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Screenings of Lactic Acid Bacteria Isolated from Dried Fruits and Study of Their Antibacterial Activity

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Abstract: The objective of this study was enumeration, identification of lactic acid bacteria LAB from dried fruits and testing their antibacterial activity against different types of bacteria. Dilution method and cultivation in selective media was used for enumeration LAB, the isolates were identified by their physiological and biochemical characteristics and their antibacterial activity was performed by the agar well diffusion method. The results showed that thirty-seven isolates of LAB were isolated from tested samples, The isolates belonged to *Lactococcus lactis* subsp *lactis* (8), *Lactococcus raffinolactis* (6), *Streptococcus thermophilus* (6), *Pediococcus acidilactici* (3), *Lactobacillus delbrueckii subsp. bulgaricus* (2), *Lactobacillus helveticus* (3), *Lactobacillus plantarum*(4), *Lactobacillus alimentarius* (1), *Lactobacillus brevis* (2) and *Lactobacillus fermentum*(2).The results of antibacterial activity showed that seven CFSs of LAB had antibacterial activity against at least four strains tested. *Lactobacillus fermentum* had the best activity, they inhibited eight strains from sixteen tested strains, such as *Streptococcus* spp, *Streptococcus sanguins, Staphylococcus epidermis, Staphylococcus aures, Proteus mirabilis, Hafnia alveie* and *Yersinia* spp. In general, CFSs wewre active against the Gram positive more than Gram negative strains. MICs values were between 25 - 100 AU/ml.

Key words: Enumeration · Identification · Antibacterial Activity · Lactic Acid Bacteria · Dried Fruit

INTRODUCTION

Microorganisms play an essential role in the food fermentations, lactic acid bacteria (LAB) has the main role, it have been involved for thousands of years in food fermentations and are one of the most ancient preservation techniques, first signs of LAB utilizations date back to 6000BC, describing the fermentation of milk and fermentation of meat 1500BC and vegetable products 300BC [1].

LAB are found in a large variety of nutrient - rich environments, including milk and dairy products, plants, cereals, meat and meat products [2].

Several studies have isolated LAB from fruit and vegetable [3-8]. Farther the effectiveness fermentation of LAB produces, many compounds products, some of them has an antimicrobial activity, such as hydrogen peroxide, organic acid, diacetyle and bacteriocin [9-12]. The activity of compounds produced by LAB has been reported by many researches against different microorganisms [13-17]. The objective of this study was enumeration, identification of lactic acid bacteria isolated from dried fruits and testing their antibacterial activity against different types of bacteria.

MATERIALS AND METHODS

Sampling: A total of 100 samples of dried fruits (raisin and fig) was collected from regions of Rabat-Casablanca in Morocco.

Enumeration and Identification of LAB: Ten gm of each sample were homogenized with 90ml of peptone water (mother solution), 1ml of mother solution was transferred into 9ml of slain solution (8.5 g NaCl, 1000 ml distilled water, pH 7.0) and serial dilutions up to 10^{-4} were made. Then, 1ml form each dilution was cultivated in the following selective media: M17 (Biokar, France) to count Streptococcus, incubation at 45°C/48h [18], MRS (Biokar, France) to count Lactobacillus and Pediococcus, incubation at 30°C/48h [19] And Elliker (Himedia, India) to count Lactococcus, incubation at 30°C/48h [20].

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Randomly picked colonies were transferred to suitable media and purification of colonies was made by repeated of streaking on suitable media. Purified strains of LAB were stored at -20°C in MRS agar + 15% glycerol. Strains of LAB were identified according to their microscopical, morphological, physiological and biochemical properties [21-23].

Extraction of Cell-free Supernatants (CFS): All of LAB isolates were cultured in MRS broth at 30°C/24h; cells were removed by centrifugation at 10,000 x g for 15 min, pH of supernatants was adjusted to 6.5, supernatants were filtered through 0.22 ìm membranes, 2-3 drop of enzyme catalase were add in cell-free supernatants (CFS) to remove the influence of hydrogen peroxide. CFS was used to as antimicrobial agents using agar well diffusion method, antibacterial activity was evaluated by measuring the zone of inhibition against the test organism [24].

Antibacterial Activity of CFS: The antibacterial activity test was done according to Nongpanga *et al*. [25] method. Sixteen bacterial strains were used as indicators of activity of LAB CFSs, strains were including: *Streptococcus* sp, *Streptococcus sanguins*, *Staphylococcus epidermidis,* Multiresistant *Staphylococcus aureus, Staphylococcus aureus, Pseudomonas aeruginosa, Acinetobacter baumannii, Pseudomonas fluorescens, Salmonella* sp1*, Salmonella* sp2*, Salmonella arizonae, Proteus mirabilis, Hafnia alvei, Yersinia* sp*, Escherichia coli* and *Klebsiella pneumoniae.* These cultures were collected in Laboratory of bacteriology medical at the National Institute of Hygiene, Rabat - Morocco.

Minimum Inhibitory Concentration (MIC): The determination of MIC of CFS against the tested bacterial strains was performed according to the micro titration technique described by Eloff [26] and expressed in terms of arbitrary units per ml (AU/ml) [27].

RESULTS AND DISCUSSION

Little information is available about isolation of lactic acid bacteria from fruits and dried fruits, for this we carried out this study to compare our results with LAB isolated from other sources.

Enumeration and Isolation of LAB Isolates: LAB was detected in low count in the most of analyzed samples. The presumptive Lactobacillus count ranged between $3x10^2$ to $l0^2$ CFU/g and presumptive Streptococcus and

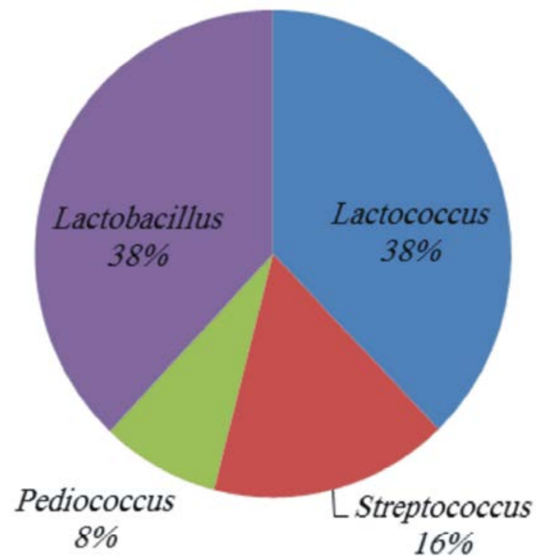

Fig. 1: Identity of 37 isolates of LAB isolated from dried fruits.

Pediococcus count was 10^2cfu/g. The presumptive Lactococcus level varied from $4x10^2$ to $2x10^2$ cfu/g. The results showed the presence of LAB in dried fruits was in low count, similar results were reported by Loveness *et al*. [28]. Several reports indicate the presence of LAB in fruits and vegetables in low count [3, 29, 30].

Distribution of LAB isolates (Figure 1) was showed as *Lactococcus* 14 isolates (38%), *Streptococcus* 6 isolates (16%), *pediococcus* 3 isolates (8%) and *Lactobacillus* 14 isolates (38%).

The isolates identified were divided in 4 groups: Group A was characterized as *Lactococcus* containing 14 isolates. Eight isolates were assigned to *Lactococcus lactis* subsp *lactis* (57%). *Lactococcus lactis* is known as one of the most important species of lactic acid bacteria by their role in many lactic industrial products [8], other studies have reported that this species is the more frequently isolated from many sources [31, 32]. The second species of this genus was *Lactococcus raffinolactis* 6 isolates (43%). This species is formerly known as *Streptococcus raffinolactis* and its lack of caseinolytic activity, for this, it’s not used in dairy industry [33, 34]. The fermentation of raffinose sugar in this species is used for differentiation from other species of *Lactococcus* [24].

Group B was identified as *Streptococcus thermophiles* 6 isolates. The first indication of this species was by oral-Jensen [35], who has described it and their ability to grow in high temperature. Bridge and Sneath [36], Driessen and Bouman [37] and Flint *et al*.

[38], have reported that the best environment for isolation of thermo - resistant streptococci was dairy manufacturing plants.

Group C was considered as *Pediococcus acidilactici* (3 isolates). *Pediococcus acidilactici* has been utilized in different varieties of food industry as meat and vegetables [24]. The bacteriocin (pediocin) produced by this species has been reported by several studies as a good bio preservation agent [39, 40].

Group D was identified as genus *Lactobacillus* 14 isolates, divided in two subgroups according to the type of fermentation. The first sub group was homo fermentative including several species. The first species was *Lactobacillus delbrueckii subsp. Bulgaricus* 2 isolates,. Until 1984 this species was known as *Lactobacillus bulgaricus*, the main use of *Lactobacillus delbrueckii subsp. Bulgaricus* is in yogurt as starter with *Streptococcus thermophiles* [41] and they have many probiotic benefits for human [42]. The second species was *Lactobacillus helveticus* 3 isolates, *Lactobacillus helveticus* have strong proteolytic system, which it's very important to cheese ripping [43, 44], also it is frequently isolated from dairy products as other sources [45]. The third species was *Lactobacillus fermentum* 2 isolates, *Lactobacillus fermentum* has a potential probiotic effect especially with fermented plant [45, 46].

The second subgroup containsed three species; the first species was identified as *Lactobacillus plantarum* 4 isolates, *Lactobacillus plantarum* has been known by their ability to grow and tolerant high acidity, for this, it's used to produce lactic acid and other related compound [47, 48]. Second species was identified as *Lactobacillus alimentarius* with one isolate. The third species was *Lactococcus brevis* 2 isolates, *Lb. brevis* is the most widely LAB species used in many fermentation system, the best environment for isolation these species was fermented olives, this species have probiotic benefits, because of their ability to tolerate low pH, bile acids and have antimicrobial activity against potentially harmful organism [49].

The final distribution of lactic acid bacteria isolated from dried fruits (raisin and fig) is presented in Table 1.

Table 1: Final distribution of lactic acid bacteria

Genus	Species	Number	Rate %
Lactococcus	Lactococcus lactis subsp *lactis*	8	21.6
	Lactococcus *raffinolactis*	6	16.2
Streptococcus	Streptococcus *thermophiles*	6	16.2
Pediococcus	Pediococcus acidilactici	3	8.1
Lactobacillus	Lactobacillus delbrueckii subsp. Bulgaricus	2	5.4
	Lactobacillus helveticus	3	8.1
	Lactobacillus *fermentum*	2	5.4
	Lactobacillus *plantarum*	4	10.8
	Lactobacillus *alimentarius*	1	2.7
	Lactobacillus brevis	2	5.4

Table 2: Antibacterial activity of of the studied lactic acid bacteria.

Bacterial strains tested	CFS of *Lb. lactis1*		CFS of *Lb.lactis2*		CFS of *Lb. P1*		CFS of *Lb.p2*		CFS of *Lb.ped*		CFS of *Lb.b*		CFS of *Lb.f*	
	mm	MIC	mm	MIC	mm	MIC	mm	MIC	mm	MIC	mm	MIC	mm	MIC
SR	12	++	0	-	20	+++	0	-	12	++	11	++	15	+++
SS	10	++	10	++	22	+++	11	++	15	+	0	-	15	++
SE	0	-	8	++	18	++	0	-	0	-	0	-	14	++
MSA	14	++	20	+++	14	++	12	++	0	-	0	-	15	+++
SA	12	+	26	+++	12	++	13	++	12	++	10	++	16	++
PA	0	-	0	-	0	-	0	-	0	-	0	-	0	-
AB	0	-	0	-	0	-	0	-	0	-	0	-	0	-
PF	0	-	0	-	0	-	0	-	0	-	0	-	0	-
SL1	0	-	18	++	0	-	12	++	11	+	10	++	0	-
SL2	0	-	0	-	0	-	0	-	0	-	15	++	0	-
SLA	0	-	0	-	0	-	9	+	8	+	7	+	0	-
PM	20	++	0	-	20	++	0	-	24	++	0	-	12	++
HA	0		0	-	0	-	0	-	0		0		10	++
Y	0		12	++	0	-	15	++	18	++	0		15	++
EC	0	-	0	-	0	-	0	-	0	-	0	-	0	-
KP	0	-	0	-	0	-	0	-	0	-	0	-	0	-

SR: *Streptococcus* sp, SS: *Streptococcus sanguins,* SE: *Staphylococcus epidermis,* MSA, multiresistant *Staphylococcus aureus,* SA : *Staphylococcus aureus,* PA : *Pseudomonas aeruginosa,* AB: *Acinetobacter baumannii,* PF : *Pseudomonas fluorescens,* SL1: *Salmonella* sp1, SL2: *Salmonella sp2,* SLA: *Salmonella arizons,*PM: *Proteus mirabilis,* HA: *Hafnia alveie,* Y: *Yersinia* spp,EC: *Escherichia coli,* KP: *Klebsiella pneumoniae,. Lb. lactis: Lactococcus lactis subsp lactis, Lb. P : Lactobacillus plantarum, Lb.ped: Pediococcus acidilactici, Lb.b: Lactococcus brevis, Lb.f : Lactobacillus fermentum,*+: 100AU/ml, ++: 50AU/ml and +++: 25AU/ml

Antibacterial activity: LAB have many antimicrobial agents which are active against closely related bacteria, they have been proved active against many other bacteria also including pathogens [50, 51].

All isolates of LAB obtained were screened for their antibacterial activity and only seven isolates were selected for detailed studies, the CFSs produced by These isolates were assayed by agar well diffusion against different Gram positive and Gram negative strains and antibacterial activity was measured in terms of mm. The results (Table 2) indicated that CFS of *Lb. fermentum* seemed to have the best antibacterial activity against eight bacterial indictors, especially against Gram positive cocci, *Staphylococcus and Streptococcus* in addition of *Hafnia* and *Yersinia.* For MIC this species showed an activity between 50-25 AU/ml. The activity of *Lb. fermentum* was reported by several authors as Aly *et al*. [52], Strompfova *et al*. [53], Mohamed *et al*. [54], Nawaz *et al.* [55] and Saba *et al.*[56] and Veeranan *et al.*[57] who reported the type of bacteriocin produce by *Lb. fermentum* is heat stable and stable at pH 2.7 and inactived by proteolytic enzymes. Probiotic effects of *Lb. fermentum* have been reported by Mohamad *et al*. [54] against spoilage of meat of chickens and by Veeranan *et al*. [57] against bovine mastitis.

The two species of *Lactococcus lactis* subsp *lactis* showed antibacterial activity against most of the Gram positive as well as three Gram negative strains and the inhibition diameter of these CFSs was between 8 - 26 mm and MIC was between 25-100 AU/ml The antimicrobial activity of *Lactococcus lactis* subsp *lactis* have been reported by many investigators [58, 59]. Niacin was isolated from this species as antimicrobial agent [60, 61].

Also two species of *Lactobacillus plantarum* showed an effect against all Gram positive and three Gram negative strains, the inhibition zone was between 9-22mm and MIC between 25-100AU/ml. Similar results of antimicrobial activity of these species were recorded by Navarro et al. [61]. Karthikeyan and Santosh [62] have suggested that the bacteriocin of *Lb. plantarum* is plasmid encoded protein molecule, also Holck *et al*. [63] reported that pediocin like bacteriocins isolated form *Lb. plantarum* may be either plasmid encoded or genomically encoded [64].

The results showed poor activity of CFS of *Lactobacillus brevis*, they showed activity against two Gram positive as well as three Gram negative strains and the diameter of inhibition zone was between 7-15 mm. with MIC 50-100AU/ml, opposite results about antibacterial activity of *Lactobacillus brevis* was reported by Ogunbanwo *et al*. [49].

Our results showed that the genus of lactic acid bacteria isolated from dried fruits had activity against Gram positive more than Gram negative strains. Aly *et al*. [52] and Strompfoval *et al*. [53] have reported that the Gram positive bacteria are more sensitive to the bacteriocin produced by the lactic acid bacteria than the Gram negative ones. There was no activity from any CFSs of lactic acid bacteria against *E. coli*, *Klebsiella pneumonae*, *Pseudonones aerugenose, Acinetobacter baunanii* and *Pseudomonas fluorescens*. The sensitivity of Gram negative bacteria to bacteriocins produced by lactic acid bacteria is not common [65, 66] because their outer membrane acts as a permeability barrier for the cell. It is responsible for preventing molecules such as antibiotics [67].

The results of this study indicate the possibility of use of lactic acid bacteria in the conservation of food products.

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