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Activity of isolated allochthonous Lactic Acid Bacteria as Reliable Probiotic Starter in Traditional Fermented Fruit Juice

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ABSTRACT

The trend of the consumer is going towards fresh-like flavor, highly nutritional value, health-promoting and rich flavor read-to-eat or drink, such as ancient fermented fruits and vegetable juices. However, we are unsure traditional fermented microorganisms responsible for fruit or vegetable fermentation as well as reliable nutritional values that can effect on our health and environments. Therefore, in this research, we screened natural lactic acid bacteria from indigenous microbial population of rambutan and tested for antibacterial activity. The screening results showed that lactic acid bacteria (at the concentration of 4-13 log cfu/ mL) have been occurred spontaneously from first week to late three months. Total 300 bacterial isolates were selected from the conventional fermented juices and 5 lactic acid bacterial isolates (LAB) expressed their high acidity production and lowering % reducing sugar as well. Only two LAB isolates from these positive isolates demonstrated high antimicrobial activities to *Escherichia coli* and *Staphylococcus aureus*. These indigenous lactic acid bacteria isolates were later identified for their species to be *Lactobacillus acidophilus* and *L. plantarum* with their DNA homological identity of 99-100%.

Key words: Fruit Juices, fermentation, , Lactic Acid Bacteria (LAB), antibacterial activity

INTRODUCTION

Fermentation is unique in that it modifies the unfermented food in diverse ways, resulting in new sensory properties in the fermented product [1,21,16]. It is a common way of preparing food traditionally as part of the cultural and traditional norm among the indigenous communities. More and more that traditional fermented beverages have been produced at home-level and at a commercial scale. The fermentation are usually taken place by lactic acid bacteria (LAB). They include alcoholic fermentation, lactic acid (non-alcoholic) fermentation, acetic acid fermentation, alkaline fermentation and aminoacid/ peptide sauce fermentations. More and more practice using LAB is because their benificial effects. Using LAB fermentation for detoxification in food is more advantageous [19,8,22,12,25,19] in that it is a milder method which improve the quality of food by increasing the availability of proteins and vitamins, preserves the nutritive value and flavor of decontaminated food [5], help boost the immune system as probiotics and thus strengthen the body in the fight against pathogenic bacterial infections, and have detoxifying effects since they irreversibly degrades mycotoxins without leaving any toxic residues through toxin binding effect [9,13,24] and possibility of an enzymatic interaction [25].

The fermenting organisms include LAB that are a large group of closely related bacteria, such as *Leuconostoc*, *Streptococcus*, *Lactobacillus*, *Enterococcus*, *Aerococcus*, and *Pediococcus* spp. [6,3,14,15,16]. However, most common problem of traditional fermentation are that these indigenous fermentation foods are locally prepared in small scale, in the village homes, and their quality depends on the skills of the household occupants, as inherited over the years [6]. Indigenous natural fermentation are taken place in a mixed colony of microorganisms such as moulds, bacteria, and yeasts [3]. These microbes are usually not harmful to the consumer and have enzymes such as proteases, amylases and lipases that hydrolyze food complexes into simple nontoxic products with desirable textures, aroma that makes them palatable for consumption. The compounds formed during fermentation include organic acids (e.g., palmitic, pyruvic, lactic, acetic,

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propionic and butyric acids), alcohols (mainly ethanol) aldehydes and ketones (acetaldehyde, acetoin, 2-methylbutanol) [7].

According to their different fermentation styles in spontaneous or natural fermentation and certain concern on their insanitary of pathogen contaminated, therefore, this research was focused on the study of the characteristics of isolated LAB cultures residing in traditional fruit fermentation style.

Methodologies:

Bacterial Strain, fermentation and Growth Conditions:

The traditional natural fermentation process was performed by following Thai traditional fermentation procedure (described in fermentation procedure). The strains of *Lactobaciius* isolates were prepared by spreading 100 µl of rambutan fermentation broth onto MRS plate. Six LAB isolates from total 300 colonies exhibited the possible bioactivity of antibacteria, bacteriocin (the procedure later described). The two bacterial species that is food spoilage bacteria and food-born pathogenic bacteria used for antimicrobial activity test were *Staphylococcus aureus* ATCC 25923 and *Eschericia coli* ATCC 25922). The strains of lactobacill were selected as bacteriocin producers were tested their characteristics and bioactive activity by cultivation in liquid or solid MRS with pH 4.5 at 30^{o}C for 48 h. The selective *Staphylococcus aureus* was carried out on Chapman medium at 37^{o}C. The other bacteria, *E. coli* were grown on nutrient agar and broth media and incubated at 37^{o}C for 18 h before spreading onto the agar plate and testing for antimicrobial activity. The isolated strains LAB were analyzed for their genetic identification baed on 16S rDNA [4] to be *Lactobacillus* spp. with their homology identity of 99.5-100%. Their rRNA sequences were amplified, sequenced, and matched with those in GenBank database as well as RDP project.

The growth characteristicof the bacteria was determined by culturing them in their selective media. The enumeration of the cultures of E. coli, LAB isolates, and S. aureus were carried out on the plates of NB, MRS, and Chapman media, respectively. Only plates that contain between 30 and 300 colonies were taken into account. The enumeration in either control or mixed cultures was done by adding of 0.1 ml of serial dilutions in two selective nutrient broth for *E. coli*, acidified MRS media for *Lactobacillus* isolates and Chapman for *S. aureus* [10].

Antimicrobial activity detection by disc diffusion test:

The production of antimicrobial substance by *Lactobacillus* spp. were detected by deferred antagonism as described by Anas et al., [1] and Ruparelia et al. Approximately 10^{7} cfu/mL of each pathogenic strain of *E. coli* and *S. aureus* was prepared by overnight inoculation them into 10 mL of MRS broth and then 100 µL of the bacterial solution (10^{6} CFU/ml) was spread uniformly over the MRS plate. An overnight culture with approximately 10^{6} cfu/ mL of a log-phase culture of *Lactobacillus* spp. isolates were also done and 1 ml of cell free supernatant of the Lactobacillus isolate was immersed with a blank disk (5 mm diameter, 1 mm thickness) with regularly shaking for 15 min. The disk was thus placed onto the surface of the MRS agar. The plates were left at room temperature for 1 h so that the absorbed supernatant and incubated for 24 h at 30^{o}C. The plates were examined for zones of inhibition surrounding the disks, as bioactive producer. LAB strains adding on the disks were recorded as positive clones if the width of the clear zone around the disk of the producer was 2 mm or larger (Tahara and Kanatani, 1996). The measured values of inhibition zone diameters excluded the diameter of the disk. The tests were carried out in triplicate.

Measurement of Acid Production:

A deduction of 10 mL of the culture was transferred in a conical flask of 100 mL and 5 drops of phenolphthalein indicator (2 mg/ mL in ethanol 60^{o}C) were added. The acidity is neutralized by NaOH 1.0 N until the appearance of a persistent pink color, the volume of the titrating solution was measured and to indicate the producing of acidity which was estimated [11,20,3,17].

Fruits and fermentation procedure:

The fruit type used in the study was rambutan, native Southeast Asia fruits, since it has low fiber (0.5%) is rich in sugar (18-20%) especially, fructose and sucrose and has an abundance of vitamin C, and certain amounts of potassium, iron, vitamin A, and calcium, magnesium, sodium zinc, niacin, fiber and protein

The traditional Thai fermentation process for fruit juice in the study was a modified method of supercheng (Thailand cultured style). In a clean container, brown sugar, rambutan fruits (only edible white flesh wrapped around the seed excluded their skin), and 5 liters of clean water without chlorine were mixed in a ratio of 1:3: 5, Firstly, the sugar was completely dissolved in water and subsequently added with the edible parts of rambutan fruits. All components were thus mixed thoroughly, put tightly with the cap, and regularly mixed everyday to keep all parts of the fruits usually sinking in the water.

Results and Discussions

After 40 weeks of natural fermentation of rambutan harboring natural common high sugar producing fruits, the LAB were enumerated from fermentation on MRS plate. The plotted graph of their growth according to the fermentation period indicated that their growth reached a plateau in 4 weeks and followed the common S shape of growth pattern; lag, log, stationary phases. Certain numbers of LAB cells were still maintained (8-9 log cfu/ mL) in the fermentation since it was possible that nutrients available in the fruit juice could be enough for their survival (figure 1). At the log phase in the fermentation process, fast increasing of the growth curve from 2 to 13 log cfu/ mL was developed since they were acquainted with this fermentation conditions. With all good optimum conditions such as sugar contents, temperature, nutrient types were available and reliable for LAB thus generated great higher acidity. LAB could possibly exploited nutrients, especially sugar moieties for their growth and thus produced acid compounds, as seen in higher acidity in the fermentation broth. From this graph, according to the fermentation period, the pH measured decreased whereas the observed LAB growth could adversely be higher detected.

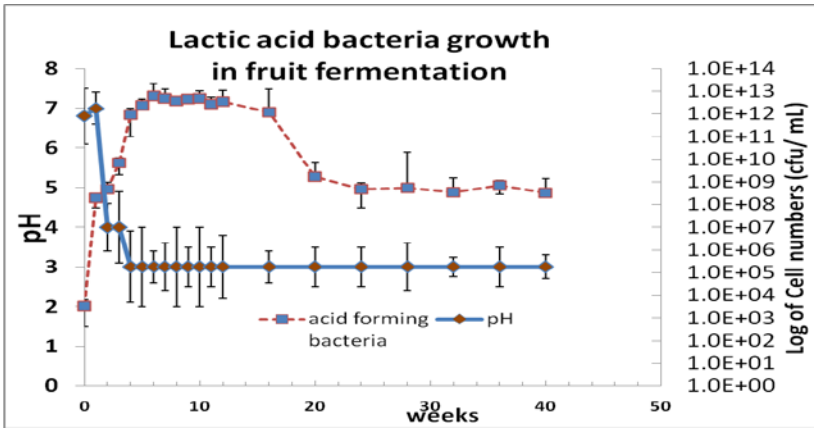

Fig. 1: The spontaneous fermentation of natural LAB in fermentation process. The cell numbers (■ symbol) and pH (◆ symbol) were periodically determined by plate counting and pH meter.

Antibacterial activity of LAB isolates:

In this study, this observation was confirmed by the study of characteristics of LAB strains that were isolated from the fermented broth during week 6. Three humdred of pure LAB isolates were selected for further antibacterial activity on pathogens of both Gram-negative *E.coli* and Gram-positive *S. aureus*. At first, these pure LAB isolates were inoculated into the rambutan juice and they expressed their high acidity or lower pH. The example of the activity of LAB3 (shown in figure 2) indicated high acidity (from 1-4.8 %) and lower pH down (from 7 to 4). These LAB isolates from rambutan could produce acid, same as many other LAB isolated from other sources that can normally generate acids such as lactic acid that are very beneficial for health.

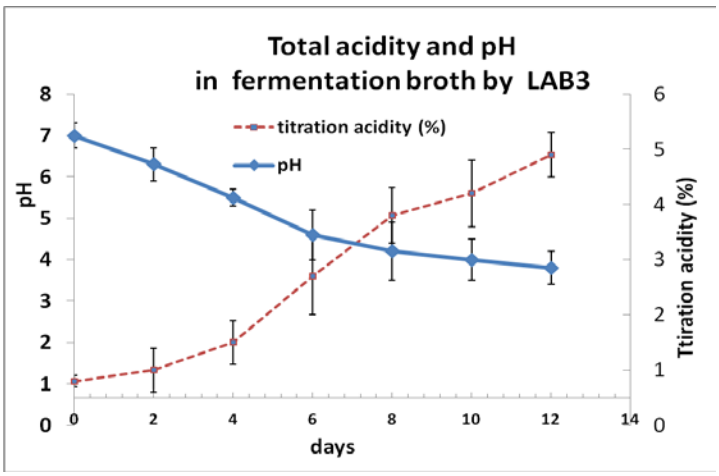

Fig. 2: Example of acidity (■symbol) and pH (◆symbol) evolution in pure culture of LAB3 isolate in the fruit fermentation broth.

From these LAB isolates, only 5 LAB isolates, LAB3, LAB28, LAB56, LAB59, and LAB72 could produce strong antibacterial activity on *E. coli* and *S. aureus* (Table 1). In particular, the highest bioactivity was from LAB56 sample that could inhibit the growth of both *E. coli* and *S. aureus* (as shown in figure 3), whereas the lower acceptable bioactive producer was LAB3 (results not shown). Both of them showed high growth activity in MRS media and a mixture of these LAB3 and LAB56 isolates were good at inhibiting both *E. coil* and *S. aureus* (the inhibition zone with the diameter size of 4 mm for *E. coli* and 3 mm for *S. aureus*). When testing the antibiotic activity in liquid culture, MRS media, a mixture of these 5 LAB isolates also had less and synergistic inhibition effect, oppositely in that they could inhibit *E. coil* less than *S. aureus*. The reduction of inhibiting effect of mixed cultures of LAB were also found by Anas et al., [1].

Table 1: Interactions of 5 selected *Lactobacillus* strains with either *E. coli* or *S. aureus* on selective solid medium. The diameter length (mm) showed the diameter of inhibition zones of the antimicrobial activity that were already excluded the disk diameter.

Code isolates	*E. coli*	*S. aureus*
LAB3	2	5
LAB28	1	3
LAB56	5	3
LAB59	2	2
LAB72	3	2
LAB3+LAB56	7	4

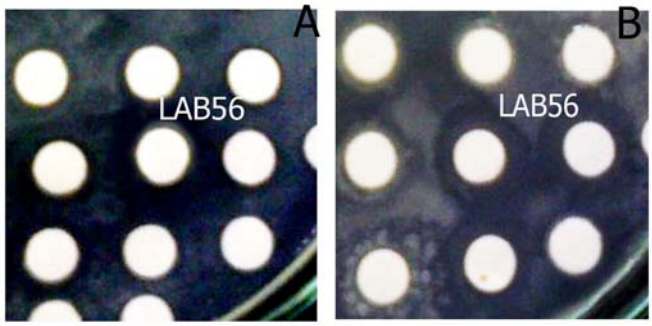

Fig. 3: The inhibition activity of LAB56 cells on 5 mm disks towards the pathogen *E.coli* and *S. aureus* by the appearance of the clear inhibition zone around the lawn of pathogenic colonies.

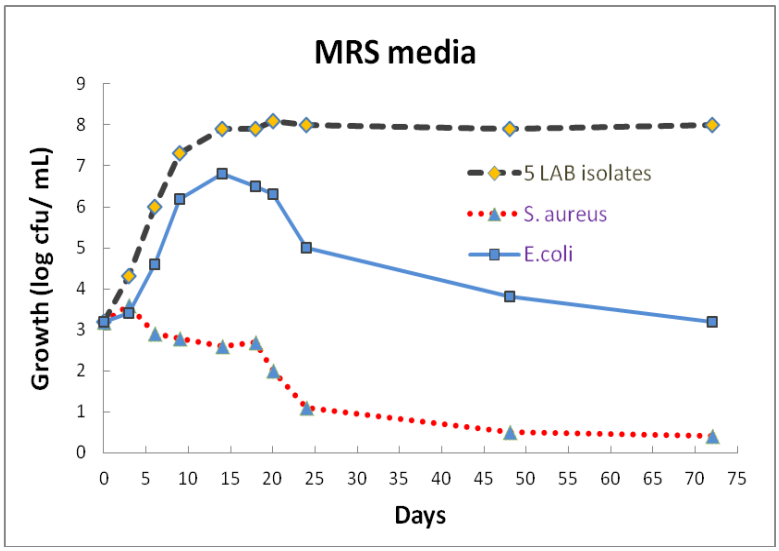

Fig. 4: Kinetics of inhibition growth of food pathogens *E. coli* and *S. aureus*. The initial cell numbers were 3.2 log were belonging to a mixture of 5 LAB cultures (control) alone (◆symbol), a mixture of 5 LAB cultures incubated with either *S. aureus* (Δ symbol) or, *E. coli* (■ symbol) in MRS medium.

Fermentation characteristic of LAB isolates:

Although it was possible that not only LAB that can produce strong acidity, but the other microorganisms such as mold, other bacteria, yeasts could also employ nutrients in the fruit juice as their substrates and thus affected pH decreased. Two LAB isolates were, therefore, tested for their capabilities in adapting themselves under fermentation conditions of rambutan fruits. From the results, in two weeks of fermentation period, the bacteria, LAB3 and LAB56 displayed very similar growth curve patterns of a fast growing line developing up. The cell numbers were beginning at 2.2 log cfu/ mL and thus ending at the cell numbers of 15.6. log cfu/ mL and 16 .2 log cfu/ mL for LAB3 and LAB56, respectively (as shown in figure 5). Both of them consumed sugar moiety in the fruit juice very fast, as shown the sugar content dropped from 40-45% to 5% in 5 days whereas the pH was also lower from pH 7 to pH 5.

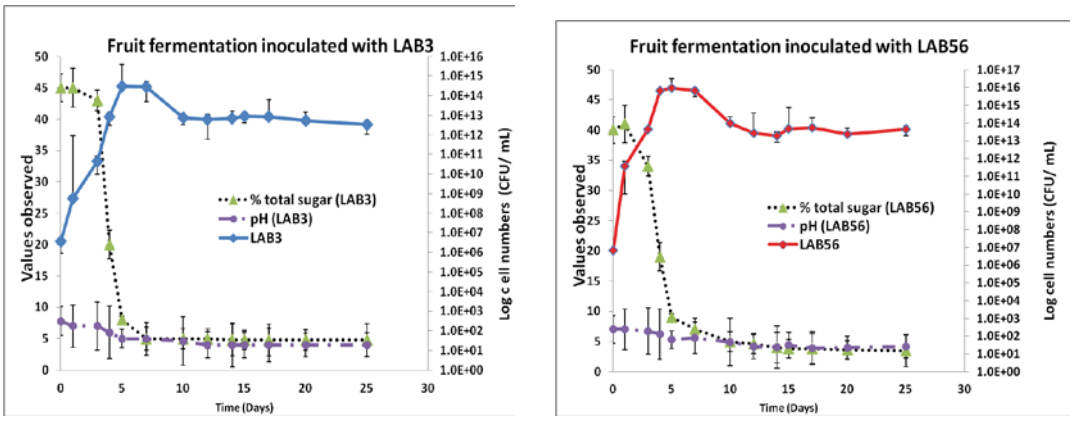

Fig. 5: The growth kinetics of fruit fermentation inoculated by either LAB3 or LAB56 (◆ symbols). Total acidity generated by pH measurement and total sugar consumption were periodically determined during 25 day incubation at 25°C.

Species identification:

The morphological characteristics of LAB3 and LAB56 observed on microscopy were rod-shaped, gram-positive, facultative anaerobic bacteria. The molecular identification for their species was applied by comparison their rDNA sequences with the nucleotide sequences database from GenBank and RDP project. The results showed the nucleotide similarity with 100% homology identity that matched with *Lactobacillus* spp. those are belong to *L. acidophilus* ATCC 4796 and *L. plantarum* KC351899.1 for LAB3 and LAB56, respectively. More biochemical characteristics could be possibly helpful for further distinguishing their different identify from the previous recorded species.

Conclusions:

The advantages of most traditional fruit fermentation not only include preservation and decontamination of toxins but should also integrate with food safety, the nutritional and flavor profile of the products to meet the expectations of all modern consumers. This research based on the understanding of LAB isolates functioning in traditional fruit fermentation process was successfully developed. Moreover, these natural microflora enhancing safety of food together with ease of application and their antibacterial products were established in the study. These convenient starter cultures of LAB3 and LAB56 those were isolated, characterized for their capability can be crucial for further development of routine beneficial fermentation procedures. Especially, it will thus bring up future study on such as nutritional and flavor profile of the fermented products. After that, the fermentation processing method using these natural LAB isolates, another alternative preservative approach, will help ensure that all consumers will obtain the benefits of indulging in fermented food with safe.

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References

1. Anas, M., H.J. Eddine and K. Mebrouk, 2008. Antimicrobial Activity of Lactobacillus Species Isolated from Algerian Raw Goat's Milk Against *Staphylococcus aureus*. World Journal of Dairy and Food Sciences, 3 (2): 39-49.
2. Aderiye, B.I. and S.A. Laleye, 2003. Relevance of fermented food products in southwest Nigeria. Plant Foods for Human Nutrition, 58: 1-16.
3. Antony, U and T.S. Chandra,1997. Microbial population and biochemical changes in fermenting finger millet (*Eleusine coracana*).

World Journal of Microbiology and Biotechnology,13(5): 533-537.
4. Badis, A., D. Guetarni, B. Moussa-Boudjema, D.E. Henni and M. Kihal, 2004. Identification and technological properties of lactic acid bacteria isolated from raw goats milk of furnace Algerianraces. Food Microbiology, 21(5): 579-588.
5. Bata, Á. and R. Lasztity, 1999. Detoxification of mycotoxin-contaminated food and feed by microorganisms. Trends in Food Science & Technology, 10(6-7): 223-228.
6. Blandino, A., M.E. Al-Aseeri, S.S. Pandiella, D.Cantero and C. Webb, 2003. Cereal-based fermented foods and beverages. Food Research International, 36(6): 527-543.
7. Campbell-Platt, G., 1994. Fermented foods- a world perspective. Food Research International, 27: 253- 257.
8. Chelule, P.K., H.P. Mbongwa, S. Carries and N.Gqaleni, 2010. Lactic acid fermentation improves the quality of amahewu, a traditional South African maize-based porridge. Food Chemistry, 122(3): 656-661.
9. El-Nezami, H., N. Polychronaki, S. Salminen and H. Mykkanen, 2002. Binding rather than metabolism may explain the interaction of two food-grade *Lactobacillus* strains with *Zearalenone* and Its derivative {alphaacute}-Zearalenol. Applied and Environmental Microbiology, 68(7): 3545-3549.
10. Kaban, G. and M. Kaya, 2006. Effect of choke culture on growth of *Staphylococcus aureus* in sucuk. Food Control, 17: 797-801.
11. Kihal, M., H. Prevost, M.E. Lhotte, D.Q. Huang and C. Divies, 1996. Instability of plasmid-encoded citrate permease in Leuconostoc. Journal of Applied Microbiology, 22: 219-223.
12. Gourama, H. and L.B. Bullerman, 1995. Antimycotic and Antiaflatoxigenic Effect of Lactic Acid Bacteria: A Review. Journal of Food Protection, 58: 1275-1280.
13. Haskard, C.A, H.S. El-Nezami, P.E. Kankaanpaa, S. Salminen and J.T. Ahokas, 2001. Surface Binding of Aflatoxin B1 by lactic acid bacteria. Appied and Environmental Microbiologyl, 67(7): 3086-3091.
14. Hirayama, K. and J. Rafter, 1999. The role of lactic acid bacteria in colon cancer prevention: mechanistic considerations. Antonie van Leeuwenhoek, 76: 391-394.
15. Holzapfel, W., 2002. Use of starter cultures in fermentation on a household scale. Food Control, 8(5-6): 241-258.
16. Leroy F and L. De Vuyst, 2004. Lactic acid bacteria as functional starter cultures for the food fermentation industry. Trends in Food Science and Technology, 15: 67-78.
17. Martinussen, J., C. Solem, A. K. Holm and P.R. Jensen, 2013. Engineering strategies aimed at control of acidification rate of lactic acid bacteria. Current Opinion in Biotechnology, 24: 124-129.
18. Mokoena, M.P, P.K. Chelule and N. Gqaleni, 2005. Reduction of Fumonisin B1 and Zearalenone by Lactic Acid Bacteria in Fermented Maize Meal. Journal of Food Protection, 68: 2095-2099.
19. Mokoena MP, Chelule PK and Gqaleni N. The toxicity and decreased concentration of aflatoxin B1 in natural lactic acid fermented maize meal. Journal of Applied Microbiology, 100(4): 773-777.
20. Moulay, M., H. Aggad, Z. Benmechernene, B. Guessas, D.E. Henni and M. Kihal, 2006. Proteolytic activity of cultivable lactic acid bacteria isolated from Algerian raw goat's milk. World Journal of Dairy FoodScience, 1: 12-18.
21. Nout, M.J.R. and P.K. Sarkar, 1999. Lactic acid food fermentation in tropical climates. Antonie van Leeuwenhoek, 76(1): 395-401.
22. Schnürer, J. and J. Magnusson, 2005. Antifungal lactic acid bacteria as biopreservatives. Trends in Food Science & Technology, 16(1-3): 70-78.
23. Tahara, T. and K. Kanatani, 1996. Isolation, partial characterization and mode of action of acidocin J1229, a bacteriocin produced by *Lactobacillus acidophilus* JCM 1229. Journal of Applied Bacteriology, 81: 669-677.
24. Turbic, A., J.T. Ahokas and C.A. Haskard, 2002. Selective in vitro binding of dietary mutagens, individually or in combination, by lactic acid bacteria. Food Additives and Contaminants, 19(2): 144-152.
25. Zinedine, A., M. Faid and M. Benlemlih, 2005. *In Vitro* Reduction of Aflatoxin B1 by Strains of Lactic Acid Bacteria Isolated from Moroccan Sourdough Bread. International Journal of Agriculture and Biology, 7(1): 67-70.