Studies on Anti-colon cancer of Marine Lactobacillus helveticus protein.

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***Abstract*—One of the world's most deadly diseases is still cancer. There were approximately 10 million deaths every year. Chemotherapy has been associated with numerous adverse effects, despite significant advances in treatment. Thereby encouraging the search for a different, less harmful treatment option. The marine environment was used to isolate the lactobacilli strains. In order to conduct in-vivo research anti-colon cell line models, the crude protein was extracted from the bacteria and used as an anticancer medication. At 24 hours of growth, *L. helveticus* had the highest free radical scavenging activity (1,1-diphenyl-2-picrylhydrazyl), but this activity significantly decreased at 48 hours when compared to the other strains and the control. Throughout the growth period, *L. helveticus* strains contained compounds with anti-colon cancer activity. After 24 hours of growth, the extract with the highest activity (19.03–50.98% inhibition of growth) significantly decreased (5.4–9.94%) at the end of the growth period. When compared to the other strains, *Lactobacillus helveticus* produced compounds that inhibited the growth of the colon cancer HT-29 cell line by 50.98 percent in the culture supernatant after 48 hours. More importantly, T4056 normal primary colon cells were not significantly inhibited by these compounds. These findings, on the other hand, suggested that *L. helveticus* strains might release bioactive compounds with important properties that can serve multiple purposes.**

***Index Terms*—Lactobacillus helveticus, colon cancer, HT-29 cell line, T4056.**

I. INTRODUCTION.

Due to their biodegradability and biocompatibility in human tissue, as well as their capacity to protect interactions in blood and tissues, the metabolites of lactic acid bacteria (LAB) and bifidobacteria (Bb) have recently received a lot of attention. Bacterial exopolysaccharides (EPS) have long been used without side effects in medical and other industrial applications. In this regard, antioxidant, antitumor, and periodontal regeneration properties of EPSs from LAB and Bb culture supernatants were identified and characterized. The ability of bacteria to make a wide range of polysaccharides is well-known. These polysaccharides can either be released as exopolysaccharides (EPSs) or remain tightly bound to the cell surface as capsular polysaccharides (CPSs). During fermentation, many bacterial taxa, especially lactic acid bacteria (LAB) and bifidobacteria (Bb), produce a variety of carbohydrate polymers (Sanalibaba and Cakmak, 2016). According to Surayot et al., LAB is generally regarded as safe microorganisms (GRAS, which stands for generally recognized as safe). They are also capable of producing EPSs with a wide variety of structures without posing any health risks. 2014). Gram-positive bacteria, which are frequently isolated from fermented natural products and are frequently utilized in industrial processes, are referred to as LAB. LAB and their metabolic items have been displayed to upgrade insusceptibility, gastro-digestive capability, protection from stoutness, cell reinforcement action, and blood glucose and cholesterol levels (Mathur et al., 2020; Wang and others, 2020). They may also have health benefits, such as the ability to fight cancer (Tukenmez et al., 2019), the ability to stimulate the immune system (Adebayo-Tayo et al., 2018).

Over 90% of the ocean's biomass is made up of marine flora, which includes bacteria, actinobacteria, cyanobacteria, fungi, microalgae, seaweeds, mangroves, and other halophytes. They are systematically different, generally useful, organically dynamic, and synthetically one of a kind contribution an incredible breadth for revelation of new anticancer medications.

Due to its probiotic property, Lactobacillus is a well-studied marine microbe. An anaerobic microbe with

gram-positive aerotolarity, lactobacillus During lactic fermentations, lactobacilli produce a variety of antibacterial compounds like organic acids, diacetyl, hydrogen peroxide, reuterin, and bacteriocin, also known as bactericidal proteins (Holzapfel et al., 2001; Hirano et al., 2003). The majority of bacteriocins delivered by grampositive microorganisms are from lactic corrosive microbes (Ennahar et al., 2000; Garneau and other, 2002). Bacteriocins are biologically active proteins or complexes proteins with antimicrobial and antitumor properties. Due to the distinct receptors that are present in various bacterial species or types, bacteriocins have a highly specific membrane interaction. The antineoplasmic movement of bacteriocin is basically credited through the acceptance of modified cell passing or apoptosis. Therefore, the research's goal is to suggest a natural anticancer drug without any side effects from marine *Lactobacillus helveticus*.

II. MATERIALS AND METHODS

A. Lactobacillus isolation:
Tests were sequentially weakened up to 10-5 with sanitized half seawater and plated with deMan-Rogosa-Sharpe (MRS) mode for lactobacilli. One milliliter of the serially diluted sediment samples was pipetted into a sterile Petri dish for plating. After that, thoroughly mixed sterile media were poured into dishes in an aseptic manner and swirled. All measurements were carried out in duplicate after the plates had solidified in an inverted position at 28±2 degrees Celsius. The number of microbial colonies was counted after the incubation period. The counts are represented as a colony-forming unit (CFU) per gram of sediment. For further research, the isolated colonies were cultured in MRS agar slant and purified using the pure culture method. The following standard morphology, physiological, and biochemical test was used to differentiate and characterize pure cultures (Bergey's manual, John Holt, 1994).

B. Identification of isolates **(***Lactobacillus helveticus***):**
The isolated lactobacilli were identified using Gram staning, Motility, biochemical test and molecular characterization.

C. Isolate bacterial sequencing using 16S rRNA:
Applied Bio-Systems, Bangalore, India, provided the facility for the sequential generation of consensus sequence for the 16S rRNA gene from forward and reverse sequence data.

D. Lactobacillus culture extract in its purest form:
MRS broth was used to culture Lactobacillus *helveticus* for 24 hours at 30°C. The cell-free supernatant was adjusted to pH 5.0 with 1M NaOH after the cells were harvested (8000x g, 10 min at 4°C), heat-treated (80°C for 10 min), and the bacteriocin was precipitated with an 80 percent saturated ammonium sulfate solution (Sambrook et al., 1989). The bacteriocin fraction was extracted, dissolved in distilled water, and dialyzed overnight at 4° C against distilled water.

E. Determination of Proteolytic Activity:
The release of free amino groups was measured using the o-phthaldialdehyde (OPA) method to determine the extent of proteolytic activity in growth time at 0, 4, 8, 12, and 24 hours of fermentation (Church et al., 1983), and by following the previously described method (Elfahri et al., 2014). To quickly precipitate large proteins, 10 mL of each sample was diluted with 10 mL of 1% (wt/vol) TCA. The mixture was centrifuged for 30 minutes at 4°C at 4,000 g, and the supernatant was vacuum-filtered with a 0.45-millimeter filter. After that, 150 mL of the collected suspension was mixed with 3 mL of OPA reagent, and it was left for two minutes at room temperature (20°C). The Spectrophotometer was used to measure each mixture's absorbance at 340 nm, and the proteolytic activity was calculated using the absorbance of OPA derivatives at 340 nm. At the beginning of growth time, the relative proteolytic activity of each sample was compared to that of control.

F. Cytotoxicity assay in vitro:
Two varieties of immortalized cells were inhibited by the produced antitumor extract; HT-29 a cell line from the colon, and T4056 a cell line. This cytotoxicity assay was carried out with the help of 3-[4,5-dimethylthiazole-2-yl]-2,5 diphenyltetrazolium bromide (MTT) [Arullappan et al., 2015].

G. Determination of Radical Scavenging Activity:
The ability of extracted samples to scavenge free radicals was assessed using the radical 1,1-diphenyl-

2-picrylhydrazyl (DPPH) (Elfahri et al., 2014). In a nutshell, 0.2 mL of each soluble extract was mixed with 800 L of 0.1 mM DPPH dissolved in 95 percent methanol in glass test tubes. After being vigorously shaken, the solutions were sealed with parafilm and left to incubate for thirty minutes in the dark at room temperature. Methanol was used for the baseline correction, and the methanolic DPPH served as the blank. Using a UV-Vis spectrophotometer (Biochrom Ltd., Cambridge, UK), the reduction in absorbance was measured at 517 nm following the incubation period. Each experiment was conducted in triplicate. Using the following formula, the radical scavenging activity was calculated as the inhibition percentage:
Radical scavenging activity (%) = [1 − (absorbance of sample/absorbance of blank)] × 100.

H. Maintenance and thawing of cells:
The human primary colon cell line, T4056, and the human colonic epithelial carcinoma cell line, HT29, colorectal adenocarcinoma, was obtained from Applied Biological lab (Bangalore). To proliferate and enact each culture, 1 mL of HT-29 or T4056 (106 /mL fixation) was immediately defrosted and quickly weakened in 20-mL aliquots of RPMI-1640 development medium containing 10% fetal cow-like serum (Invitrogen, Waltham, Mama), and the cell societies were pelleted by centrifugation at 200 × g for 5 min at 20°C. After being pelleted, the cells were resuspended in 20 milliliters of complete growth medium with one percent penicillin-streptomycin and incubated for one week in 75 cm2 cell culture flasks. After this, the aggregated colon cancer and primary colon cells were dissociated by washing them with PBS and replacing the medium with 5 mL of trypsin/EDTA and incubating them for 5 minutes at 37°C. The complete growth medium was added to 15 mL to inactivate the trypsin solution. In a 50-mL falcon tube, the trypsinized cell suspension was removed by centrifugation (200 g for 5 minutes at 20°C), and the cells were resuspended in complete medium. Before being counted using a hemocytometer (Sigma Aldrich, St. Louis, MO), the cells were stained with trypan blue in a 1:1 ratio for three minutes. Using a 400-magnification light microscope (Olympus CH-2, model CHT, Olympus Optical Co. Ltd., Tokyo, Japan), cells were counted in the four outer quadrants of the hemocytometer. The diluted cells were divided into two parts: one was used for passingaging into a new flask for continued propagation (1.0 $x10^6$ /mL), and the other was used for an antiproliferative assay (1.0 $x10^3$ /100 L).

I. Assay for MTT Proliferation:
The MTT (CellTiter 96 AQueous One Solution Cel Proliferation Assay) [3-(4,5-dimethylthiazol-2-yl)-5-(3 carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] Assay was carried out in accordance with the directions provided by the manufacturer. In a nutshell, cells (HT-29 or T4056) were first passed through and counted using a hemocytometer to determine the appropriate seeding. The MTT assay was used to measure the inhibition of cell proliferation after cells were plated at a concentration of 1.0x 10^3 cells per well. In a nutshell, the cells received 20 mL of the soluble extract sample (1.0 $x10^3$ in 80 mL of complete medium). The MTS reagent was added after the sample had been exposed to the cells for 72 hours, and it was incubated for an additional 4 hours in the same conditions. Formazan's absorbance was measured at 495 nm, and cells that had not been stimulated served as a control. The results of each assay were presented as mean values standard error. Each assay was carried out in triplicate. The percentage of normal colon cells inhibited by colon cancer cells was calculated (Kim et al., 2000) from the following ratio of treatment values to controls:
Proliferative inhibition (%) = (Treatment A495/Control A495) × 100,
where A495 = absorbance at 495 nm.

J. Statistical Analysis:
Each bacterial culture was the subject of three separate experiments. The obtained results were examined using a blocked split plot in a time design and two primary factors: time as a subplot and strains and replications as the main plot. As a block, the replications were used. The general linear model of SPSS (version 13.5) was utilized for the statistical analysis of the data. ANOVA was used to look for significant differences between treatments, and Fisher's least significant difference method was used to compare treatments with a level of significance of P 0.05.

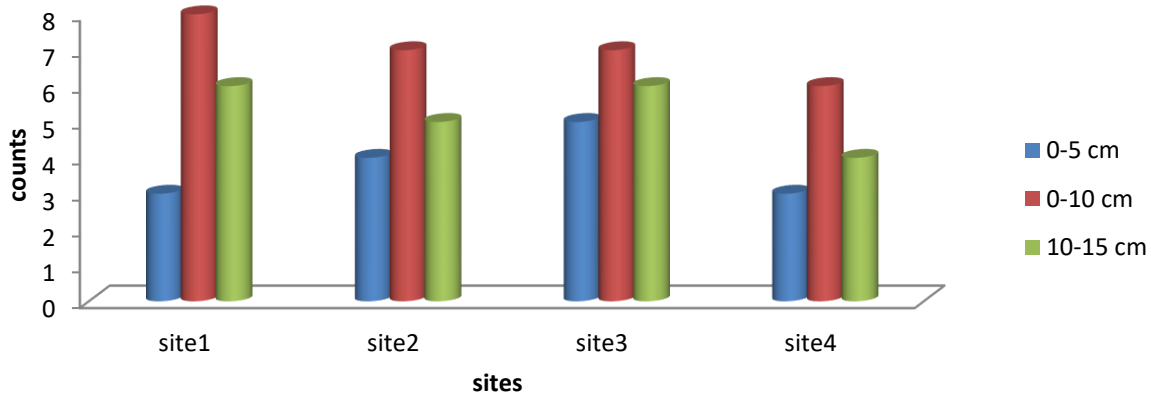
III. RESULT AND DISCUSSION.

The study areas had a variety of marine lactobacilli counts, ranging from $5.0x10^2$ to $285x10^2$ CFU. g^{-1} at various depths and sediments. The counts were generally higher at sediment depths of 10-15 cm than at any other depth (0-5, 6-10). Lactobacilli counts were higher in subsurface sediments than in surface sediments in this study table1 and fig-1.

Table-. Lactobacilli counts in different depth and site of marine biotopes of Pattukkottai Coast

Pattukkottai coastal area	Lactobacilli (cfu× 102. g-1 of sediment)			
	0-5 cm	0-10 cm	10-15 cm	Average
Site1	3	8	6	5.6^d
Site2	4	7	5	5.3^d
Site3	5	7	6	6.0^d
Site4	3	6	4	4.3^d

Fig-1. Lactobacilli counts in different depth and site of marine biotopes of Pattukkottai Coast.



Since, lactose is available just in endlessly nutrients, it is conceivable that these strains have developed from conditions related with warm blooded creatures, as was recommended for other lactose positive lactobacilli (Garvie, 1984). Lactose might be available in the climate as a waste; resulting from the production of livestock and dairy factories' wastewater. Their DNA guanine plus cytosine (G+C) (fig-2) content ranges from 32 to 54%, indicating a wide range of genetic diversity. According to Kendall and Weiss (1986), this is about twice as big as what is typically considered to be a well-defined genus (Schleifer and Stackbrandt, 1983). The resolved precipitate with 80% saturation of ammonium sulfate contained the most antibacterial activity when the precipitation of bacteriocin was attempted at various ammonium saturation levels (20 percent, 30 percent, 40 percent, 50 percent, 60 percent, 70 percent, 80 percent, and 90 percent). However, even when 90% saturation of ammonium sulfate was added, it continued to exhibit high activity. Bacteriocin was sanitized 1.57 crease and explicit action of the to some degree cleaned planning was 280 U/mg protein, addressing an all-out recuperation of 28.07 (Table 2). Bacteriocins are proteins or protein complexes with biological activity that have a bactericidal mode of action against typically closely related species. In the last two decades, numerous strains of organisms that produce bateriocin from various ecological niches have been isolated from various sources.

Fi-g-2 16 s RNA Sequence of Lactobacillus helveticus

aagcatgggt tgcgtagata tatggaagaa caccagtggc gaaggcggct gtctggtctg caactgacgc tgaggctcga aagcatgggt agcgaacagg attagatacc aatcatcatg ccccttatga cctgggctac acacgtgcta caatggatgg tacaacgagt cgcgagaccg cgaggttaag ctaatctctt aaaaccattt tcagttcgga atgtaggctg caacttgcct acacgaagtc ggaatcgcta gtaatcgtgg atcagcatgc cgcggtgaat acgttcccgg gccttgtaca caccgcccgt 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

Table 2. Partial purification of bacteriocin produced by Lactobacillus helveticus

Purification Stages	Volume (ml)	Activity (Au100µl/)	[a]Total activity	Protein (µg/100 µl)	[b]Total Protein (mg)	Specific activity[c]	Purification factor[d]	Recovery (%) [e]
Culture supernatant (crude bacteriocin)	50	22	10100	63	63	179	1.08	100
(NH4)2SO4 precipitation 40% -	20	25	5000	24	25	240	1.38	41.3
(NH4)2SO4 precipitation 80%-	10	28	5600	13	13	280	1.57	28.07

It has been demonstrated that cell wall proteases are the primary cause of the high proteolytic activity of the *L. helveticus* strain chosen for our research (Virtanen et al., 2007; Nielsen and other, 2009; Elfahri and other, 2014) and metabolic processes that result in the release of a variety of peptides. The primary metabolite production of organic acids, as measured by a decrease in pH, was used to evaluate the culture's performance (Table 3). Viable cell counts were used to measure the growth of the selected strains (Figure 3), which significantly (P 0.05) increased after 24 hours of growth culture compare than 48 hours. Indicating strain dependence and acid resistance (Elfahri et al., 2014). A starter culture needs highly developed proteolytic and glycolytic systems that are able to provide essential compounds for the growth of the culture (AA, glucose; Kunji and co., 1996). While glucose is primarily needed to meet energy needs, AA is needed to support a sufficient growth rate. A cell wall-bound protease is the starting point for a complex proteolytic apparatus that yields these AA (Elfahri et al., 2014). Oligopeptides that have been liberated by this enzyme are transported across the cell wall and into the cytoplasm, where they are further degraded into simpler peptides and, as a result, AA. Even oligopeptides appear to have a very potent physiological activity, but it is unclear which enzymes are responsible for the release of bioactive peptides (Ashar and Chand, 2004). According to Donkor et al., apparent bioactivity in water extracts was probably time-dependent, indicating that some of these peptides may be further degraded, losing or gaining potency. 2007). The ability of OPA to react with primary amines to form fluorescent moieties was used to first assess the proteolytic activity of a few strains of L. helveticus in different duration. All strains outperformed the control in terms of proteolytic activity, as shown in Table 3.

Table 3. During the growth of *Lactobacillus helveticus* strains in sterile reconstituted modified MRS medium for up to 48 hours at 37°C, the o-phthaldialdehyde (OPA) method measured the decline in pH and the extent of proteolysis.

Incubation time (h)	Strain code	pH	OPA at 340 nm
0	Control	6.61 ± 0.01^{A}	0.31 ± 0.01^{B}
	Lh	6.48 ± 0.06^{B}	0.33 ± 0.02^{B}

8	Control	6.61 ± 0.01^{A}	0.33 ± 0.01^{E}
	Lh	6.29 ± 0.03^{B}	0.37 ± 0.01^{D}
12	Control	6.58 ± 0.01^{A}	0.34 ± 0.01^{D}
	Lh	4.91 ± 0.26^{B}	0.83 ± 0.02^{B}
24	Control	6.54 ± 0.01^{A}	0.35 ± 0.03^{C}
	Lh	6.85 ± 0.03^{C}	0.90 ± 0.01^{B}
48	Control	6.53 ± 0.07^{A}	0.35 ± 0.02^{C}
	Lh	6.46 ± 0.1^{B}	1.52 ± 0.16^{BA}

A–CMeans in the same column at particular incubation time with different uppercase letters are significantly different ($P < 0.05$). Values are mean of 3 replicates (mean ± SE).

By measuring the decrease in the concentration of DPPH radicals when crude peptide extracts encountered radical scavengers, the antioxidant activity of the extracts was evaluated. From 0 to 48 hours, the samples' free radical scavenging activity significantly changed (P 0.05) in comparison to the control. This change varied between specific strains of L. helveticus. This may be because antioxidant capacity is likely related to strain selection and proteolytic enzyme specificity rather than continuing protein hydrolysis or bacterial growth (Virtanen et al., 2007). In the present investigation, antioxidant activity peaked at 12 hours, but significantly decreased (P 0.05) after 48 hours of fermentation (table-4).

Antioxidant activity was highest in L. helveticus (0.526% at 0 h to 7.67 % at 24h) (P 0.05). At 48 h, *L. helveticus* showed similar trends (7.67, 5.97 and 4.59 percent, respectively) table-4. The DPPH radical scavenging activity might also be attributed to fermentation progresses (37°C, final pH of 3.7; Nishino et al., 2000).

Table-4 During the growth of *Lactobacillus helveticus* strains in sterile reconstituted modified MRS medium for up to 48 hours at 37°C the DPPH assay.

Lactobacillus helveticus	Average optical density 230nm	Concentration (µg/µl)
Blank	0.143	0
12 hrs	0.526	4.59 ± 0.137
24 hrs	0.371	7.67 ± 0.114
48 hrs	0.354	5.97 ± 0.111

DNA damage and mutation, which can increase the incidence of cancer, can result from an imbalance between the presence of antioxidants and the formation of free radicals. As a result, cancer prevention and treatment may benefit from antioxidants;

By minimizing reactions that influence cell proliferation induction not only in vitro, they may reduce the incidence of cancer (Kim et al., 2000), in vivo in rats, and in clinical trials on humans (Tsuda et al., 2002; Kozu et al., 2009). The cell viability (MTT) assay was used to evaluate in vitro the inhibition of both cell lines (HT-29 and T4056) after treatment with crude peptide extract. The current study evaluated the inhibition effect of crude peptide extracts during fermentation on the proliferation of colon cancer cell (HT-29) and healthy colon cell line (T4056).

The effect of incubation period the strains of *L. helveticus* on the proliferation of the HT-29 colon cancer cell line is depicted in table-5. When compared to the control, all samples displayed antiproliferative effects at varying levels. The sample taken after 12 hours of ioincubation showed the greatest (P 0.05) decrease in HT-29 proliferation. When the 24 h after incubation sample was used, the effect was significantly reduced. After 12 hours of incubation with *L. helveticus*, the percentage of proliferative inhibition increased significantly (P 0.05) and was higher than that of other strains. The normal T4056 cells exhibited no significant inhibition of any soluble extract. The increased concentration of a potential bioactive compound in the medium in which certain cell death mechanisms, such as apoptosis (programmed cell death), were activated could have contributed to the HT-29 cell line's decrease in proliferation. Colon cancer cell lines (T 4056 and HT29) were found to be dose-dependently cytotoxic when helveticin, a peptide derived from *L. helveticus* protein, was used at various concentrations (50 and 100, g/mL) (Mader et al., 2006).

Table-5 Anticancer activity of marine L. helveticus

We can conclude that *Lactobacillus helveticus* strains have a statistically significant effect on the release of great potential antioxidant and anti-colon cancer compounds during different culture time.

Lactobacilus helveticus	Conc. (µg/ml)	HT-29	T 4056
	Growth inhibition (%)		
0	50	-	-
	100	-	-
12	50	34	64
	100	82	93
24	50	83	68
	100	96	79
48	50	66	14
	100	89	41
Mito-C	-	-	-
Adriamycin	-	-	-

IV. CONCLUSION

In this study, four proteolytic strains of L. helveticus were used as potential functional dairy starter cultures that could extract numerous bioactive compounds from modified MRS medium. Under acidic conditions, strains of Lactobacillus helveticus thrived in modified MRS medium. Medium appears to have released compounds that are anti-oxidative and anti-colon cancer as a result of their growth. However, fermentation time, temperature, pH, and the concentration of released bioactive compounds all appeared to have an impact on the level of bioactivities, which appeared to be strain-specific.

V. ACKNOWLEDGEMENT

The authors acknowledge to college management. This study was in supported by the Maruthupandiyar College affiliated to Bharathidasan University; kind help and support of the technical staff at Microbiology department was greatly appreciated.

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