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Research Article

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Isolation and characterization various microbes and their antimicrobial products, from various samples

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ABSTRACT

Probiotics are living micro organism used as food supplements, which provide health benefit when consumed, by improving intestinal microbial balance of host. Probiotics have high antagonistic activity, antimicrobial activity. The probiotic strain Lactobacillus casei, Lactobacillus rahmnosus, Lactobacillus fermentum and were used as pure culture and standard strains. Probiotics reduce the risk of intestinal disease like diarrhea and typhoid, probably due to their role in suppressing the activity of certain bacterial enzymes with the production of bacteriocins or with the help of lowering the pH by producing certain acids like lactic acid and hydrogen peroxide. Thus probiotic treatment will offer a promising alternative to the use of antibiotics in healthcare.

Keywords: Probiotics, *Escherichia coli*, *Salmonella typhi*, *Lactobacillus casei*, *Lactobacillus rahmnosus*,& *Lactobacillus fermentum*, *Staphyllococcus. aureus*, *Aspergillus. niger*, *Pseudomonas. aeroginosa.*

INTRODUCTION

Probiotics and prebiotic formulations are proving very popular and it has been documented on television,in consumer press and inshelvs of the health food stores. This is because, unlike many nutrition trends, the evidence that they promote good health is strong [1]. Consumption of food containing live bacteria is the oldest and still most widely used way to increase the number of advantageous bacteria called "probiotics" in the intestinal tract[2]. Not only do they help us digest our food, but they may also help reduce the severity of food poisoning and reduce effects of food intolerance. Users report that formulations also help improve general well-being and they may help improve performance in sports due to improved digestion of food and, therefore increased availability of nutrients. Diarrhea occurs in about 20% of patients who receive antibiotics. Antibiotics may directly affect the indigenous gut micro biota by compromising colonization resistance and favoring the growth of pathogenic micro-organisms. Indigenous intestinal bacteria protect the host from infection by exogenous pathogens and opportunistic bacteria that are present in the gut. The mechanism of protection is termed colonization resistance. The quest to find food ingredients with valuable bioactive properties has encouraged interest in lactic acid bacteria (LAB) with probiotic attributes such as antimicrobial activity against pathogenic microorganisms [3], antiviral activity [4], anti-yeast property [5], antimutagenic [6], antiplatelet aggregation [7], and antioxidant attributes etc. The antimicrobial activity of starter cultures anprobiotic bacteria has been attributed to the production of metabolites such as organic acids (lactic and acetic acid),hydrogen peroxide, ethanol, diacetyl, acetaldehyde, other low molecular mass molecular mass compounds and bacteroicins.[8]

EXPERIMENTAL SECTION

Media Required: MRS Agar (Hi Media) at pH 7±2,

Microbial culture: Four probiotic strains; *Lactobacillus casei*, *Lactobacillus rahmnosus*, *Lactobacillus fermentum* were obtained from the National Collection of Dairy cultures, National Dairy Research Institute, Karnal (Haryana). Two strains of pathogens were included in study *E.coli, S. typhi, S.aureus, A.niger, P.aeroginosa.*

PREPARATION OF INOCULUM- Growth of organism appeared on Lactobacillus MRS Agar Medium after 24-48hrs of incubation, and was inoculated with inoculation loop, transferred to 5% peptone water. The inoculum strength was checked at 540nm and set at OD 0.3 to be used as inoculums when OD is 1.0 the dry weight is 0.28 (Rosemarei *et al*, 1989).

ISOLATION OF VARIOUS MICROBES FROM VARIOUS SAMPLES-Various samples were used for the isolation of *lactobacillus* like milk and their various products(curd, cheese, baby milk and whey) by using streak plate method. And their pure cultures were generated.

IDENTIFICATION AND CHARACTERIZATON- The isolated bacteria were identified as *Lactobacillus* spp. by observing their morphological characteristics by means Gram staining, motility test, catalase test, endospore test, IMVIC, starch hydrolysis, fat hydrolysis and salt tolerance.

PREPARATION FOR ANTIMICROBIAL PRODUCTS-

Determination of Bacteroicin production-

All the cultures were inoculated in their respective broths and incubated for 20 days. After incubation, cells were removed from the growth medium by centrifugation (10,000×g for 15 min, 4°C). The resultant supernatants are bacteriocins and can be stored at -20°C for further studies.

ESTIMATION OF BACTERIOCIN PRODUCED USING BRADFORD METHOD

A standard curve was prepared by taking BSA as a standard protein. The standard curve was prepared by taking the following amounts of water, BSA and bradford reagent:

Table 1- Standard curve for protein estimation

Standard(BSA) µg	Standard(BSA) µl	Water(µl)	Bradford reagent (ml)	Optical density
Blank	0	200	3	0
10	20	180	3	0.06
20	40	160	3	0.26
30	60	140	3	0.32
40	80	120	3	0.34
50	100	100	3	0.49
60	120	80	3	0.88
80	160	40	3	0.95

Now after we have prepared a standard curve we check the OD of all the samples by putting them in volume.

3ml bradford reagent + 50µl sample + 150µl water. The O.D of supernatants were observed.

Determination of quantity of Lactic acid-For these measurements Lactobacillus were grown on MRS broth for 72 hrs and samples taken at 12 hr interval. To 25 ml of broth culture of organisms, 3 drops of phenolphathein were added as an indicator. From burette 0.1 N was slowly added to sample until pink color appears. Each ml of 0.1 N NaOH is equivalent to 90.08 mg of Lactic acid.It was also determined by using thin layer chromatography.

Determination of quantity of Hydrogen peroxide- 5 ml of dil. Sulphuric acid was added to 5ml of broth culture of *Lactobacillus*. Titrationwas carried out with 0.1N potassium permagnate. A decolorisation of sample from pink colour was regarded as end point. Each ml of 0.1 N potassium permanganate is equivalent to 1.07 mg.

DETERMINATION OF ANTIMICROBIAL ACTIVITY OF PROBIOTICS – TEST PATHOGENS ORGANISMS BY WELL DIFFUSION METHOD AND OVER LAY METHOD.

Well Diffusion Method- The strains of pathogens were included in study (*E.coli, S. typhi, S.aureus, A.niger, P.aeroginosa.*) selective media were used to test the anti microbial activity against these pathogens. 0.1 ml dilution of each pathogen were tested by pour plate method , four holes was made by using sterile cork borer and then pure cultures were added and results were recorded for 3 days incubation with 24 hrs interval .

Overlay method: The strains of pathogens were included in study (*E.coli, S. typhi, S.aureus, A.niger, P.aeroginosa.*) selective media were used to test the anti microbial activity against these pathogens. 0.1 ml dilution of each pathogen were tested by pour plate method , incubated anaerobically for 4 days for growth , then an another layer of media having either pure culture and isolated culture was over laid over it , results were recorded after 4 days anaerobic incubation.

RESULTS

ISOLATION OF VARIOUS MICROBES FROM VARIOUS SAMPLES- The organisms were isolated from different milk products such as curd, cheese, yoghurt, baby milk,etc: 15 culture were isolated in pure their pure form.

Table 2- Cultures isolated from various samples

S. No	SAMPLE	CULTURE
1	RAW MILK	R,R1, R2, R3, R4
2	CHEESE	CH1, CH2, CH3, CH4
3	CURD	C1, C2
4	WHEY	W1,W2
5	YOGHURT	Y
6	BABY MILK	B

IDENTIFICATION AND CHARACTERIZATON- After staining it was observed that bacteria were rod shaped, gram positive and non motile in nature. Out of 15 positive colonies picked from MRS agar 12 were observed having large colony size and 6 were of small colony size. All colonies were opaque. All cultures are gram positive, endospore negative, catalase negative, MR positive, maximum of the cultures are showing fermentation (shown in table 3). Out of 15 cultures only 2 cultures that were isolated from the curd (C1, C2) were showing starch hydrolysis and culture from whey W2 showed fat hydrolysis. Salt tolerance was maximum in culture isolated from curd C2 i.e. 9%.

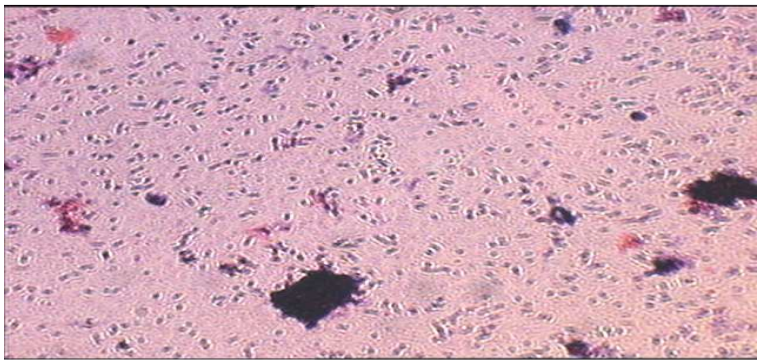


Figure 1 - Gram staining of *Lactobacilli* isolated from milk

Table3- Morphological and biochemical characterization of isolated microbes from various samples

Culture / Characteristics	R	R1	R2	R3	R4	CH 1	CH 2	CH 3	CH4
Gram Staining	+	+	+	+	+	+	+	+	+
Endospore staining	-	-	-	-	-	-	-	-	-
Catalase	-	-	-	-	-	-	-	-	-
MR	+	+	+	+	+	+	+	+	+
VP	-	-	-	-	-	-	-	-	-
Citrate utilization	-	-	-	-	-	-	-	-	-
Indole	-	-	-	-	-	-	-	-	-
Glucose fermentation	+, G	+,G	-	+,G	+	+, G	+	-	+,G
Lactose fermentation	-	+	+	+	+	-	-	-	+
Sucrose fermentation	+	+	+	+	+	-	-	+	+
Mannitol fermentation	+	-	-	-	+	+	+	+	-
Fructose fermentation	-	-	+	+	+	+	+	+	+
Galactose fermentation	+	+	+	+	+	+	+	+	+
Motility	-	-	-	-	-	-	-	-	-
Starch hydrolysis	-	-	-	-	-	-	-	-	-
Fat hydrolysis	-	-	-	-	-	-	-	-	-
Arginine hydrolysis	+	+	+	+	+	+	+	+	+
% NaCl tolerance	1%	8%	8%	5%	4%	3%	5%	8%	8%

Table4)- Morphological and biochemical characterization of isolated microbes from various samples

Culture / Characteristics	C 1	C 2	W 1	W 2	Y	B
Gram Staining	+	+	+	+	+	+
Endospore staining	-	-	-	-	-	-
Catalase	-	-	-	-	-	-
MR	+	+	+	+	+	+
VP	-	-	-	-	-	-
Citrate utilization	-	-	-	-	-	-
Indole	-	-	-	-	-	-
Glucose fermentation	+, G	+	-	+,G	+	+, G
Lactose fermentation	+, G	+	+	-	-	-
Sucrose fermentation	-	+,G	+	+	+	-
Mannitol fermentation	+	-	+	+	-	-
Fructose fermentation	+	+	+	+	+	+
Galactose fermentation	+	+	+	+	+	+
Motility	-	-	-	-	-	-
Starch hydrolysis	+	+	-	-	-	-
Fat hydrolysis	-	-	-	+	-	-
Arginine hydrolysis	_	-	+	+	+	+
% NaCl tolerance	4%	9%	8%	8%	6%	8%

Table 5- Anti microbial substances production

Bacterial cultures	LACTIC ACID PRODUCTION AMOUNT OF NaOH USED (ml)	H_2O_2 PRODUCTION AMOUNT OF $KMnO_4$ USED (ml)	Bacteriocin produced O.D
R	55	*16*	0.35
R1	47.4	22	0.36
R2	60	16	0.36
R3	80	15	0.30
R4	75	11	0.30
CH1	14	14	0.37
CH2	37	16	0.39
CH3	30	21	0.38
CH4	38	16	0.33
C1	57	13	0.38
C2	72.5	12	0.34
W1	42	16	0.31
W2	50	14	0.37
Y	39	22	0.38
B	16	17	0.33

Determination of Antimicrobial products- Results were recorded with an interval of 24 hrs compared with reference to control shown in table given below. Out of 15 cultures culture R3 was observed to show maximum lactic acid production, R1 &Y produced maximum H_2O_2 and CH1 showed maximum bacteroicin production.

DETERMINATION OF ANTIMICROBIAL ACTIVITY OF PROBIOTICS–TEST PATHOGENS ORGANISMS BY WELL DIFFUSION METHOD AND OVER LAY METHOD.

Data presented in table given below involves the use of agar well method with reference to sensitivity of test pathogens in presence of pathogens.Antimicrobial sensitivity observed as zone of inhibition against pathogens. Result of the agar overlay method showed that all of the probiotic strains were showing inhibiton against test microbial isolates .The spectrum of their antimicrobial activity varied.

Table6- Zone of clearance (mm)against various test pathogens

Culture / Test pathogens	R	R1	R2	R3	R4	CH1	CH2	CH3	CH4
E.COLI	1.6	1.8	2.3	2.0	1.6	1.8	2.0	1.8	2.0
ASPERGILLUS NIGER	1.9	1.3	3.0	3.0	1.8	1.8	1.7	2.0	2.5
S.AUREUS	4.2	2.5	2.6	2.2	2.6	2.9	2.2	2.8	1.9
S.TYPHI	2.7	1.3	1.5	2.7	2.7	1.5	1.6	2.4	2.5
P.AEROGINOSA	2.0	1.8	1.5	1.7	1.5	2.2	1.5	1.6	1.7

Table7 - Zone of clearance against various test pathogens

Culture / Test pathogens	**C1**	**C2**	**W1**	**W2**	**Y**	**B**
E.COLI	2.5	1.6	2.0	-	2.5	1.2
ASPERGILLUS NIGER	1.9	1.8	1.8	1.8	1.9	2.3
S.AUREUS	2.9	2.6	1.5	2.1	2.9	2
S.TYPHI	1.5	2.7	1.6	1.6	1.5	2.7
P.AEROGINOSA	2	1.5	1.5	2	2	3

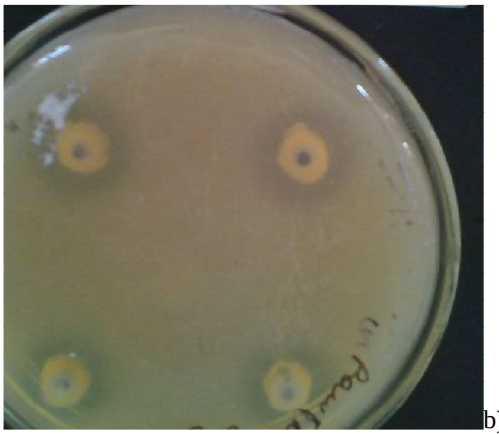
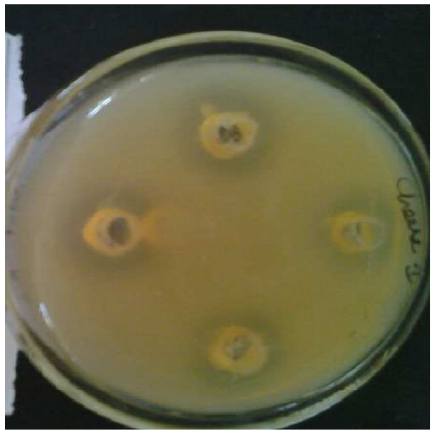
a)  b) 

Figure 2: a) zone of clearance shown by cultures isolated cheese against *Aspergillus niger*and Figure b) zone of clearance shown by cultures isolated raw milk against *Salmonella typhi*

All cultures were showing inhibition against the test pathogens may be due to the antimicrobial productsbut maximum zone of clearance was observed by the culture R1 against *S.aureus*.

CONCLUSION

The concept of probiotics in dietary supplements is unique to have scientifically proven effects on human health promoting potential in conjunction with prebiotics, probiotics and synbiotics has generated global debate by medical experts. The positive effect of the probiotics hasbeen observed against pathogens. Results of study showed that antimicrobial activity of probiotics, isolated from different milk products. This may be due to following reasons.

Competitiveadherence in intestine with pathogens and competed for nutrients, lactic acid, hydrogen peroxideproduction and bacteriocin production that killed pathogens as observed in results.

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