAB (CFU ml^{-1})	*Bacillus* (CFU ml^{-1})
S01	86.10^5	36.10^5	30.10^5	ABS	ABS	ABS	ABS	20.10^5
S02	55.10^5	17.10^5	34.10^5	ABS	ABS	ABS	20.10^5	25.10^5
S03	76.10^5	ABS	ABS	ABS	ABS	ABS	47.10^5	29.10^2
S04	ABS	25.10^5	36.10^5	ABS	ABS	ABS	60.10^0	72.10^5

* ABC, absence.

decreased the pH of products and increased also the CO_2 production; these conditions inhibit the growth of contaminants in the tomato paste (Buta and Moline, 1998; Benkeblia, 2004). Also, this LAB and *Bacillus* have antibacterial and antifungal activities against a variety of Gram-negative and Gram-positive bacteria and fungi. Related to fungal development, the highest counts were observed in control samples (Siboukeur, 2011).

A total of 60 isolates were obtained from fermented tomato paste and screened for antimicrobial spectrum against the Gram-positive, Gram-negative bacteria, and fungi using the well diffusion method. The average diameter of the inhibition zone measured ranged from 2 to 4 mm size. One strain of LAB was selected for further studies, because LBc03 contained antimicrobial compound with wide spectrum that inhibited the growth of three indicator strains *E. coli*, *S. aureus*, and *Aspergilus* sp but did not inhibited *Penicillium* sp. and *Clostridium* sp. On the basis on their positive Gram reaction, non-motility, absence of catalase activity and of spore formation, the rod or coccal shape, physiological and biochemical characters as well as sugar utilization pattern, LBc03 was identified as *Leuconostoc* spp. (Table 4). The isolation of LAB species from healthy tomato fruits is in accordance with findings of Sajur et al. (2007) and Settanni and Corsetti, (2008). The presence of LAB in tomato fruit is attributed to their high survival in post harvest conditions of tomatoes (Trias et al., 2008; Ogunbanwo et al., 2014).

The LAB ability to produce antimicrobial compounds is due the absence of true catalyses to break down hydrogen peroxide generated which accumulates and becomes inhibitory to some organisms. The antagonistic activity of LAB metabolites against the spoilage bacteria and fungi agrees with the findings of Trias et al. (2008). The inhibitory effect of lactic acid is due to undissociated forms of the acids which penetrates the pathogen's membrane and liberate hydrogen ion in the neutral cytoplasm thus inhibiting vital cell functions (Corleh and Brown, 1980; Adeniyi et al., 2006). Diacetyl is known to be very effective against fungi and this is due to the interference with the utilization of arginine (De Vyust and Vandamme, 1994) and in addition to a strong oxidizing effect on the organisms cell especially bacteria (Condon, 1987; Sangorrin et al., 2014).

The *Leuconostoc* spp. (LBc03) bacteriocin-like substance produced was inactivated by the tested enzymes (Table 5). However, since the activity of the filtrate was not completely inhibited, it is possible that the bacteriocin may also be bound to other molecules like a lipid or a carbohydrate moiety. These data clearly show that the bacteriocin-like substance was of a proteinaceous nature.

Similar results were obtained (Joshi et al., 2006; Diop et al., 2008; khalil et al., 2009; Chanforan et al., 2010a and b; Rakshita, 2011).

Bacteriocin-like substance produced by LBc03 resulted in the decrease of *E. coli* from an initial population of 10^7 to 05.10^3CFU mL^{-1} and 1.10^3 CFU mL^{-1} in tomato paste and sauce respectively (Figure 1). This reveals a possible potential of the LAB metabolites in the retardation of food spoilage which agrees with the findings of Ogunbanwo et al. (2008).

The bio-preservative potential of LAB metabolites has been tested on other food product like suya (Adesokan et al., 2008) and chicken meat (Ogunbanwo and Okanlawon, 2006). A major advantage in the use of lactic acid bacteria and their metabolites is that they are considered as generally recognized as safe (GRAS) and comply often with the recommendations for food products (Stiles and Holzapfel, 1997). Unlike some chemical preservatives, LAB metabolites have not been reported to have residual effect on the food product or the consumer's health.

Conclusion

Our bacteriocin-like substance revealed interesting properties that justifies it importance regarding food safety and protection.

Conflict of interests

The author(s) did not declare any conflict of interest.

Table 4. Morphological, physiological and biochemical properties of LAB isolate (LBc03).

Test	Isolate LBc03
Morphology	**Rod, circular and white colonies 3.0mm**
Growth at temperature (°C)	
10	+
15	+
30	+
37	+
45	+
Growth at pH	
3.5	-
4.5	+
5.5	+
6.5	+
7.5	+
8.5	+
9.5	w
Growth at NaCl %	
4	+
6.5	+
10	+
15	+
CO2 from glucose	+
CO2 from gluconate	+
ADH	+
Citrate	+
Thermoresistance at 60°C for 30 min at 45°C	+
Fermentation Type	He
Fermentation of: glucose, saccharose, and lactose.	+
Lait Sherman (1% BM) at 42°C	+
Lait Sherman (3% BM) at 42°C	-
Hydrolyse of caseine	+
Fructose	+
Mannane	+
Maltose	+
Trehalose	+
Manose	+
Melibiose	-
Palmitine	-
Raffinose	+
Xylose	-
Mannitol	+

+, Growth; -, no growth; w, weak growth; He, hetero-fermentation.

Table 5. Effect of enzymes treatment (1 mg/mL) on bacteriocin activity against *S. aureus*. Results are expressed as % of means values of growth reduction (n= 3) ± standard deviations.

Enzymes	Enzyme concentration (1 mg mL^{-1})
Pepsin	38.50 ± 0.5
Trypsin	40.30 ± 0.5
α- Amylase	41.80 ± 0.3
Lipase	40.80 ± 0.5

Figure 1. Reduction of *E. coli* population in paste and sauce tomato treated with natural antimicrobial of LBc03 isolate and stored at 4°C for 03 days.

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