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PROBIOTIC AS ANTIFUNGAL AGENT AND INHIBITOR OF MYCOTOXIN PRODUCTION

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Abstract:

The present study was carried out to probiotic as an antifungal agent and inhibitor of mycotoxin production. Lactic acid bacteria (LAB) used as probiotics isolated from different curd samples using MRS media. These sample were further studied for probiotic characterization such as pH 2, 4, 7 and11, temperature at 37, 40, 60 and 80°C, salt concentration NaCl 1, 2, 3 and 4% and Bile 0.5, 1, 1.5 and 2% were analyzed. Isolates were showed probiotic characteristics and used further for antifungal and antitoxigenic activity against *Aspergillus flavus* and *Aspergillus niger*. *Aspergillus flavus* and *Aspergillus niger* were isolated from these infected groundnuts A total four samples of groundnuts were collected from storehouse of grocery shop using PD media.Probiotics also effectively inhibit the growth of mycotoxin produced by fungi. Agar well diffusion bioassay was performed on PDA plate against the crude and supernatant MRS broth. Probiotics L5 strain was found prominent probiotic strain identify by gene sequencing and showed strong inhibitory activity against *Aspergillus spp*. Using HPLC and TLC mycotoxin identification was done.

Key words: LAB, Probiotics, Mycotoxin, Antifungal agent, *Aspergillus* spp.

Introduction:

Probiotic are live microorganisms which when administered in adequate amounts confer a health benefit on the host‖(2). Yogurt is the most common source of probiotics. Yogurt consists of milk fermented by bacteria that modify lactose into lactic acid (1). Lactic acid bacteria (LAB) are a group of Gram-positive, non-spore forming, cocci or rods, catalase-negative. For an organism to be a probiotic, it must essentially be nonpathogenic, be generally regarded as safe (GRAS), tolerate low pH, tolerate high concentrations of bile salts (3). Lactobacillus plantarum, Lactobacillus pentosus and Pediococcus acidilactici, which possess catalase activity are active in meat products as these substrates contain heamin in abundance The mode of action of the lactoperoxidase system, foundin milk, has specific antimicrobial effects against both bacteria and fungi (Ammor et al.,2007). LAB are normally found in nutrient-rich environments. Even if they grow in a variety of habitats, they are fastidious and amongst others nutrients they require fermentable carbohydrates, amino acids, fatty acids, salts and vitamins for their growth (Björkroth et al., 2003). Aspergillus is a large genus composed of more than 180 accepted anamorphic species , with teleomorphs described in nine different genera. The genus is sub divided in 7 sub genera. As with fungi in general, Aspergillus taxonomy is complex and ever evolving. The genus is easily identified by its characteristic conidiophores, Macro morphological features which are considered include conidial and mycelia color, colony diameter, colony

reverse color, production of exudates and soluble pigments, presence of sclerotic and cleistothecia. Micro morphology characterization is mainly dependent on seriation, shape and size of vesicle, conidia and stipe morphology. Mycotoxins are secondary metabolite of a mold that exert toxic effects on animals and humans, is referred as mycotoxicosis which can cause serious health problems. Mycotoxins are chemically stable and cannot be destroyed during food processing and heat treatment. To date, more than 300 mycotoxin, possessing varying degrees of toxicity, have been identified, although only a relatively few of these are widely accepted as presenting a significant (Jon et al., 2003). These mycotoxin are reported to be carcinogenic, immunotoxic, teratogenic, neurotoxic, nephrotoxic .Fungal growth is one of the causes of spoilage in vegetables and baked foods causing significant reduction in their quality and quantity. In addition, allergenic fungal spores and mycotoxin such as aflatoxins, fumonisins, ochratoxins, patulin, trichothecenes and zearalenone (2). LAB have antifungal properties and may be used to eliminate fungal spoilage and formation of mycotoxin (1). The bactericidal mode of action usually acts on the bacterial cytoplasmic membrane, there is no cross resistance to antibiotics, and their genetic determinants are usually plasmid-encoded, facilitating genetic manipulation. Probiotic bacteria produce various compounds, which are inhibitory to the pathogen's growth, which include bacteriocin , organic acids such as lactic, acetic, propionic and phenyl lactic, have frequently been involved in the antifungal activity of LAB.Bacteriocins are ribosomal-synthesized peptides and biologically active proteins or protein complexes with antimicrobial activity against closely related species which are produced by different groups of bacteria. Four classes of bacteriocins (I, II, III and IV) have been defined based on chemical structure, molecular weight and thermal stability. Recently, new classifications of bacteriocins have been proposed to divide the bacteriocins into two different categories: the lantibiotics containing lanthionine (Class I) and the non-lanthibiotics (Class II). Class III is reclassified as bacteriolysines, and Class IV has to be withdrawn. The bacteriocins have several properties that make them suitable for use in food preservation: Apart from antifungal activity, LAB has also been found to bind and remove aflatoxin both in vitro and in vivo. Studies have reported that certain cell wall components of the LAB are responsible for binding and removal of aflatoxin from solution (4)

This study aims to evaluate the antifungal activity and mycotoxin production inhibiting ability of probiotic organisms belonging to the genus Lactobacillus.

MATERIALS AND METHODS

The research work was conducted in Department of Microbiology at Sanjivani Arts Commerce and Science College, Kopergaon, Maharashtra.

Isolation of Lactobacillus:

The different samples of fresh curd were collected in sterilized screw capped bottles.1 loopful of curd samples were inoculated in 50 ml of MRS broth separately in an aseptic condition and incubate flask at 37◦ c for 24hrs.After sufficient growth was observed in MRS broth were streak on sterile MRS agar plate. Incubation was carried out at 37 ˚C for 24 – 48 hours.

Identification of lactic acid bacteria:

The identification was done mainly on the basis of morphological, biochemical & cultural characteristics. Morphology was examined by Gram staining, motility was observed by hanging drop method. Biochemical characterization was done by sugar fermentation test, Indole, MR-VP & citrate utilization test and catalase test. Cultural characterization based on colony characteristics.

Screening of LAB for probiotic characteristics:

1. Acid tolerance:

From the curd sample isolate 5 LAB were screened for the acid tolerance activity of different pH. MRS broths were prepared such as pH 2, 4, 7, 11.

2. Bile tolerance:

From the curd sample isolate 5 LAB were screened for the bile tolerance activity of different range. MRS broths were prepared with bile salt concentrations such as 0.5%, 1%, 1.5%, 2%.

3 .Salt tolerance:

From the curd sample isolate 5 LAB were screened for the salt tolerance activity of different range. MRS broths were prepared such as 1%, 2%, 3%, and 4%.

4. Temperature tolerance:

From the curd sample isolate 5 LAB were screened for the temperature tolerance activity of different range.MRS broth were prepared and screened for temperature such as 27^0 C, 37^0C, 40^0C, 60^0C, 80^0C.

Isolation and Identification of fungi

A total 4 infected groundnuts samples were collected from storehouse of grocery shop for isolation of fungi. Infected groundnut inoculated in 50 ml PD broth separately in an aseptic condition and incubate flask at 37◦ c for 72 hrs. After sufficient growth was observed in PD broth were streak on sterile PD agar plate. Incubation was carried out at 37 ˚C for 72 hours. Identification was done by morphological characteristics by using Lacto phenol cotton blue stain.

Extraction and Identification of Mycotoxin

Aspergillus differential medium (ADM) Methyl- β- cyclodextrin + bile salts (0.6%) used for production of mycotoxin. Mycotoxin detection by UV light under 365nm and identification was performed by TLC & HPLC.

Antifungal activity of crude & supernatant:

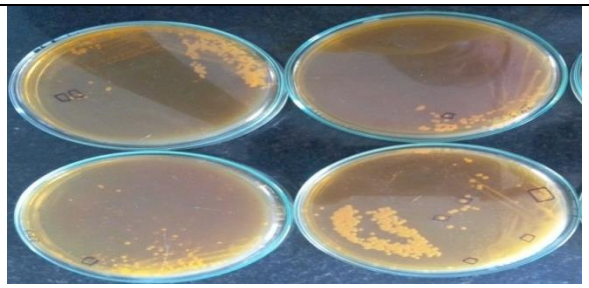
Antifungal activity of crude & supernatant performed by agar diffusion method.

Results and Discussion:

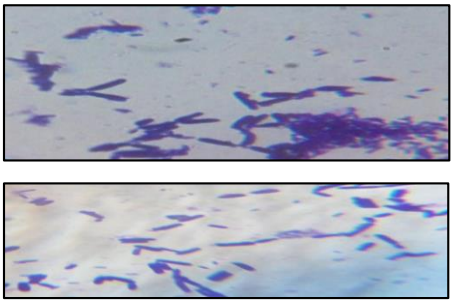
Isolation Identification and characterization of lactic acid bacteria:

The Characterization of LAB isolates was carried out by study of cultural and morphological characters. The morphological characters observed are shown in table. The isolate L1, L2, L3, L4 and L5, produced, white colored, entire, flat, opaque, smooth, colonies. All isolates were detected Gram positive (Photo Plate 1), non- motile and circular shaped bacteria. Spore staining of all LAB was performed by Schaeffer's and Fulton's method and all isolates were found to be non spore former.

Photo Plate 1



Isolation of Lactic Acid Bacteria on MRS Agar Plate



Gram Staining of Isolated Lactic Acid Bacteria

After the cultural and morphological study of LAB, all LAB isolates such as L1, L2, L3 L4 and L5 were examined

for sugar fermentation of Glucose, Mannitol, Maltose and Sucrose. From the results (Table 2), it was observed that all these LAB were ferment sugars with acid production in Glucose, Dextrose, Maltose except Mannitol and Sucrose while no gas formation was observed.

Table 2: Biochemical Characterization of LAB

Sr.no	Isolate code	Biochemical character									
		Sugar fermentation									
		Glucose		Sucrose		Maltose		Mannitol		Dextrose	
		Acid	Gas	Acid	Gas	Acid	Gas	Acid	Gas	Acid	Gas
1	L1	+	-	-	-	+	-	-	-	+	-
2	L2	+	-	-	-	+	-	-	-	+	-
3	L3	+	-	-	-	+	-	-	-	+	-
4	L4	+	-	-	-	+	-	-	-	+	-
5	L5	+	-	-	-	+	-	-	-	+	-

Screening of Lactobacilli for probiotic characteristics:

Isolate code	Growth in NaCl				Growth in Bile salt				Growth at pH				Growth at temperature			
	1%	2%	3%	4%	0.5%	1%	1.5%	2%	2	4	7	11	37°C	40°C	60°C	80°C
L1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
L2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
L3	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
L4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
L5	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-

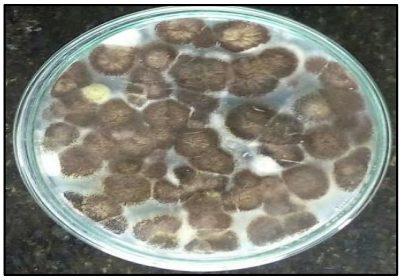
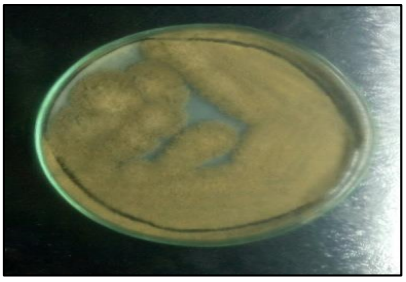
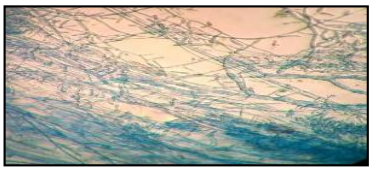
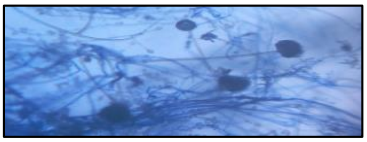
(+ : Growth , - : No growth)

Here all the isolate of Lactic acid bacteria L1, L2, L3, L4 and L5 tolerated the extreme condition of various parameter such as NaCl 4%, bile salt 2%, pH 2 and temperature of 60°C and showed their growth. From the results (Table no 3), it was observed that these entire LAB showed probiotic potential.

Isolation of Fungi:

Aspergillus *Aspergillus* isolated and with the help and *niger* and *flavus* were identified of cultural morphological characteristics. The morphological characters observed are shown in photo plate.

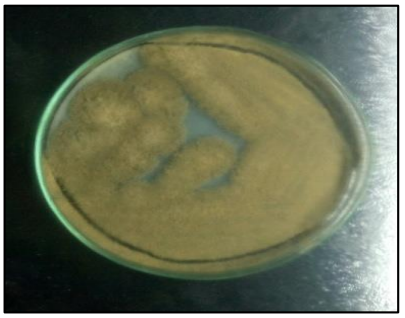
Photo Plate: **Isolation and Identification of fungal spp.**

	
Aspergillus niger	*Aspergillus flavus*
	
Aspergillus niger :Lacto phenol cotton blue stain	*Aspergillus flavus* :Lacto phenol cotton blue stain

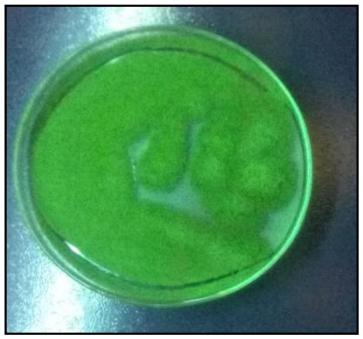
Identification and characterization of Mycotoxin:

The Aspergillus differential medium *Aspergillus spp*. inoculated and incubated for 7 days and observed under the UV light and in that green fluorescence showed presence of mycotoxin.

Photo Plate: Mycotoxin detection by UV light under 365nm



Visible light



UV light

Antifungal activity of probiotics:

The testing of isolates for their ability to produce antifungal compound was conducted of crude by agar well diffusion method. The isolated fungi such as *Aspergillus niger* and *Aspergillus flavus* were used as test organisms in diffusion assay. After incubation the zone of inhibition were obtained against all test organisms. The diameters of zone of inhibitions were measured and recorded which are summarized in (Table 4). Isolate code L1, L2, L3, L4 and L5 found ability to exerted antifungal activity against all pathogenic test organisms.

Table 4: Antifungal activity of probiotics

Sr. no	Isolate code	Fungal pathogen (Zone of inhibition in mm)	
		Aspergillus niger	***Aspergillus flavus***
1	L1	10	20
2	L2	18	14
3	L3	17	17
4	L4	17	14
5	L5	19	24

Crude extract obtained from isolates L1 to L4 found ability to exerted antifungal activity against both pathogenic test fungi. L1 to L4 crude extract inhibited the growth of Aspergillus niger moderately with 10, 18, 17, 17 mm diameter of zone of inhibition respectively and inhibited the growth of Aspergillus flavus with 20, 26, 19, 18mm diameter of zone of inhibition. L5 isolate show more potential that is 19 and 24 of the both fungi respectively.

Antifungal activity of bacteriocin of probiotic:

The testing of isolates for their ability to produce antifungal compound was conducted of supernatant by agar well diffusion method. The isolated fungi such as *Aspergillus niger* and *Aspergillus flavus* were used as test organisms in diffusion assay. After incubation the zone of inhibition were obtained against all test organisms. The diameters of zone of inhibitions were measured and recorded which are summarized in (Table no 5). Isolate code L1, L2, L3, L4 and L5 found ability to exerted antifungal activity against all pathogenic test organisms.

Table 10: Antifungal activity of bacteriocin of probiotic			
Sr. no	**Isolate code**	**Fungal pathogen (Zone of inhibition in mm)**	
		Aspergillus niger	***Aspergillus flavus***
1	L1	20	22
2	L2	24	25
3	L3	17	24
4	L4	18	22
5	L5	26	28

Supernatant obtained from isolates L1 to L4 found ability to exerted antifungal activity against both pathogenic test fungi. L1 to L4 crude extract inhibited the growth of *Aspergillus niger* moderately with 20, 24, 17, 18m diameter of zone of inhibition respectively and *Aspergillus flavus* show 22, 25, 24, 22 mm diameter of zone of inhibition.

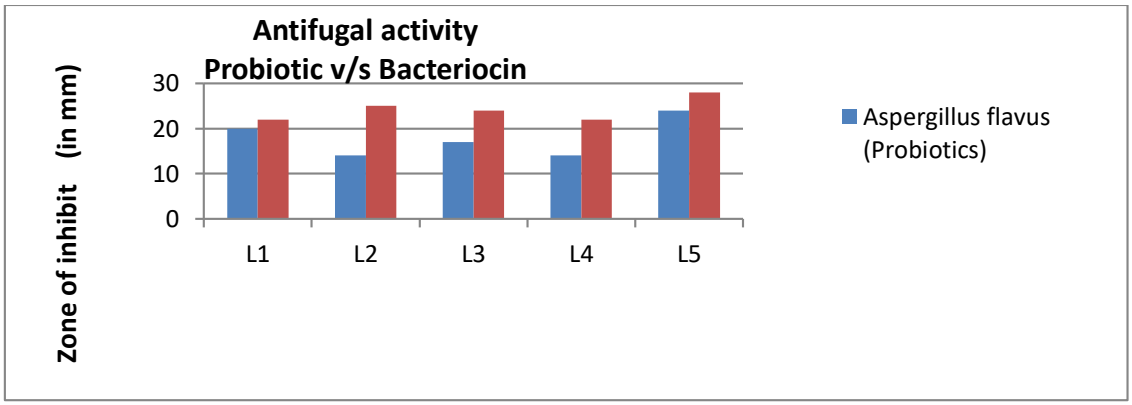
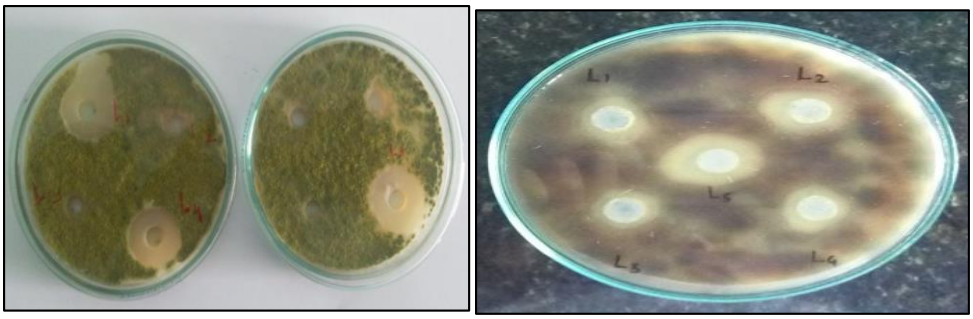


Photo Plate 3: Antifungal activity of probiotics against *Aspergillus* spp.



Confirmation of isolate by sequencing 16S rRNA:

The potential strain of Probiotic L5 was selected for sequencing and identified as Lactobacillus plantarum. Lactobacillus plantarum subsp. plantarum strain LB1-2 chromosome, complete genome Sequence ID: CP025991.1 Length: 3359427 Number of Matches: 1

Conclusion:

This thesis presents studies of antifungal and antitoxigenic properties of LAB. The overall conclusion from this work is that many LAB-species seem able to inhibit fungal growth by many organic acid, bacteriocin, fatty acid, aroma compound, etc. However, it must be considered that the substances produced by LAB probably act synergistically and that other metabolic products, especially bacteriocin, also contribute to the overall inhibition. Lactic acid bacteria are known to inhibit bacterial pathogens which disturb the normal microbial flora of gastrointestinal tract by sequence identified LAB strain Lactobacill

us plantarum. This strains of LAB able to prevent fungal contamination and great inhibitor of mycotoxin is of great interest since they are an inexpensive and safe choice to make food grains safe for human consumption.

Acknowledgement:

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