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Growth Inhibitory Effect of Lactocare on *Vibrio cholerae*

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ABSTRACT

Background and Objective: Acute microbial diarrheal diseases are the major public health problems in the developing countries. People affected by diarrheal diseases have the lowest financial resources and poorest hygienic facilities. Children under five, primarily in Asian and African countries, are mostly the subjects affected by microbial diseases transmitted through water.

The current study aimed at investigating the comparative inhibitory effect of Lactocare (commercial probiotic) on clinical samples and standard strains of *Vibrio cholerae*.

Methods: A total of 20 clinical samples and a standard strain *(ATCC 14035*) were provided by Health Reference Laboratory and Biotechnology Institute, respectively. In order to confirm the samples, biochemical analysis and the polymerase chain reaction (PCR) were performed on intergenic space. Afterward, agar well diffusion method was performed in order to measure the minimum inhibitory concentration to monitor the antimicrobial activity of Lactocare.

Results: Colony count of V. *cholerae* for the standard strain in 30% and mean for clinical samples in 50% concentration of Lactocare treatment revealed that it would propel to death phase. Since the number of colonies decreased to 100, it was considered that higher concentrations of Lactocare would completely inhibit the growth of *V. cholera*.

Conclusion: Probiotics are employed to develop new pharmaceutical preparations and functional foods in order to promote the public health.

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Introduction

Accessibility to safe drinking water can significantly result in improvement of health and quality of life; therefore, efforts should be made to acquire safe drinking water. Waterborne infections are common due to the difficulty of accessing any kind of sanitary or clean water. According to the World Health Organization(WHO), about 2.5 billion people do not have access to sanitary water, and more than 1.5 million children die from diarrheal diseases each year (1). Also, it is reported that over 5 million people die per year due to water borne diseases, which more than 50% of them are microbial intestinal infections, and cholera is in the first place (2).

Probiotics plays an important role in the health of the host; a change in their balance and levels affects the health and digestion ability of the host (3). Probiotics could be employed as an effective tool for its potency to control the balance of pathogens growth, which results in the prevention of infections (4, 5). Indeed, multiple studies performed on various strains of probiotic bacteria revealed that they can participate in decreasing the number of pathogens and their virulence mechanisms (6, 7). The potency of lots of probi-

otic bacteria in inflammatory bowel diseases is studied in many clinical trials (8, 9). Since World War I, the non-pathogenic *Escherichia coli* strain is isolated and proposed for the maintenance therapy of Crohn's disease and ulcerative colitis (9-11). Moreover, purposeful transduction of E. coli strain to newborns can prevent the colonization of the microbial intestine pathogens. The current study aimed at investigating the inhibitory effect of commercial Lactocare on *Vibrio cholerae.*

Materials and methods

Preparation of bacteria

The bacterial strains used in the current study were *V. cholerae* ATCC14035 provided by Biotechnology Institute (Iranian Research Organization for Sciences and Technology, Tehran) and 20 clinical samples provided by the Health Reference Laboratory.

Preparation of *Vibrio cholerae* Suspension

To prepare bacterial suspension, bacterial colonies grown on thiosulfate-citrate-bile salts-sucrose (TCBS) agar were stirred in 1 mL phosphate-buffered saline (PBS) for 0.5 McFarland turbidity (1-1.5 × 10^8 colony-forming unit; CFU). In order to measure the accuracy of turbidity, samples were evacuated by spectrophotometer at 620 nm wavelength range and absorbance was set at the range 0.08-0.1 nm.

Biochemical analysis

All clinical isolates were screened by the biochemical tests including motility status using (sulfide-indole-motility) SIM agar, oxidase reaction, triple sugar iron (TSI) agar for sucrose, mannose, and arabinose fermentation tests, ornithine decarboxylase, arginine dihydrolase, and growth rate in cultures containing different amounts of NaCl (2%, 4%, 8%, and 16%) (12, 13).

DNA extraction and the polymerase chain reaction

DNA genome of each isolate was purified using a DNA extraction kit according to manufacturer's instructions (CinnaGene, Iran) and PCR was performed according to the protocol of Chun J. et al., for 16S-23S mRNA genes. The primer set was as follows: VC-Forward 5'-AGTCACTTAACCATTCAACCCG-3' and VC-Reverse 5'-TTAAGCGTTTTCGCTGAGA-ATG-3' (14, 15).

Preparation of Lactocare Suspension

The full contents of two capsules of commercial probiotics (Lactocare) purchased from ZistTakhmir Company were dissolved in 9 mL of PBS. One milliliter of the obtained suspension was added to each falcon containing MRS (de Man, Rogosa and Sharpe) broth and incubated for about 24 to 48 hours at 37°C, then centrifuged at 4000 g for 10 minutes and the supernatant was transferred under sterile conditions.

Agar well diffusion method

Agar well diffusion method was followed to determine the antimicrobial activity. Mueller-Hinton agar plates were swabbed with *V. cholerae* suspension and a 10-mm diameter well was punched in each center of plates. Afterward, 100 µL of Lactocare suspension was added into the well of each plate and incubated at room temperature for two hours. In collateral, 100 µL of MRS broth was fielded in case of control sample. The plates were incubated at 37°C for 24 hours for bacterial growth. Finally the inhibition zone was measured and the activity index was also calculated.

Kinetic activity of Lactocare

To obtain the kinetics of death, a certain amount of diluted bacterial and Lactocare solution was mixed in 1:1 ratio. Bacterial concentrations were defined in a manner that contained 10%, 20%, 30%, and 40% of V. *cholerae*, equally well applied to the Lactocare and the remaining volume was filled up by MRS broth.

Kinetic assay and colony counting were performed at 0 to 420 minute treatment time; in collateral adding, MRS agar culture was performed in order to stimulate the optimal growth conditions of pathogenic and lactobacilli at 37°C.

Statistical Analysis

The mean ± standard deviation (SD) were calculated for clinical samples and differences between clinical samples and ATCC strain were analyzed by one-way analysis of variant (ANOVA) with SPSS version 19.0. Statistical significance was considered P <0.05.

Results

Biochemical Tests and Bacterial Culture Results

V. cholerae produced smooth yellow colonies with 2-4 mm in diameter, an opaque center, and transparent periphery on TCBS agar, after 16 to 18 hours of incubation. The results of oxidase, TSI, Voges-Proskauer (VP), motility, indole, and string of pearls test were positive. The bacteria were capable of fermenting carbohydrate on TSI with no gas or hydrogen sulfide production. *V. cholerae* was confirmed for all the isolates by studying the test results.

PCR assay

The PCR results revealed that all 20 clinical isolates were positive for *V. chlore*a (Figure 1) and the nucleotide sequence of 16S-23S mRNA genes revealed an amplicon of 300 bp.

Agar well diffusion method

The inhibition zone of 22 mm was significantly larger than that of CLSI guidelines (16 mm) as shown in (Figure 2).

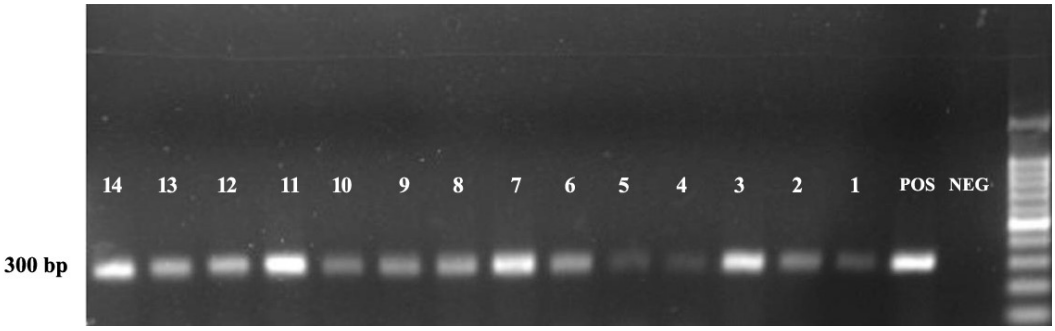

Figure 1. Gel Electrophoresis of the PCR Products of 16S-23S mRNA Genes

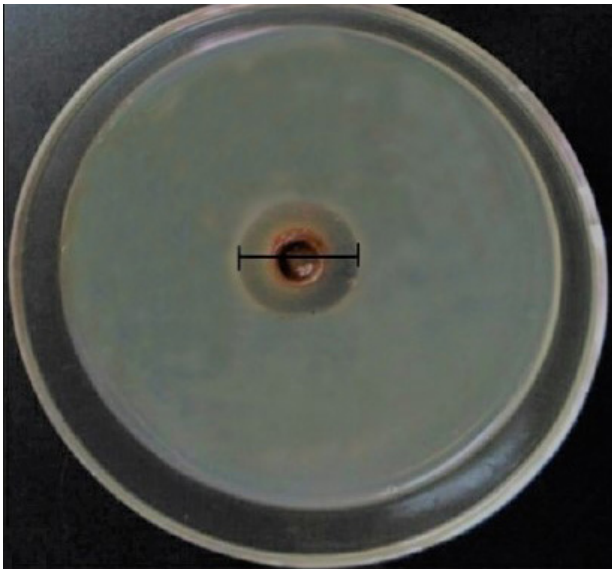

Figure 2. Growth Inhibition Zone by Lactocare

Kinetic activity of Lactocare

V. *cholerae* colonies were counted and recorded in all concentrations and treatment time to obtain the kinetics of death as mentioned in Table 1.

The average results from all clinical samples compared to the standard strain against each concentration of Lactocare at the incubation period is depicted in Figure 3.

Statistical Analysis

A significant difference was observed between the inhibitory growth of ATCC strain and those of clinical samples at certain concentrations. As a result of the applied different treatment time and concentrations, a treatment time and concentration dependent decrease in inhibition was observed when ATCC and clinical samples were exposed to uni-colonies, where increas-

ing treatment time resulted in reduction of bacterial growth by an average of 53% at second hour of 40% concentration for ATCC and 46% at second hour of 60% concentration for clinical samples ($P \leq 0.05$).

Table 1. Number of Colonies During the Incubation With Lactocare Based on Lactocare Concentration

	Time (h)						
Concentration (%)	1	2	3	4	5	6	7
	ATCC14035 Strain						
10	2038	1950	1800	1779	1597	1521	1480
20	1424	1260	1080	972	624	552	540
30	1100	871	680	549	321	267	128
40	920	642	343	156	72	35	12
	Mean Number of Clinical Isolates						
10	2293	2194	1944	1913	1765	1694	1603
20	1938	1785	1597	1486	1235	1098	1076
30	1526	1428	1214	1100	883	751	717
40	1260	1143	935	816	630	517	475
50	1025	889	526	361	225	148	125
60	875	784	363	164	75	31	16

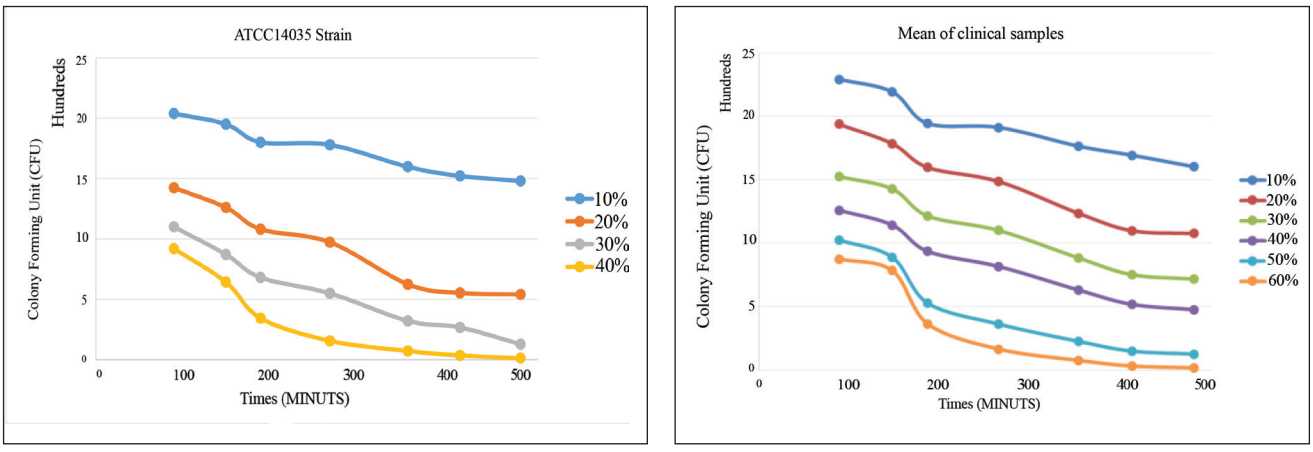

Figure 3. Downward Trend of Vibrio *cholerae* Colonies at Various Concentrations

Discussion

Since bacteria play an important role in the health of the host, a change in their balance and levels affects the health and ability to digest the host food. Antibiotics can be taken either directly or by consumption of meat products and cause diarrhea; by applying a change in the intestinal flora and providing the pathogenic ones. On the other hand, antibiotics can increase the number of resistant bacteria, which makes treatment more difficult. Also, decrease in the levels

of intestinal flora reduces the ability of carbohydrates to ferment and metabolize bile acids and cause diarrhea by absorbing a lot of water. Also, it ceases the inhibitory effect of the pathogenic bacteria growth. Finally, taking antibiotics increases the individual's need for vitamins. Probiotics are powerful dietary supplements that help human beings to preserve the balance of beneficial gastrointestinal microorganisms in chronic conditions. Therefore, the food industry needs to carefully evaluate the safety and effects of new species and probiotic strains before combining with food products (16). Based on the European Food Safety Authority (EFSA) guidelines, microorganisms presenting minimum inhibitory concentrations (MICs) similar or less in comparison with EFSA's breakpoint are appointed as susceptible to this antimicrobial (17).

Producing antimicrobial compounds is an important screening tool for probiotics in intestinal health; in addition to the production of metabolites such as organic acids, probiotic bacteria may produce antimicrobial agents such as bacteriocins. Probiotic bacteria may impose their effects on intestinal pathogens by reducing environmental pH. The consumed commercial Lactocare was composed of *Lactobacillus casei*, *L. acidophilus, L. rhamnosus, L. bulgaricus*, Bifido*bacterium breve, B. longum, Streptococcus thermophiles*, and fructooligosaccharides (FOS) with certain colony count (10^{-9} CFU) as the company claimed.

Prema et al., revealed that *L. plantarum* strain shows an inhibitory potency by producing bacteriocin, which was active against the indicator starins. According to Figure 2, the zone of growth inhibition was 22 mm, which was more effective in comparison with CLSI guidelines (16 mm), but could not completely inhibit the pathogen's growth. The maximum zone of inhibition in the study by Prema was recorded about 24 mm against *Salmonella typhi* as an indicator strain (18); which almost confirmed the current study.

A study by Tambekar et al., used standard and commercial bacterial strains to make probiotics from cow and goat milk; the results showed that both the standard and commercial strains could tolerate the gastric acid at pH 2 and the bile salt at concentration of 2%. Tambekar et al., revealed that these bacterial strains have antibacterial activities against antibiotic-resistant enteric pathogens, which maintains their bacteriocin stability at 121°C and pH 3-9. Also, a comparative study on goat milk probiotics evolved that probiotic bacteriocins has stronger antibacterial activities versus the commercial probiotics. Although probiotic products made from domestic animals' milk can be considered as a candida for oral therapy, the low access to such products should be considered (19).

In an in vitro study by Meshref et al., the potency of probiotic bacterial strains used for growth inhibition of *E. coli, S. aureus* and *L. monocytogenes* was reported. They also revealed that after 15 days of storage at 4°C, both *L. acidophilus* La-5 and B. longum ATCC15707 remained viable at $>10^7$ CFU/g, which was appropriate in yoghurt (20). The current study revealed the downward trend in the number of counted colonies at various concentrations of Lactocare versus V. *cholerae* in different intervals. The number of V. *cholerae* colonies in 30% for standard strain and mean of clinical isolates in 50% concentrations of Lactocare treatment from the first to the last hour was 10^3 to 10^2 colonies, which revealed that it could propel to death phase. According to Figure 3, since the number of colonies decreased to 100, higher concentrations of Lactocare would completely inhibit the growth of *V. cholera.*

Conclusion

Probiotics are employed to develop new pharmaceutical preparations and functional foods in order to promote the public health.

Conflict of Interest

The authors declare that there was no conflict of interest

References

1. Fenwick A. Waterborne Infectious Diseases—Could They Be Consigned to History? Science. 2006;313(5790):1077-81. https://doi.org/10.1126/science.1127184 PMID:16931751
2. Seas C, Alarcon M, Aragon JC, Beneit S, Quinonez M, Guerra H, et al. Surveillance of bacterial pathogens associated with acute diarrhea in Lima, Peru. International journal of infectious diseases : IJID : official publication of the International Society for Infectious Diseases. 2000;4(2):96-9. https://doi.org/10.1016/S1201-9712(00)90101-2
3. Saarela M, Mogensen G, Fonden R, Matto J, Mattila-Sandholm T. Probiotic bacteria: safety, functional and technological properties. Journal of biotechnology. 2000;84(3):197-215. https://doi.org/10.1016/S0168-1656(00)00375-8
4. Fooks LJ, Gibson GR. Probiotics as modulators of the gut flora. The British journal of nutrition. 2002;88 Suppl 1:S39-49. PMID: 12215180
5. Shanahan F. Inflammatory bowel disease: Immunodiagnostics, immunotherapeutics, and ecotherapeutics. Gastroenterology. 2001;120(3):622-35. https://doi.org/10.1053/gast.2001.22122 PMID:11179240
6. Arvola T, Laiho K, Torkkeli S, Mykkanen H, Salminen S, Maunula L, et al. Prophylactic Lactobacillus GG reduces antibiotic-associated diarrhea in children with respiratory infections: a randomized study. Pediatrics. 1999;104(5):e64. PMID: 10545590
7. Hudault S, Lievin V, Bernet-Camard MF, Servin AL. Antagonistic activity exerted in vitro and in vivo by Lactobacillus casei (strain GG) against Salmonella typhimurium C5 infection. Appl Environ Microbiol. 1997;63(2):513-8. PMID:9023930 PMCid:PMC168342
8. Gionchetti P, Rizzello F, Venturi A, Brigidi P, Matteuzzi D, Bazzocchi G, et al. Oral bacteriotherapy as maintenance treatment in patients with chronic pouchitis: a double-blind, placebo-controlled trial. Gastroenterology. 2000;119(2):305-9. https://doi.org/10.1053/gast.2000.9370 PMID:10930365
9. Kruis W, Schutz E, Fric P, Fixa B, Judmaier G, Stolte M. Double-blind comparison of an oral Escherichia coli preparation and mesalazine in maintaining remission of ulcerative colitis. Alimentary pharmacology & therapeutics. 1997;11(5):853-8. https://doi.org/10.1046/j.1365-2036.1997.00225.x
10. Malchow HA. Crohn's Disease and : A New Approach in Therapy to Maintain Remission of Colonic Crohn's Disease?Escherichia coli: A New Approach in Therapy to Maintain Remission of Colonic Crohn's Disease?. Journal of clinical gastroenterology. 1997;25(4):653-8. https://doi.org/10.1097/00004836-199712000-00021
11. Rembacken BJ, Snelling AM, Hawkey PM, Chalmers DM, Axon AT. Non-pathogenic Escherichia coli versus mesalazine for the treatment of ulcerative colitis: a randomised trial. Lancet. 1999;354(9179):635-9. https://doi.org/10.1016/S0140-6736(98)06343-0
12. Choopun N, Louis V, Huq A, Colwell RR. Simple procedure for rapid identification of Vibrio cholerae from the aquatic environment. Applied and environmental microbiology. 2002;68(2):995-8. https://doi.org/10.1128/AEM.68.2.995-998.2002 PMID:11823252 PMCid:PMC126716
13. Ramamurthy T, Bag PK, Pal A, Bhattacharya SK, Bhattacharya MK, Shimada T, et al. Virulence patterns of Vibrio cholerae non-O1 strains isolated from hospitalised patients with acute diarrhoea in Calcutta, India. Journal of medical microbiology. 1993;39(4):310-7. https://doi.org/10.1099/00222615-39-4-310 PMID:8411093
14. Chun J, Rivera IN, Colwell RR. Analysis of 16S-23S rRNA intergenic spacer of Vibrio cholerae and Vibrio mimicus for detection of these species. Methods in molecular biology. 2002;179:171-8. PMID:11692861
15. Ghatak A, Majumdar A, Ghosh RK. Molecular phylogenetic analysis of Vibrio cholerae O1 El Tor strains isolated before, during and after the O 139 outbreak based on the inter-genomic heterogeneity of the 16S-23S rRNA intergenic spacer regions. Journal of biosciences. 2005;30(5):619-25. https://doi.org/10.1007/

BF02703562 PMID:16388136

16. Parvez S, Malik K, Ah Kang S, Kim HY. Probiotics and their fermented food products are beneficial for health. Journal of applied microbiology. 2006;100(6):1171-85. https://doi.org/10.1111/j.1365-2672.2006.02963.x PMID:16696665

17. Bories G, BrantomP, et al. Technical guidance - Update of the criteria used in the assessment of bacterial resistance to antibiotics of human or veterinary importance. The EFSA Journal. 2008;732:1-15. https://doi.org/10.2903/j.efsa.2008.732

18. Prema P. In vitro antagonistic activity of a probiotic Lactobacillus plantarum against water borne pathogens. Int J Pharm phar Sci. 2013;5(4):175-8.

19. Bhutada S, Tambekar D. An evaluation of probiotic potential of Lactobacillus sp. from milk of domestic animals and commercial available probiotic preparations in prevention of enteric bacterial infections. Recent Research in Science and Technology. 2010;2(10).

20. El-Kholy A, El-Shinawy S, Meshref A, Korny A. Screening of Antagonistic Activity of Probiotic Bacteria against Some Food-Borne Pathogens. Journal of Applied & Environmental Microbiology. 2014; 2(2):53-60.

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