International Journal of Probiotics and Prebiotics Vol. 1, No. 1, pp. 27-32, 2006
ISSN 1555-1431 print,

IN VITRO PRESELECTION CRITERIA FOR PROBIOTIC LACTOBACILLUS PLANTARUM STRAINS OF FERMENTED OLIVES ORIGIN

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[Received December 10, 2006 ; Accepted February 12, 2006]

ABSTRACT: ***The aim of this research was to evaluate some probiotic traits of Lactobacillus plantarum strains previously isolated from fermented olives. For this purpose, 11 strains were tested for their in vitro antibiotics susceptibility, tolerance to bile, resistance to low pH values, acidifying activity, proteolytic activity, haemolytic activity, lactic acid and exopolysaccharide production. Collectively, the strains were susceptible to the most of antibiotics tested, showed the survivability (11 ± 2.2 to 65 ± 1.8%) at high bile salt concentration (2% oxgall) and resistance at pH 2. Most strains have showed fast (1.035 ± 0.29 to 0.912 ± 0.21 mmol/l±sd of lactic acid) or medium (0.556 ± 0.29 to 0.692 ± 0.18 mmol/l±sd) acidification activity with a good proteolytic activity (1.49± 0.25 and 5.25± 0.11 mg l^{1} tyrosine at 72 h). None of the strains produced exopolysaccharides or haemolysin.***

KEY WORDS: Fermented olives, Lactic acid bacteria, *Lactobacillus plantarum,* Probiotics

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INTRODUCTION

Probiotics have been defined as "live microbial food supplements which beneficially affect the host by improving the intestinal microbial balance" (Fuller, 1989). Among these microorganisms, lactic acid bacteria are regarded as a major group of probiotic bacteria (Collins et al., 1998). They are non-pathogenic, technologically suitable for industrial processes, acid fast; bile tolerant, adhere to the gut epithelial tissue and produce antimicrobial substances, including, organic acids, hydrogen peroxide and bacteriocins (biologically active proteins) (Dunne et al., 1999).

However, among the lactic acid bacteria, antibiotic resistance has been reported for strains isolated from animals or human (Salminen et al., 1998). This is due to the use of the antimicrobial agents for therapy and prophylaxis of bacterial infections and in some cases to the abuse of antibiotics in general. Tolerance of lactic acid bacteria to antibiotics is interest due to their possible use to reconstitute the intestinal microflora of patients suffering from antibiotic-associated colitis. On the other hand, the transmission of antibiotic resistance genes to unrelated pathogenic or potentially pathogenic bacteria in the gut is a major health concern, with obvious ramifications for the selection and safety of probiotic strains (Danielsen and Wind, 2003).

Lactobacillus plantarum isolated from fermented olives has been extensively studied with the aim of its use in starter cultures for olive or other vegetable fermentations (Leal-Sánchez et al., 2002 Kacem et al., 2004), but little is known about its antibiotic resistance and its probiotic effects. In recent study, Lavermicocca et al. (2005) have selected one potential probiotic strain of *Lactobacillus* that was used to validate table olives as a carrier for transporting bacterial cells into the human gastrointestinal tract.

In previous study, a total of 11 *L. plantarum* strains have been isolated from the fermented green olives produced in Algeria (Kacem et al. 2004). Of these, *L. plantarum* OL15 and OL9 strains produce bacteriocins towards Gram-positive and negative bacteria (Kacem et al., 2005, 2006). We aimed in this work to evaluate the resistance of these strains to a range of antibiotics, and to screening them for some criteria for selecting probiotic strains.

MATERIALS & METHODS

1. Bacterial strains and media

As mentioned above, strains of *L. plantarum* designated: OL2, OL7, OL9, OL12, OL15, OL16, OL23, OL33, OL36, OL40 and OL53 used in this study were isolated from fermented green olives by Kacem et al. (2004). They were maintained as a frozen stock at - 20°C in distilled water plus 20 % (v/v) glycerol and propagated twice in Man Rogosa Sharpe (MRS) broth (Oxoid Ltd., UK) (de Man et al., 1960) at 30°C before use.

2. Testing for resistance to antibiotics

Bacterial antibiotic resistance was determined on solid MRS medium by the use of 11 different antibiotic discs (bioMérieux, Marcy-l'Etoile, France) (Table 1). The result (average of 3 reading) was expressed as sensitive (S) or resistant (R) thanks to the standard disc diffusion method (National Committee for Clinical Laboratory Standards, 1999). Two strains with known antibiotic resistances (*Staphylococcus aureus* ATCC 25923 and *Enterococcus faecalis* ATCC 29212) were used as the control strains.

3. Bile tolerance

Strains were cultivated in MRS broth enriched with 2% (w/v) of oxgall (dehydrated fresh bile, Becton and Dickinson) at 37 ºC for 24 h. The growth was checked by spreading of 100µl of cultures of appropriate dilutions onto MRS agar (Oxoid). Control cultures were without oxgall and cells counts were compared with those after 24 h. Bacterial growth was expressed in colony forming units per milliliter (cfu/ml) and the survival percentage (% ± sd) of strains to bile was then calculated. Reported data are means for duplicate samples and analyses

4. Lactic acid production

Production of lactic acid was measured by precipitate method according to Pryce (1969) and expressed in mmol/l after three replicates.

5. Acidifying activity

Acidification was measured by the change in pH (ΔpH) during time according to Lombardi et al. (2002) and Ayad et al. (2004) methods. Fifty millilitres of MRS (Oxoid) was inoculated with 2% of culture (in order to standardized the assay, the inoculums were approximately 10^6 cfu/ ml) and incubated at 37°C. The pH was measured at 0, 2, 4 and 6 h using a pH-meter (Micro pH 2002, Crison, Barcelona, Spain). The acidification values were expressed as pH decrease, calculated as the difference between the value immediately after inoculation and values at 0, 2, 4 and 6 h ($\Delta pH=pH_{at\ time}-pH_{zero\ time}$). The cultures were considered as fast, medium or slow acidifying when a ΔpH of 0.4 U (pH units) was achieved after 3, 3-5 and > 5 h, respectively (Ayad et al., 2004).

6. Tolerance to acidic pH values

Strains were grown in MRS broth (Oxoid) at 30°C overnight, then subcultured into fresh MRS broth and incubated for another 24 h. The cultures were centrifuged at 5000 x g for 10 min. at 4°C. The pellets washed in sterile phosphate-buffered saline (PBS) (Oxoid), pH 7 and resuspended in PBS. Each strain was diluted 1/100 in PBS at pH 1, 2 and 3. Incubation times were 2, 4 and 6 h. Bacteria were then transferred to MRS broth (Oxoid) and incubated at 37°C overnight (El-Naggar, 2004). Counts of surviving cells were determined by plating on MRS agar (Oxoid). Bacterial growth was expressed in colony forming units per milliliter (cfu/ml) and the survival percentage (% ± sd) of strains to different pH values was then calculated. The experiment was repeated twice and each reading represents the means of three observations.

7. Proteolytic activity

The proteolytic activity of strains was determined in skimmed-milk medium (reconstituted skimmed milk powder 10% w/v) by the tyrosine method (Hull, 1947), in accordance with International Dairy Federation (IDF) standard 149A (1997). Milk was inoculated at 0.2% with each strain precultured in MRS broth (Oxoid) at 37°C for 18 h, to obtain approximately 10^6 cfu / ml and then incubated at 30°C for 24 h. The IDF method is based on the reaction of the amino acids tyrosine and tryptophan realised from the milk substrate at 72 h with a phenol reagent, yielding a blue colour that is measured at 650 nm. The results were calculated from a calibration curve obtained from dilutions of tyrosine in distilled water and expressed as mg tyrosine l^{-1} of milk.

8. Exopolysaccharide production

Exopolysaccharide production was evaluated as reported by Mora et al. (2002). Overnight cultures were streaked on the surface of plates containing ruthenium red milk (10% w/v, skim milk powder, 1% w/v, sucrose and 0.08 g/l ruthenium red, 1.5% w/v agar) (Oxoid). After incubation at 37°C for 24 h, non-ropy strains gave red colonies due to the staining of the bacterial cell wall, while ropy strains appeared as white colonies.

9. Haemolytic activity

Blood haemolysis was evaluated on Columbia agar plates (Oxoid) supplemented with 5% sheep blood which were incubated at 37°C for 24 h (Lombardi et al., 2004)

RESULTS

Table 1 shows the results obtained for antibiotic susceptibility of the 11 strains tested. All strains were susceptible to penicillin G, ampicillin, vancomycin, cloramphenicol, clindamycin, rifampicin and ciprofloxacin. Three strains (OL16, OL23 and OL53) were totally susceptible to all antibiotics tested. Most strains showed resistance to 4 of the 11 antibiotics tested, *i.e.* to cefoxitin (2 strains: OL12 and OL40), oxacillin (3 strains: OL12, OL40 and OL15), tetracycline (4 strains: OL2, OL7, OL9 and OL15) or kanamycin (8 strains; OL2, OL7, OL9, OL12, OL15 OL33, OL36 and OL40). Three strains (OL12, OL15 and OL40) have showed a multiple resistance to 3 different antibiotics (both *L. plantarum* OL12 and OL40) resist to cefoxitin, oxacillin and kanamycin (Table 1).

Table 1. Antibiotic susceptibility of isolates of lactic acid bacteria

Antibiotic	OL2	OL7	OL9	OL12	OL15	OL16	OL23	OL33	OL36	OL40	OL53
Penicillin G (10µg)	S	S	S	S	S	S	S	S	S	S	S
Ampicillin (10 µg)	S	S	S	S	S	S	S	S	S	S	S
Cefoxitin (30 µg)	S	S	S	**R**	S	S	S	S	S	**R**	S
Oxacillin (1 µg)	S	S	S	**R**	**R**	S	S	S	S	**R**	S
Vancomycin (30 µg)	S	S	S	S	S	S	S	S	S	S	S
Cloramphenicol (30 µg)	S	S	S	S	S	S	S	S	S	S	S
Clindamycin (2 µg)	S	S	S	S	S	S	S	S	S	S	S
Rifampicin (5 µg)	S	S	S	S	S	S	S	S	S	S	S
Tetracycline (30 µg)	**R**	**R**	**R**	S	**R**	S	S	S	S	S	S
Kanamycin (30 µg)	**R**	**R**	**R**	**R**	**R**	S	S	**R**	**R**	**R**	S
Ciprofloxacin (5 µg)	S	S	S	S	S	S	S	S	S	S	S

(R): Resistance (S): Sensitive

Table 2. Proteolytic activity, lactic acid production and bile tolerance of the 11 *L. plantarum* strains studied.

Strains	Proteolytic activity (mg l^{-1} ±sd tyrosine/72 h)	Lactic acid production (mmol/l ± sd)	Survival percentage (% ± sd) of strains to bile (2% of oxgall)
L. plantarum OL2	4.20± 0.22	0.997 ± 0.21 (F)*	22 ± 3.4
L. plantarum OL7	3.56± 0.35	0.692 ± 0.18 (M)*	43 ± 2.1
L. plantarum OL9	3.13± 0.41	1.035 ± 0.29 (F)	11 ± 2.2
L. plantarum OL12	5.25± 0.11	0.999 ± 0.11 (F)	35 ± 1.9
L. plantarum OL15	4.49± 0.18	0.998 ± 0.32 (F)	59± 2.1
L. plantarum OL16	3.44± 0.15	0.589 ± 0.29 (M)	65 ± 1.8
L. plantarum OL23	1.49± 0.25	0.456 ± 0.23 (S)*	23 ± 2.1
L. plantarum OL33	3.28± 0.15	0.912 ± 0.21 (F)	41 ± 0.9
L. plantarum OL36	2.12± 0.33	0.399 ± 0.29 (S)	18 ± 2
L. plantarum OL40	2.55. ± 0.22	0.556 ± 0.29 (M)	40 ± 0.8
L. plantarum OL53	4.56± 0.14	0.923 ± 0.29 (F)	22± 1.7

***** The cultures were considered as fast, medium or slow acidifying when a ΔpH of 0.4 U was achieved after 3, 3-5 and > 5 h, respectively (Ayad et al., 2004); sd = standard deviation

Table 3. Survival percentage of *L. plantarum* strains at pH values.

	Survival percentage (% ± sd) after incubation						
at:	pH 1.0	pH 2.0			pH 3.0		
For:	2 h	2 h	4 h	6 h	2 h	4 h	6 h
Strain							
L. plantarum OL2	0±0.0	40±2.0	30±2.1	15±0.1	62±2.3	52±0.3	47±3.3
L. plantarum OL7	0±0.0	44±3.1	39±0.1	20±2.1	65±0.2	61±3.2	60±0.2
L. plantarum OL9	0±0.0	49±0.5	40±0.2	19±2.2	76±0.3	72±3.3	65±2.3
L. plantarum OL12	0±0.0	55±2.7	44±0.7	26±1.7	84±0.4	74±1.4	73±1.2
L. plantarum OL15	0±0.0	65±0.3	53±2.3	28±1.4	73±2.1	70±3.1	52±2.1
L. plantarum OL16	0±0.0	54±3.0	24±2.3	11±3.3	64±1.3	56±3.3	51±0.3
L. plantarum OL23	0±0.0	33±2.0	18±2.6	12±2.6	58±2.3	50±0.3	42±1.4
L. plantarum OL33	0±0.0	57±0.6	36±1.6	21±1.0	76±0.6	68±0.4	56±2.4
L. plantarum OL36	0±0.0	52±3.1	24±2.1	11±2.1	71±1.1	55±1.7	51±1.1
L. plantarum OL40	0±0.0	52±0.3	44±0.6	24±0.4	68±0.3	58±2.3	44±1.3
L. plantarum OL53	0±0.0	43±1.2	29±0.2	16±2.2	65±0.4	53±0.1	47±2.1

sd :standard deviation

Among the 11 strains tested for lactic acid production, 6 (*L. plantarum* OL2, OL9, OL12, OL15, OL33 and OL53) were fast acidifying isolates and produced between 1.035 ± 0.29 and 0.912 ± 0.21 mmol/l ± sd of lactic acid, 3 (*L. plantarum* OL7, OL16 and OL40) showed a medium acidification activity (with 0.692 ± 0.18, 0.589 ± 0.29 and 0.556 ± 0.29 mmol/l ± sd of lactic acid respectively) and only 2 strains (*L. plantarum* OL23 and OL36) showed a slow acidification activity (0.456 ± 0.23 and 0.399 ± 0.29 mmol/l ± sd) (Table 2).

As shown in Table 2, the values of proteolytic activity of the 11 strains studied ranged between 1.49± 0.25 and 5.25± 0.11 mg l^{-1} tyrosine at 72 h. *L. plantarum* OL12 strain showed the highest activity (5.25± 0.11 mg l^{-1} tyrosine) while, *L. plantarum* OL23 strain showed the lowest (1.49± 0.25 mg l^{-1} tyrosine). However, for the other lactobacilli, tyrosine content varied from 2.12± 0.33 to 4.56± 0.14 mg l^{-1} tyrosine. *L. plantarum* OL9 and OL15 strains showed good proteolysis (3.13± 0.41 and 4.49± 0.18, respectively) in comparison with the other lactobacilli (Table 2).

Tolerance to bile salts is considered to be a prerequisite for colonization and metabolic activity of bacteria in the small intestine of the host (Havenaar et al., 1992). Therefore, when evaluating the potential of using lactic acid bacteria as effective probiotics it is generally considered necessary to evaluate their ability to resist the effects of bile acids (Lee and Salminen, 1995). In this study, bile tolerance of the 11 strains of *L. plantarum* was investigated (Table 2). Strains demonstrated variable susceptibility to 2% oxgall concentration. *L. plantarum* OL9 and *L. plantarum* OL36 were the more sensitive strains with survival percentage of 11 ± 2.2 and 18 ± 2.1%, respectively. Respectively *L. plantarum* OL16 and OL15 strains showed the highest tolerance (65 ± 1.8 and 59±2.1 %). The other strains have showed variable survival percentage ranged between 22± 1.7 and 43 ± 2.1%.

Before reaching the intestinal tract, probiotic bacteria must first survive transit through the stomach where the pH can be as low as 1.5 to 2 (Dunne et al., 2001). Table 3 shows the results of acid tolerance (survival percentage of *L. plantarum* strains at various pH values). All tested strains survived an incubation periods of 2h to 6h at pH 2.0 and pH 3.0 with decrease in survival percentage when the exposure time progresses for strains. Generally, *L. plantarum* OL12, OL15, OL16 and OL33 strains survived acidic conditions better than the rest of strains. At pH 2.0, *L. plantarum* OL15 strain showed the highest survival percentage (65±0.3%, 53±2% and 28±1.4%) after 2, 4 and 6 h incubation period, respectively. No growth occurred after incubation at pH 1 for 2 h.

Finally, none of the strains produced exopolysaccharides or haemolysin on sheep blood.

DISCUSSION

Results indicated that *L. plantarum* strains (originate of fermented olives) were susceptible to the most of antibiotics tested and low multiple resistances were observed. This is not in accordance with various reports indicating that lactic acid bacteria are normally resistant to the principal antibiotics, such as penicillin G, ampicillin, vancomycin, cloramphenicol or ciprofloxacin (Halami et al., 2000 and Coppola et al., 2005). In similar study conducted by Herreros *et al.* (2005) showed that most of the tested *L. plantarum* strains from different sources were resistant to antibiotics used. In addition, our results showed that 4 strains were tetracycline resistant which is in accordance with other reported studies (Halami et al., 2000 and Coppola et al., 2005). It is well known that vancomycin is an antibiotic belongs to glycopeptide antibiotics inhibits the peptidoglycan synthesis which is an important structural component of the bacterial cell wall. Therefore, Gram-positive bacteria, including lactic acid bacteria are especially vulnerable to vancomycin treatment (Reynolds, 1989). In our case, all strains tested were sensitive to vancomycin. This result not confirms the finding of Salminen et al. (1998) who reported that vancomycin resistance is an intrinsic property of lactobacilli.

Most strains showed a fast or medium acidification activity. These results are not in accordance with those reported by Ayad et al. (2004) who indicated that most strains of *L. plantarum* isolated from different sources have showed a slow acidification rate.

Also, strains showed good proteolysis. Similar results were obtained by other authors (Usman and Hosono, 1999 and El-Naggar, 2004).

The 2% oxgall (bile salt) used for testing our strains represents the extreme concentration obtained in animal or human intestine during the first hour of digestion (Gotcheva et al., 2002). Afterwards the normal level of bile salt in intestine is around 0.3%. It is also mentioned that the resistance to bile salts varies a lot among the lactic acid bacteria species and even between strains themselves (Xanthopoulos, 1997). Bile resistance of some strains is related to specific enzyme activity-bile salt hydrolase (BSH) which helps hydrolyse conjugated bile, thus reducing its toxic effect (Du Toit et al., 1998). Failure to do so may explain the sensitivity of some *L. plantarum* strains tested in this study. BSH activity has most often been found in organisms isolated from the intestines or faeces of animals (Bateup et al., 1995; Tanaka et al., 1999). This is not in accordance with our results, since the strains tested here were isolated from fermented olives.

All strains survived an incubation periods of 2 h to 6 h at pH 2.0 and pH 3.0. Similar were reported by Dunne et al. (2001) and El-Naggar (2004).

CONCLUSION

Results obtained in the present study showed the survivability of *L. plantarum* strains tested in the conditions of high bile salt concentration and low pH values. This will help strains to reach the small intestine and colon and contributing the balance of the intestinal microflora. Most strains were fast lactic acid producing, have a good proteolytic activity and with no haemolytic activity. In addition, most strains were susceptible to antibiotics tested, which belonged to the major classes of antibiotics used in human clinical therapy. The absence of antibiotic resistance can be considered a positive trait for bacteria used in probiotic food productions.

Among strains, *L. plantarum* OL15 and OL9 also produced bacteriocins with the inhibitory activity against Gram-positive and negative bacteria (Kacem et al., 2005, 2006). This result suggests that these two strains are favourable for use as probiotics. Additional experiments concerning the adhesive capability and the role of *L. plantarum* OL15 and OL9 in the intestinal health as are in progress.

ACKNOWLEDGEMENTS

This study was supported by a grant of the "Ministère de l'Enseignement Supérieur" of Algeria (Project: F3101/04/97) and a grant of ANDRS (Project: 02/12/01/99038).

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