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RESEARCH ARTICLE

PROBIOTIC POTENTIALS OF LACTOBACILLUS AND ITS ANTI CANCER ACTIVITY

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ABSTRACT

Cancer is still a challenging diseases of human community mainly because of the inability of the modern medicine. We can state several reasons for the inability of modern medicine. The most challenging aspects are the development of resistance to the cytotoxic agents, side effects of the drugs used to treat cancer, the ability of cancer cells to behave like stem cells acquiring mesenchymal characteristics during metastasis. So our aim of the research is to formulate a natural drug, which acts as a potential anticancer agent without any side effects. The lactobacilli strains were isolated from the marine environment. The strains were sequenced for 16srRNA and compared with available data in NCBI. Among the strains isolated 7 of them proved to be new. The crude protein was isolated from the bacteria and treated as anticancer drug for *in-vivo* studies in tumour induced hamster models. Promising results were obtained; the drug decreased the tumor volume to a considerable amount and increased the life span of the animal model without any side effects when compared to the control models.

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INTRODUCTION

Cancers figure among the leading causes of morbidity and mortality worldwide, with approximately 14 million new cases and 8.2 million cancer related deaths in 2012.More than 60% of world's total new annual cases occur in Africa, Asia and Central and South America. These regions account for 70% of the world's cancer deaths (WHO cancer report 2012). Among cancer Oral cancer is as an important global health disease totalizing over 275.000 cases and 128.000 deaths per year. (Liu *et al*., 2008) The oral cancer is called as squamous cell carcinoma. Oral SCC arises from within a field of precancerized epithelium either from a pre-existing potentially malignnant lesion, or *de novo*. The use of tobacco and betel liquid, heavy drinking of alcoholic beverages and a diet low in fresh fruits and vegetables are the major risk factors for oral SCC. (Liviu Feller *et al*., 2012) According to International Agency for Research on Cancer of the World Health Organization (IARC–WHO), cancer rates are expected to increase at an alarming rate: from 10 million new cases globally in 2000 to 15 million in 2020. (Mignogna *et al*., 2004) Even though there are several drugs available in the market due to its side effects, in affordability and drug resistance property we are urgently in need of a natural drug.

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To counter the development of the resistance, side effects, metastasis property, mesenchymal characteristics of the cancer cells, there is an urgent need for the development of potential natural drugs which avoid side effects. Nowadays marine microflora is gaining more and more importance due to its unique metabolic pathways.

Marine floras, such as bacteria, Actinobacteria, cyanobacteria, fungi, microalgae, seaweeds, mangroves, and other halophytes are extremely important oceanic resources, constituting over 90% of the oceanic biomass. They are taxonomically diverse, largely productive, biologically active, and chemically unique offering a great scope for discovery of new anticancer drugs. The marine floras are rich in medicinally potent chemicals predominantly belonging to polyphenols and sulphated polysaccharides. The chemicals have displayed an array of pharmacological properties especially antioxidant, immune stimulatory, and anti tumour activities. (De Vries *et al*., 1995)Marine peptides induce cell death via the following pathways, apoptosis, affecting the tubulin-microtubule equilibrium and angiogenesis pathway.

Among marine microbes, Lactobacillus is very well studied microbe due to its probiotic property. Lactobacillus is a gram positive aerotolarent anaerobic microbe. Lactobacillus produce a variety of antibacterial compounds such as organic acids, diacetyl, hydrogen peroxide, reuterin and bacteriocin or bactericidal proteins during lactic fermentations (Holzapfel

et al., 2001; Hirano *et al*., 2003). Most of bacteriocins produced by grampositive bacteria are from lactic acid bacteria (Ennahar *et al*., 2000; Garneau *et al*., 2002). Bacteriocins are proteins or complexed proteins biologically active with antimicrobial and antitumor activity. Bacteriocins are highly specific in their membrane interaction which is related to the unique receptors found in different bacterial species or types. The antineoplasmic activity of bacteriocin is mainly attributed through the induction of programmed cell death or apoptosis. So aim of the research is propose a natural drug with anticancer potentials.

MATERIALS AND METHODS

Animals

Male golden Syrian hamsters, aged 8-10 weeks, weighing 80-120 g, were purchased from the National Institute of Nutrition, Hyderabad, India and were maintained in the Animal House, Srimad Andavan Arts and Science College, Trichy. The animals were housed in polypropylene cages and provided with a standard pellet diet (Agro Corporation Pvt. Ltd., Bangalore, India) and water ad libitum. The animals were maintained under controlled conditions of temperature (27±2^{o}C) and humidity (55±5%) with a 12 h light/dark cycle.

Chemicals

The carcinogen, 7, 2-dimethylbenz(a) anthracene was obtained from Sigma-Aldrich Chemical Pvt. Ltd., Bangalore, India.

Sample collection and pre-treatment

Sediments from the pichavaram mangroves environment and coral reef environment Kurusadai Island of the Gulf of Mannar Biosphere Reserve, south east coast of India, were collected with a corer. The collected samples were transferred to a sterile polythene bag and taken immediately to the laboratory.

Isolation of Lactobacillus

Samples were serially diluted up to 10^{-5} with sterilized 50% seawater and plated with deMan- Rogosa- Sharpe (MRS) medium for lactobacilli. For plating, one milliliter of the serially diluted samples of sediment was pipetted out into sterile Petri-dish. Then sterile media was poured into dishes aseptically and swirled for thorough mixing. After solidification, the plates were incubated in an inverted position at 28±2˚C. All the determinations were carried out in duplicates. After the incubation period, microbial colonies were counted. The counts are expressed as Colony Forming Unit (CFU) per gram of the sediment sample. The isolated colonies were purified using pure culture technique, and culture was maintained in MRS agar slant for further studies. Pure cultures were differentiated and characterized by the following standard morphology physiological and biochemical test (Bergey's manual John Holt, 1994)

Identification of isolates

The isolated lactobacilli were identified using Gram staning, Motility, biochemical test and molecular characterization.

16S rRNA sequencing of bacterial isolate

Consensus sequence of 16S rRNA gene was generated from forward and reverse sequence data using aligner software and sequential using the facility available at Applied Bio-Systems, Bangalore, India.

Purified culture extract of Lactobacillus

Lactobacillus sp was cultured in MRS broth for 24 h at 30°C. The cells were harvested (8000x g, 10 min at 4°C) and the cell-free supernatant was adjusted to pH 5.0 with 1M NaOH, heat-treated (80°C for 10 min) and the bacteriocin was precipitated with 80% saturated ammonium sulphate solution (Sambrook *et al*., 1989). The bacteriocin fraction was removed, dissolved in distilled water and dialyzed against distilled water overnight at 4° C.

Experimental design

The institutional animal ethics committee (Register number 790/03/ac/CPCSEA), Bharathidasan University, Palgalaiperur, India, approved the experimental design. The animals were maintained as per the principles and guidelines of the ethical committee for animal care of Bharathidasan University in accordance with Indian National Law on animal care and use. A total number of 52 hamsters were randomized into nine groups of six hamsters in each.

Group 1 : Normal control
Group 2 : Carcinoma chemical agents (DMBA) control
Group 3 : DMBA + (strain A) crude culture extract of Lactobacillus 250 mg.kg.-1.
Group 4 : DMBA+(strainA)Partial purified bacteriocin of Lactobacillus 250 mg.kg.-1.
Group 5 : DMBA + Standard–5-fluorouracil (5-FU) (20mg/ kg.bw).
Group 6 : DMBA + (strain A) crude culture extract of Lactobacillus 250 mg.kg.-1.
Group 7 : DMBA + (strain B) Partial purified bacteriocin of Lactobacillus 250 mg.kg.-1.

The experiment was terminated at the end of 16th week and all hamsters were sacrificed by cervical dislocation.

Tissue homogenate

The required amount of tumour sample was weighed and homogenized using a Teflon homogenizer. Tissue homogenate (10%) was prepared in 0.1 M Tris Hcl buffer (pH 7.4) and used for the estimation of various biochemical parameters.

Histological studieds

The tumour, liver and kidney tissues were fixed in 10% normal saline for 72 h after which the tissues were sliced to a thickness of 2.1mm each.

These were dehydrated using alcohol of graded concentration. They were further treated with paraffin wax and cast into blocks; sections of the tissues were cut on a microtome to

5µm. These were later attached to a slide and dried. The samples slides were viewed on a photographic microscope to find out histological changes.

Isolation of saliva from lactobacillus

After 14 weeks of experiment just before the sacrifice of the animal, saliva samples were collected fron the right buccal region of the hamster rats using a swab with the help of the Whatman no.1 filter paper (with a diameter of 5 mm) The saliva was diluted and plated on MRS media and indubated for 36 at room temperature at 37 degree centigrade. The colonies formed on the plates were enumerated and its expressed as colony forming units (CFU) per ml

Measurement of Tumor volume

Tumor volume was calculated by the formula (Suresh *et al*., 2010):

$$V = \frac{4}{3} \pi \left(\frac{D_1}{2}\right)\left(\frac{D_2}{2}\right)\left(\frac{D_3}{2}\right)$$

where D_1 , D_2 , and D_3 are the three diameters (mm) of the tumors.

Biochemical analysis

Activity of SGOT (AST)

The serum SGOT was estimated by the method of Reitman and Frankel (1957)

Activity of SGPT (ALT)

The serum SGPT was estimated by the method of Reitman and Frankel (1957)

Estimation of Urea

Urea was estimated by the method of Natelson (1957).

Estimation of Creatinine

Serum creatinine was carried out by alkaline picrate method (Boneses and Taussy, 1954).

Assay of alpha-fetoprotein

The tumor marker alpha-fetoprotein was measured by radioimmunoassay techniques (Eiken Chemical Co., Japan).

RESULTS

Morphology and physiology of *Lactobacillus*

Marine lactobacilli counts in the study areas varied with a range from 3.0×102 to 310 ×102 CFU. g-1 at different sediments in depths. In general the counts were higher at 10-15 cm depths of sediments than other depth (0-5, 6-10). In the present study, the lactobacilli counts were higher at sub-surface than surface sediments (Table 1).

Table 1. Lactobacilli counts in different mangrove biotopes of east coast of India

S. No	Study sites	Lactobacilli (CFU× 10^2 .g-^{1} of sediment)			
		0-5 cm	6-10 cm	11-15 cm	Average
1	Pichavaram-1	4	9	5	5.6^d
2	Pichavaram-2	3	8	6	5.6^d
3	Pichavaram-3	4	7	5	5.3^d
4	Pichavaram-4	5	7	6	6.0^d
5	Pichavara m-5	3	6	4	4.3^d

Lactobacillus sp. TLMP5 16S ribosomal RNA gene, partial sequence 921 bp linear DNA GenBank: Accession: KF406348.1

aagtcgagcg aacagataag gagcttgctc ctctgacgtt agcggcggac gggtgagtaa cacgtggata acctacctat aagactggga taacttcggg aaaccggagc taataccgga taatatattg aaccgcatgg ttcaatagtg aaagacggtt ttgctgtcac ttatagatgg atccgcgccg tattagctag ttggtaaggt aacggcttac caaggcaacg atacgtagcc gacctgagag ggtgatcggc cacactggaa ctgagacacg gtccagactc ctacgggagg cagcagtagg gaatcttccg caatgggcga aagcctgacg gagcaacgcc gcgtgagtga tgaaggtctt cggatcgtaa aactctgtta ttagggaaga acaaatgtgt aagtaactgt gcacgtcttg acggtacctg atcagaaagc cacggctaac tacgtgccag cagccgcggt aatacgtagg tggcaagcgt tatccggaat tattgggcgt aaagcgcgcg taggcggttt tttaagtctg atgtgaaagc ccacggctca accgtggagg gtcattggaa actggaaaac ttgagtgcag aagaggaaag tggaattcca tgtgtagcgg tgaaatgcgc agagatatgg aggaacacca gtggcgaagg cgactttctg gtctgtaact gacgctgatg tgcgaaagcg tggggatcaa acaggattag ataccctggt agtccacgcc gtaaacgatg agtgctaagt gttaggggtt ttccgccct tagtgctgca gctaacgcat taagcactcc gcctggggag tacgaccgca aggttgaaac tcaaaggaat tgacgggacc cgcacaagcg gtggagcatg tggtttaatt cgaagcaacg c

Lactobacillus sp. TLMP3 GenBank: KF406346.1

tgcgtagata tatggaagaa caccagtggc gaaggcggct gtctggtctg caactgacgc tgaggctcga aagcatgggt agcgaacagg attagatacc ctggtagtcc acgccgtaaa cgatgattac taagtgttgg agggtttccg cccttcagtg ctgcagttaa cgcattaagt aatccgcctg gggagtacga ccgcaaggtt gaaactcaaa agaattgacg ggggcccgca caagcggtgg agcatgtggt ttaattcgaa gctacgcgaa gaaccttacc aggtcttgac atcttttgac cgtctaagag attagaggtt cccttcgggg acagaatgac aggtggtgca ggttgtcgt cagctcgtgt cgtgagatgt tgggttaagt cccgcaacga gcgcaaccct tatttctagt tgccagcatt aagttgggca ctttagtgag actgccggtg acaaaccgga ggaaggtggg gaagacgtca aatcatcatg ccccttatga cctgggctac acacgtgcta caatggatgg tacaacgagt cgcgagaccg cgaggttaag ctaatctctt aaaaccattt tcagttcgga atgtaggctg caacttgcct acacgaagtc ggaatcgcta gtaatcgtgg atcagcatgc cgcggtgaat acgttcccgg gccttgtaca caccgcccgt c

The biochemical characteristics used for identification of lactobacilli may suggest some ideas in relation to the occurrence of the strains in nature. About 80% of Lactobacilli examined in this study had the capacity to ferment lactose and galactose (Table 2). Generally, most lactobacilli are able to ferment lactose, by uptake of this disaccharide by a specific permease and splitting it by ß-galactosidase for further phosphorylation of galactose and glucose (Kandler, 1983).

Table 2. Partial purification of bacteriocin produced by *Lactobacillus* sp.

Purification Stages	Volume (ml)	Activity (Au100 µl/)	[a]Total activity	Protein (µg/100 µl)	[b]Total Protein (mg)	Specific activity[c]	Purification factor[d]	Recovery (%)[e]
Culture supernatant (crude bacteriocin)	50	21	10500	62	62	169	1	100
(NH4)2SO4 precipitation -40%	20	24	4800	22	22	218	1.28	42.3
(NH4)2SO4 precipitation -80%	10	25	2500	8	10	250	1.47	23.07

The values given are the means from at least three experiments
a-activity x quantity, b- protein x quantity, c- a/b.

Table 3. Biochemical analysis of oral cancer sample

S.NO	Group I Normal Control	Group II Crude lactobacillus Strain A	Group III Partially purified bacteriocin Strain A	Group IV Crude lactobacillus Strain B	Group V Partially purified bacteriocin Strain B	Group VI Treated With DMBA Alone	Group VII Treated With 5 Fluro Uracil
Tumour weight (gm)	--	1.25±0.09	2.15±0.15	3.69±0.26	1.750±0.13	1.69±0.11	4.20±0.30
Tumour Length (cm)	--	3.3±0.30	5.6±0.39	6.3±0.44	4.8±0.33	5.6±0.39	6.6±0.46
Tumour Volume (mm^3)	--	16.33±1.28	17.24±1.49	37.08±2.87	11.04±0.98	18±1.48	42.94±3.14
Tumour Size (cm)	--	0.4 × 3.3	0.3 × 4.6	0.5 × 5.3	0.4 × 2.8	0.4 × 4.6	0.5 × 5.6
SGOT (IU/L)	20.27±3.35	42.92 ±3.35	32.85 ±2.38	25.32 ±1.98	39.39 ±2.89	35.03 ±2.59	29.67 ±2.28
SGPT (IU/L)	18±2.34	44.33 ±3.59	36.88 ± 2.72	31.66 ±2.28	42.11 ±3.15	38.88 ±2.72	34.22 ±2.39
Urea (mg/dl)	16.20±1.39	42.57 ±3.39	35.61 ±2.49	31.85 ±2.19	39.00 ±2.8	31.28 ±2.39	29.42 ±2.19
Creatinine (mg/dl)	0.7±1.39	1.07 ±0.14	1.50 ±0.10	1.26 ±0.08	1.51 ±0.11	1.28 ±0.09	1.03 ±0.07

Because, lactose is present only in milk and milk derivatives, it is possible that these strains have evolved from environments related with mammals, as was suggested for other lactose positive lactobacilli (Garvie, 1984). Lactose may be present in the environment as a waste; resulting from livestock production, and disposed effluents from dairy factories. They are genetically quite diverse and their DNA guanine plus cytosine (G+C) content ranges from 32 to 54%. This is about twice as large as that normally accepted for a well-defined genus (Kandler and Weiss, 1986; Schleifer and Stackbrandt, 1983). When precipitation of bacteriocin with different saturation levels of ammonium (20%, 30%, 40%, 50%, 60%, 70%, 80 % & 90 %) was attempted, the maximum antibacterial activity was found in the resolved precipitate with 80% saturation of ammonium sulfate. However, it still showed high activity when 90% saturation of ammonium sulfate was added. Bacteriocin was purified 1.47 fold and specific activity of the partially-purified preparation was 250 U/mg protein, representing a total recovery of 23.07% (Table 2). Bacteriocins are biologically active proteins or protein complexes that display bactericidal mode of action towards usually closely related species. Numerous strains of bateriocin producing organisms have been isolated from different sources in the last two decades from different ecological niches.

Histopathological Observations

The tumour formation is evident by histological observations. There was well developed squamous cell carcinoma, along with well-defined epithelial and keratin pearls in the connective tissue with cellular pleomorphism of the control model which is treated with DMBA alone. However, the animals treated with DMBA with crude culture of lactobacillus strain A exhibited only reddish oral mucosa. No pathological observations were noted in control animals. The animal model treated with partially purified protein exhibits dysplasia. However, the animals treated with DMBA with crude culture of lactobacillus strain B also exhibited only reddish oral mucosa. The animal model treated with partially purified protein exhibits dysplasia. For this study the reference drug used was 5-Flurouracil, it considerably decreased tumour volume but oral mucositis and abscess were observed in animal models treated with 5-Fluro uracil.

Body mass index

The body mass index of the experimental animals was examined every day. The animal models treated with crude culture of lactobacillus remained healthy and increased in BMI. But the animal model treated with 5-flurouracil considerably decreased in weight. The animals treated with partially purified protein remained healthy but there is no significant change in weight.

Biochemical analysis of oral cancer sample: (refer Table 1)
Estimation of SGOT and SGPT

The normal range of SGOT and SGPT for hamster is 30.27±3.35 and 28±2.34. The SGOT and SGPT levels of all the groups were significantly high when compared to normal models.

Urea and Creatinine

The normal level of urea and creatinine for hamster model is 26.20±1.39 and 0.7±1.39.The urea level and creatine level was high among all the groups when compared with normal model.

Assay of alpha-fetoprotein

The level of Assay of alpha-fetoprotein was analysed for all the hamster groups. Alfa fetoprotein of all groups remained high when compared to normal.

DISCUSSION

The results of present study indicated that the crude culture from Lactobacillus Strain A and strain B effectively prevented the DMBA induced carcinogenesis. The amount lactobacillus present in the saliva clearly indicates that the tumour volume was controlled due the presence of lactobacillus and it's by product. More over the Lactobacillus is a very efficient antimutagen it binds with the mutagens through cell wall and peptidoglycon and exhibit Anticancer activity (Rhee *et al*., 2001). The colonising ability of Lactobacillus inhibits other pathobiotic organisms to colonize the mucosal layer. *Lactobacillus* has an ability to produe latic acid there by it is decreasing the Ph of the stomach. It aids in digestion by converting non digestible carbohydrate to short chain fatty acids which helps in hoemeostatis. Several reviews interpret that *Lactobacillus* has an ability to provoke immune response and IL-12 and TNF alpha (Rhee et al., 2001) which as an ability to suppress tumour growth.so todays review states that Lactobacillus is a potential probiotic which can act as a potential Anticancer agent.

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REFERENCES

Boneses RN and Taussk HA 1945. On the colorimetric determination of creatinine by the Jaffe reaction. *J. Biol. Chem*., 158: pp 581-591.

De Vries D.J., Beart P.M. Fishing for drugs from the sea: Status and strategies. *Trends Pharmacol. Sci*., 1995, 16, 275–279.

Ennahar, S., Sashihara, T., Sonomoto, K., & Ishizaki, A. 2000. Class IIa bacteriocins: biosynthesis, structure and activity. *FEMS Microbiology Reviews*, 24(1), 85-106.

Garneau, Sylvie, Nathaniel I. Martin, and John C. Vederas. "Two-peptide bacteriocins produced by lactic acid bacteria."*Biochimie*., 84.5 (2002): 577-592.

Garvie EI 1984. Genus Leuconostoc. In: Sneath PH, Mair NS, eds, Bergey's Manual of Systematic Bacteriology, 2:1071-1075.

Hirano, J., Yoshida, T., Sugiyama, T., Koide, N., Mori, I., & Yokochi, T. 2003. The effect of Lactobacillus rhamnosus on enter hemorrhagic Escherichia coli infection of human intestinal cells in vitro. *Microbiology and Immunology*, 47(6), 405-409.

Holzapfel, W. H., Haberer, P., Geisen, R., Björkroth, J., & Schillinger, U. 2001. Taxonomy and important features of probiotic microorganisms in food and nutrition. *The American Journal of Clinical Nutrition*, 73(2), 365s-373s.

Kandler, O. 1983. Carbohydrate metabolism in lactic acid bacteria Ant. v. Leeuwenhoek, vol. 49, pp. 209–224

Kandler, O. 1983. Carbohydrate metabolism in lactic acid bacteria Ant. v. Leeuwenhoek, vol. 49, pp. 209–224

Liu L, Kumar SKS, Sedghizadeh PP, Jayakar AN, Shuler CF. Oral squamous cell carcinoma incidence by subsite among diverse racial and ethnic populations in California. *Oral Surg Oral Med Oral Pathol Radiol & Endon*., 2008;105:470-80.

Liviu Feller, Johan Lemmer.Oral Squamous Cell Carcinoma: Epidemiology, Clinical Presentation and Treatment, *Journal of Cancer Therapy*, 2012, 3, 263-268

Mignogna MD, Fedele S, Lo Russo L. The World Cancer Report and the burden of oral cancer. *Eur J Cancer Prev*., 2004; 13(2):139–42.

Natelson S. 1957. Micro-techniques of clinical chemistry for the routine laboratory. C.C.Thomas, Spring-Field, Illinois, p: 381.

Reitman S. and Frankel S. 1957. A colorimetric method for the determination of serum glutamic oxaloacetic and glutamic pyruvic transaminase. *Am J Clin Path.,* 25, pp: 56.

Rhee, S. G., Kang, S. W., Chang, T. S., Jeong, W. and Kim, K. 2001. Peroxiredoxin, a novel family of peroxidases. IUBMB Life 52, 35-41.

Sambrook, J. et al. 1989. Molecular Cloning: A Laboratory Manual, 2nd Edition Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Suresh K, Manoharan S, Vijayaanand AM, Sugunadevi G. 2010. Chemopreventive and antioxidant efficacy of (6)-paradol in 7,12-dimethylbenz(a)anthracene induced hamster buccal pouch carcinogenesis. *Pharmacological Reports*, 62; 1178-85.

WHO cancer report 2012.

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