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Antifungal Activity of Lactic Acid Bacteria, Isolated from Bulgarian Wheat and Rye Flour

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Abstract: The economic losses and the health hazards of the mycotoxins produced by spoilage fungi are the main concerns of the food industry. The spoilage of bakery products by fungi is more common in countries with a high humidity and temperature. About 5-10% of food production is spoiled by the growth of yeast and fungi in food materials. Similarly, in Western Europe, the growth of the spoilage fungi of bread is estimated to reach more than 200 million Euros per year. The history conditions of the food can be a major factor in determining any fungal spoilage—for example, stored and processed foods are more sensitive to spoilage when compared with fresh and prepared foods. Lactic acid bacteria isolated from Bulgarian wheat and rye flour were used in the present study to check their antifungal properties against pathogenic yeast and fungi imperfecta using standard disc diffusion method in *vitro*. A broad spectrum of antifungal activity of the six newly identified as *L. plantarum* strains e Ts1, Ts2, Ts3,Ts4 and Ts5, and *L. helveticus* Ts6 was estimated. Our in *vitro* studies were performed with wheat and rye sourdough, in order to simulate a real product and to assess the bio-protective potential of the tested lactobacilli. The used test-cultures are representatives of carcinogenic, toxigenic, deteriorative and allergenic fungi from the genera *Aspergillus* and *Penicillium*. The all tested strains completely suppress the growth of against *C. glabrata* 72. Strains *L. plantarum* Ts1 and Ts3 completely suppress the growth against *S. cerevisae*. While, in the sample with *L. plantarum* strains e Ts1, Ts2, Ts3,Ts4 and Ts5, and *L. helveticus* Ts6 , a retarded and weak growth of *A. niger* and *P. claviforme* was observed. However, the spore germination and the colony growth started only on the fifth day of the mould lactobacilli co-cultivation, which also should be considered as a good result. In this study six isolates Ts1,Ts2, Ts3, Ts4, Ts5 and Ts6, from the traditional Bulgarian wheat and rye flour have been identified as *L. plantarum* and *L. helveticus* and characterized as cultures with promising antifungal activity. Obtained results from the combined molecular identification (16S rRNA gene sequencing) approach contribute to give new data on the microbial biodiversity of this not well-studied niche. The antifungal activity of our new isolates, identified as *L. plantarum* and *L. helveticus*, seems to be a promising advantage of these six strains, suggesting their potential applications in different food technologies. However, more experiments have to be conducted to clarify the nature and the mechanisms of the reported antifungal activity and they are still in progress. The combination of dairy origin and strong inhibitory activity of the lactobacillus strains is a prerequisite for their possible application as starters and/or bioprotective antifungal adjuncts.

Key words: Lactic acid bacteria, antifungal activity, Bulgarian wheat and rye flour, pathogenic yeast, fungi imperfect.

1. Introduction

Fungi have a profound biological and economic impact as food spoilage agents, decomposers, plant and animal pathogens [1]. Their ability to grow anywhere, on anything, makes them both beneficial and harmful recyclers of carbon and nitrogen. Fungal spoilage of food and feed is a common and global phenomenon [2]. In addition to the negative financial consequences, fungal spoilage of food and feed also poses a serious health concern. Fungal growth on food stuffs can result in the production of mycotoxins which are known to be toxic to human and animals [3, 4]. The most widespread species of fungi that contaminate bakery products belong to the genera *Aspergillus*, *Penicillium*, *Eurotium* [5-7], *Monilia*, *Mucor*, *Endomyces*, *Cladosporium*, *Fusarium* and *Rhizopus* [8, 9].

LAB (Lactic acid bacteria) has traditionally been used as natural biopreservatives in food and animal

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feed, sauerkraut and silage. Their preserving effect relates mainly to the formation of organic acids and hydrogen peroxide, competition for nutrients and production of antimicrobial substances [10]. Biopreservation refers to extended shelf-life and enhanced safety of foods obtained by the natural or added microflora or their antimicrobial products [11]. The preserving capacity of bacteria naturally occurring in food has gained increasing interest during the recent years, due to consumers demand for reduced use of chemical preservatives. Moulds and yeasts are common spoilage organisms of food products, such as cheese. Benzoic acid and sodium benzonate are primarily used as antifungal agents as well as natamycin produced by *Streptomyces natalensis* [12]. However, moulds and yeasts are becoming resistant to antibiotics but also to sorbic and benzoic acids [13, 14]. LAB may produce compounds with antifungal activity, such as proteinaceous compounds [15], phenyllactic acid， cyclic dipeptides [16] and hydroxylated fatty acids [17]. Bacteriocin-like substances and other low and medium molecular weight mass compounds produced by LAB have been reported as antifungal [18-20]. However, studies on the effect of LAB on fungi are complicated by the sensitivity of most fungi to metabolites, lactic and acetic acids [21].

In this paper, the antifungal activity of LAB isolates from Bulgarian wheat and rye flour were used to check their antibacterial properties against pathogenic yeast and fungi using standard disc diffusion method *In vitro*.

2. Materials and Methods

2.1 Test Microrganisms

Aspergillus niger, *Penicillium claviforme*, *Saccharomyces cerevisae*, *Candida albicans 8673 and Candida glabrata 72* were obtained from the National Bank for Industrial Microorganisms and Cell Cultures, Sofia, Bulgaria. All the isolates were checked for purity and maintained in slants of Nutrient agar.

2.2 Media

They were maintained on PDA (Potato Dextrose Agar, Oxoid, Hampshire, UK) plates at 30 °C and subcultured on a monthly basis until sporulation. The spores were harvested after establishing a good growth rate of each of the fungal cultures and were filtered with sterile cotton filter, to avoid the presence of conidia and mycelia. The spore's suspensions in PDA (pH e 7.0) were adjusted to the final concentrations in the range of 10^5-10^6 spores/mL.

2.3 Lactic Acid Bacteria Isolates

The LAB strains was isolated from Bulgarian wheat and rye flour. Molecular analysis in LAB was performed by molecular identification (16S rRNA gene sequencing) in GeXP Genetic analysis system (Beckman Coulter, USA) [22]. The strains cultivated in media of MRS (de Mann Rogosa Sharpe, Biolife 272-20128, Milano, Italia) in composition, per liter: Tween 80—1; pepton from casein—10.0; meat extract—8.0; yeast extract—4.0; K_2HPO_4—2.0; sodium acetat—5.0; amonium citrate—2.0; $MgSO_4.7H_2O$—0.2 and $MnSO_4$—0.05 g/L. The pH of media was adjusted to 6.5 with 1M NaOH. The basic media was sterilized by autoclaving at 121 °C for 20 min, and carbohydrates (glucose–20g/L) supplemented were sterilized using 0.22 μm filters (Manisart ®).

2.4 Assay for Antifungal Activity

Antifungal assay was performed by the well disc diffusion method using soft 0.8% agar. Agar medium was added to sterile Petri dishes seeded with 100 μL of each test yeast and fungi strains. Wells of equal distance were dug on the seeded plates. Before the assays, the strains LAB were twice pre-cultured in MRS broth, for 24h at 37 °C. Exponential Lactobacillus cultures in MRS broth were used as inoculum for the antifungal tests. The plates were incubated at 37 °C for 48 h. The antifungal activity was assayed by measuring the diameter of the inhibition zone formed around the well [23]. All

experiments were performed in triplicate.

3. Results and Discussion

Following the classical microbial methods were isolated 10 strains from 4 different natural sourdoughs prepared from wheat and rye flour. The data show that 90% of the isolates are of the type *L. plantarum* and 10% are the type *L. helveticus* [22]. In the present study, the effects of LAB on five pathogenic fungi were evaluated.

The effects of LAB on the pathogenics fungi were summarized in Table 1.

Varying degrees of inhibition were detected against the isolates of *Aspergillus niger*, *Penicillium claviforme*, *Saccharomyces cerevisae*, *Candida albicans 8673 and Candida glabrata 72.*

The sensitivities of the test organisms to infusions were indicated by clear zone around the wells (Fig. 1).

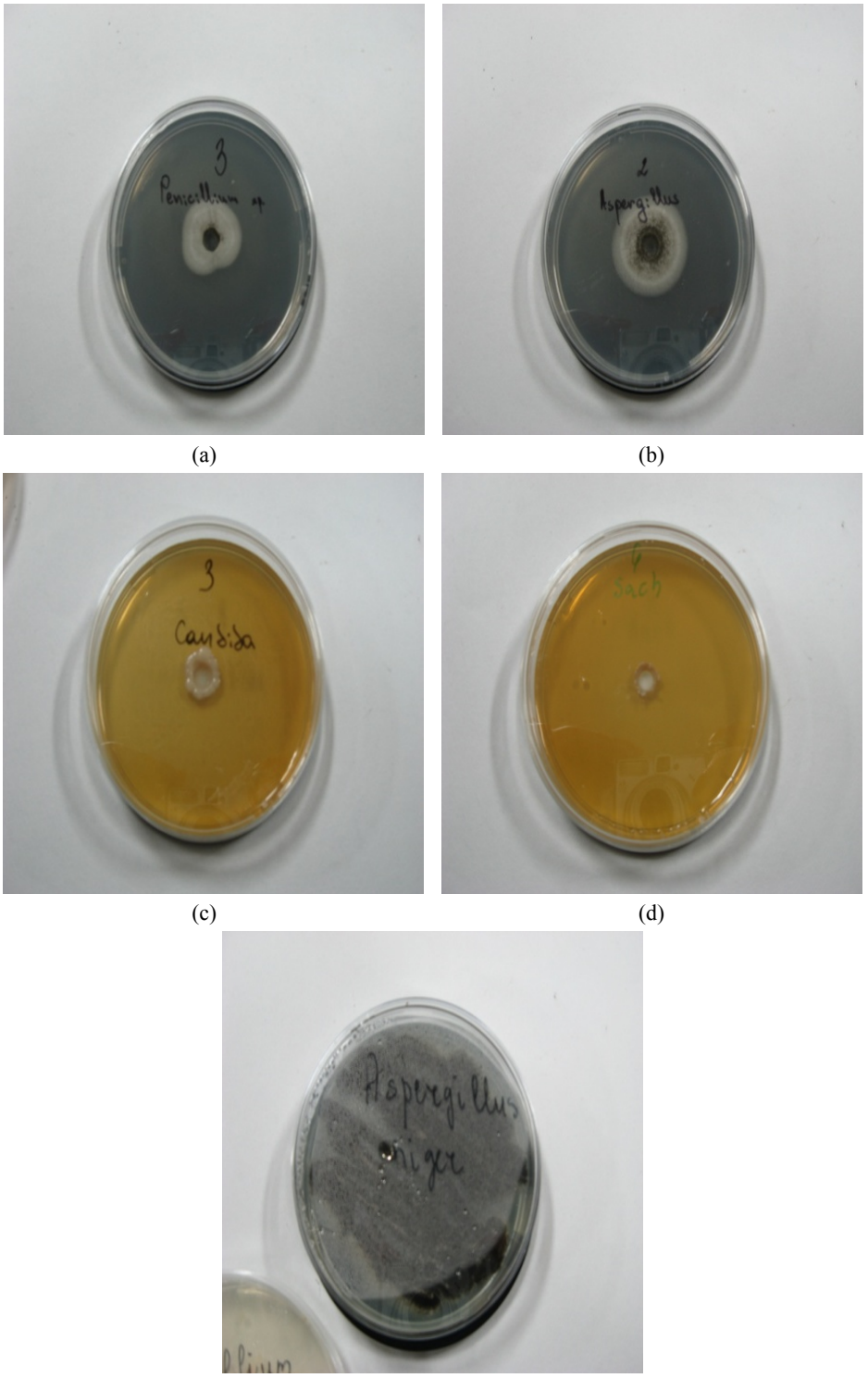
The tested strains completely suppressed the growth of against *C. glabrata 72* (Table 1). Strains of *L. plantarum* Ts1 and Ts3 completely suppressed the growth of *S. cerevisae* (Table 1 and Fig. 1d). While, in the sample with *L. plantarum* strains e Ts1, Ts2, Ts3, Ts4 and Ts5, and *L. helveticus* Ts6, a retarded and weak growth of *A. niger* and *P. claviforme* (Figs. 1a and 1b) was observed. However, the spore germination and the colony growth started only on the fifth day of the mould lactobacilli co-cultivation, which also should be considered as a good result. In the literature, most of the active antifungal strains in fermented milk products were related to the *L. casei* group [24]. The displayed strong ability to inhibit species of genus *Aspergillus* and *Penicillium* (Figs. 1a and 1b) is a good testimonial to the *L. plantarum* and *L. helveticus* isolates from sourdough since these moulds often show resistance [25] and the inhibition of their growth is usually a difficult task. In the experiments, it was found that the presence of six *Lactobacillus* strains, inhibit the *P. claviforme* growth (Fig. 1b).

There are limited published data on the inhibition of *Penicillium* spp. by *L. brevis* [26, 27]. Only a few papers report a broad spectrum of inhibitory effects of LAB against micromycetes [15, 16, 28]. Most of the studies revealed a strain-specific antifungal activity against species of one, maximum two major mould genera [29−31]. There are several reports on antifungal activity of LAB. Okkers et al. [19] found that *L. pentosus* exhibited fungistatic effects against *Candida albicans*. Lavermicocca et al. [32] reported the production of antifungal compounds by a sourdough *L. Plantarum* strain. The same was observed for *Lactobacillus coryneformis* Si3 [28] as well as isolates of *L. plantarum*, *L. coryneformis*, *Lactobacillus salivarius*, *Lactobacillus sake*, *E. hirae*, and *Enterococcus durans* from various sources [15]. Ström et al. [16] also found that *L. plantarum* MiLAB 393 produced antifungal substances. Addis, Fleet, Cox, Kolak, and Leung [33] reported that LAB inhibited the growth at several yeasts with *L. plantarum* being active against *S. cerevisiae* and other yeast species. Durlu-Ozkaya, Karabiзak, Kayali, and Essen [34] found LAB, such as *L. plantarum*, *L. paracasei*, *subsp. paracasei* and *Lactobacillus lactis* isolated from a Turkish traditional cheese to inhibit *S. cerevisiae*, several species of *Candida*, *Torulopsis glabrata* and

Table 1 Inhibition zones (mm), of lactobacilli isolated from Bulgarian wheat and rye flour.

Target strains	Zone of inhibition (mm) by strains LAB					
	L. plantarum TS1	*L. plantarum TS2*	*L. plantarum TS3*	*L. plantarum TS4*	*L. plantarum TS5*	*L. helveticus TS6*
A. niger	35.85 ± 0.01	33.66 ± 0.19	32.92 ± 0.02	32.49 ± 0.02	32.59 ± 0.20	33.28 ± 0.15
P. claviforme	27.34 ± 0.15	26.81 ± 0.17	27.01 ± 0.03	26.35 ± 0.02	25.29 ± 0.03	26.10 ± 0.21
S. cerevisae	-	7.85 ± 0.03	-	8.64 ± 0.15	10.23 ± 0.19	8.99 ± 0.02
C. albicans 8673	13.69 ± 0.03	17.58 ± 0.01	14.34 ± 0.02	12.55 ± 0.17	14.24 ± 0.01	18.27 ± 0.19
C. glabrata 72	-	-	-	-	-	-

Data are presented as average values ± standard deviation in mm.



(a) (b)

(c) (d)

(e)

Fig. 1 Showing Zone of inhibition with LAB along without LAB as control after seven days. a) Strain *L. plantarum* Ts3 cultivated with *P. claviforme*; b) Strain *L. plantarum* Ts2 cultivated with *A. niger*; c) Strain *L. plantarum* Ts3 cultivated with *C. albicans* 8673; d) Strain *L. helveticus* Ts6 cultivated with *S. cerevisae*; e) Control *A. niger* cultivated without LAB.

Rhodotorula rubra, isolates from the same cheese. Several LAB isolates from sourdough bread cultures were screened for antifungal activity against a battery of moulds and the most inhibitory was *L. paracasei subsp, tolerans* [25]. Also, LAB with potential to eliminate fungal spoilage in foods were obtained from malted cereals [20].

4. Conclusions

In this study seven isolates Ts1, Ts2, Ts3, Ts4, Ts5 and Ts6, Ts7 from the traditional Bulgarian wheat and rye flour have been identified as *L. plantarum* and *L. helveticus* and characterized as cultures with promising antifungal activity. Obtained results from the combined molecular identification (16S rRNA gene sequencing) approach contribute to give new data on the microbial biodiversity of this not well-studied niche. The antifungal activity of our new isolates, identified as *L. plantarum* and *L. helveticus*, seems to be a promising advantage of these six strains, suggesting their potential applications in different food technologies. However, more experiments have to be conducted to clarify the nature and the mechanisms of the reported antifungal activity and they are still in progress. The combination of dairy origin and strong inhibitory activity of the lactobacillus strains is a prerequisite for their possible application as starters and/or bioprotective antifungal adjuncts.

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References

[1] Oranusi, S., Braide, W., and Oguoma, O. I. 2013. "Antifungal Properties of Lactic Acid Bacteria (LAB) Isolated from Ricinus Communis, Pentaclethra Macrophylla and Yoghurts." *Global Advanced Research Journal of Food Science and Technology* 2 (1): 1-6.

[2] Pitt, J. J., and Hocking, A. D. 1999. *Fungi and Food Spoilage.* 2nd edn. Gaithersburg, M. D. Aspen publication.

[3] Kabak, B., Dobson, A., and Var, I. 2006. "Strategies to Prevent Mycotoxin Contamination of Food and Animal Feed: a Review." *Crit. Rev. Food Sci. Nutr.* 46: 593-619.

[4] Sweeney, M., and Dobson, A. 1998. "Mycotoxin Production by *Aspergillus*, *Fusarium* and *Penicillium* Species." *Int. J. Food Microbiol*. 43: 141-158.

[5] Abellana, M. L., Torres, V. S., and Ramos, A. J. 1997. "Caracterizatión of Differents Products from Pastries Industrial II." *Estudio de la Microflora. Alimentaria* 287: 51-56.

[6] Batish, V. K., Roy, U., Lal, R., and Grower, S. 1997. "Antifungal Attributes of Lactic Acid Bacteria e a Review." *Critical Rev. Biotechnol.* 17 (3): 209-225.

[7] Guynot, M. E., MarÍn, S., Setu, L., Sanchis, V., and Ramos, A. J. 2005. "Screening for Antifungal Activity of Some Essential oils Against Common Spoilage Fungi of Bakery Products." *Food Science and Technology International* 11: 25-32.

[8] Lavermicocca, P., Valerio, F., and Visconti, A. 2003. "Antifungal Activity of Phenyllactic Acid Against Molds Isolated from Bakery Products." *Applied Environmental Microbiology* 69: 634-640.

[9] Samson, R. A., Seifert, K. A., Kuijpers, A. F. A., Houbraken, J. A. M. P., and Frisvad, J. C. 2004. "Phylogenetic Analysis of *Penicillium* Subgenus *Penicillium* Using Partial B-Tubulin Sequences." *Studies in Mycology* 49: 175-200.

[10] Stiles, M. E. 1996. "Biopreservation by Lactic Acid Bacteria." *Antonie van Leeuwenhoek* 70: 331-345.

[11] Schnürer, J., and Magnusson, J. 2005. "Antifungal Lactic Acid Bacteria as Biopreservatives." *Trends in Food Science and Technology*. 16: 70-78.

[12] Davidson, M. P. 2001. "Chemical Preservatives and Natural Antimicrobial Compounds." In *Food Microbiology: Fundamentals and Frontiers*, edited by Doyle, M. P., Beuchat, L. R., and Montville, T. J. Washington: ASM Press.

[13] Brul, S., and Coote, P. 1999. "Preservative Agents in Food. Mode of Action and Microbial Resistance Mechanisms." *International Journal of Food Microbiology* 50: 1-17.

[14] Viljoen, C. B. 2001. "The Interaction Between Yeasts and Bacteria in Dairy Environments." *International Journal of Food Microbiology* 69: 37-44.

[15] Magnusson, J., and Schnürer, J. 2001. "Lactobacillus Coryniformis subsp. Coryniformis Strain Si3 Produces a Broad-Spectrum Proteinaceous Antifungal Compound." *Appl Environ Microbiol* 67 (1): 1-5.

[16] Ström, K., Sjögren, J., Broberg, A., and Schnürer, J. 2002. "Lactobacillus Plantarum MiLAB 393 Produces the

Antifungal Cyclic Dipeptides Cyclo (L-Phe-L-Pro) and Cyclo (L-Phe-trans-4-OH-L-Pro) and 3-Phenyllactic Acid." *Applied and Environmental Microbiology* 68: 4322-4327.

[17] Sjogren, J., Magnusson, J., Broberg, A., Schnürer, J., and Kenne, L. 2003. "Antifungal 3-Hydroxy Fatty Acids FROm Lactobacillus Plantarum MiLAB 14." *Applied and Environmental Microbiology* 46: 62-67.

[18] Niku-Paavola, M. L., Laitila, A., Matilla-Sandholm, T., and Haikara, A. 1999. "New Types of Antimicrobial Compounds Produced by Lactobacillus Plantarum." *Journal of Applied Microbiology* 86: 29-35.

[19] Okkers, D. J., Dicks, L. M. T., Silvester, M., Joubert, J. J., and Odendaal, H. J. 1999. "Characterization of Pentocin TV35b, a Bacteriocin-Like Peptide Isolated from Lactobacillus Pentosus with a Fungistatic Effect on Candida Albicans." *Journal of Applied Microbiology* 87: 726-734.

[20] Rouse, S., Harnett, D., Vaughan, A., and van Sinderen, D. 2008. "Lactic Acid Bacteria with Potential to Eliminate Fungal Spoilage in Foods." *Journal of Applied Microbiology* 104: 915-923.

[21] Bonestroo, M. H., Dewit, J. C., Kusters, B. J. M., and Rombout, F. M. 1993. "Inhibition of the Growth of Yeasts in Fermented Salads." *International Journal of Food Microbiology* 17: 311-320.

[22] Ignatova-Ivanova, Ts., Ananieva, M., Ivanov, R., Iliev I., and Ivanova, I. 2014. "Biodiversity of Lactic Acid Bacteria in Bulgarian Wheat and Rye Flour." *J. BioSci. Biotech* SE/ONLINE: 101-105.

[23] Muhialdin, B. J., Zaiton, H., and Nazamid, S. 2013. "Lactic Acid Bacteria in Biopreservation and the Enhancement of the Functional Quality of Bread." *INTECH*. Chapter 6.

[24] Delavenne, E., Ismail, R., Pawtowski, A., Mounier, J., Barbier, G., and Le Blay, G. 2012. "Assessment of Lactobacilli Strains as Yogurt Bioprotective Cultures." *Food Control* 30: 206-213.

[25] Hassan, Y. I., and Bullerman, L. B. 2008. "Antifungal Activity of Lactobacillus *Paracasei* spp. Tolerans Isolated from a Sourdough Bread Culture." *International Journal of Food Microbiology* 121: 112-115.

[26] Falguni, P., Shilpa, V. I. J., and Mann, B. 2010. "Production of Proteinaceous Antifungal Substances from Lactobacillus Brevis NCDC 02." *Int. J. Dairy Technol.* 63 (1): 70-76.

[27] De Muynck, C., Leroy, A.I., De Maeseneire, S., Arnaut, F., Soetaert, W., and Vandamme, E. J. 2004. "Potential of Selected Lactic Acid Bacteria to Produce Food Compatible Antifungal Metabolites." *Microbiol Res*. 159 (4): 339-346.

[28] Magnusson, J., Streom, K., Roos, S., Sjeogren, J., and Schnürer, J. 2003. "Broad and Complex Antifungal Activity among Environmental Isolates of Lactic Acid Bacteria." *FEMS Microbiol Lett*." 219 (1): 129-135.

[29] Gerez, C. L., Torino, M. I., and Rollan, G. 2009. "Font de Valdez G. Prevention of Bread Mould Spoilage by Using Lactic Acid Bacteria with Antifungal Properties." *Food Control* 20 (2): 144-148.

[30] Gourama, H. 1997. "Inhibition of growth and mycotoxin production of *Penicillium* by Lactobacillus species." *LWT e Food Sci Technol* 30 (3): 279-283.

[31] Laitila, A., Alakomi, H. L., Raaska, L., Mattila-Sandholm, T., and Haikara, A. 2002. "Antifungal Activities of Two Lactobacillus Plantarum Strains Against *Fusarium* Moulds in vitro and in Malting of Barley." *J. Appl. Microbiol.* 93 (4): 566-576.

[32] Lavermicocca, P., Valerio, F., Evidente, A., Lazzaroni, S., Corsetti, A., and Gobetti, M. 2000. "Purification and Characterization of Novel Antifungal Compounds from the Sourdough Lactobacillus Plantarum Strain 21B." *Applied and Environmental Microbiology* 66: 4084-4090.

[33] Addis, E., Fleet, G. H., Cox, J. M., Kolak, D., and Leung, T. 2001. "The Growth, Properties and Interactions of Yeasts and Bacteria Associated with the Maturation of Camembert and Blue-Vined Cheeses." *International Journal of Food Microbiology* 69: 25-36.

[34] Durlu-Ozkaya, F., Karabiɜak, N., Kayali, R., and Essen, B. 2005. "Inhibition of Yeasts Isolated from Traditional Turkish Cheeses by *Lactobacillus* spp." *International Journal of Dairy Technology* 58: 111-114.