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Evaluation of Probiotic *L. rhamnosus* GG as a Protective Culture in Sea Buckthorn-Based Beverage

Srijita Sireswar, Gargi Dey *, Kinjoll Dey and Arkasish Kundu

School of Biotechnology, KIIT University, Patia, Bhubaneswar, Odisha PIN-751024, India; sireswar.srijita@gmail.com (S.S.); srijita.ksbt@gmail.com (K.D.); gargi.dey@kiitbiotech.ac.in (A.K.)

* Correspondence: drgargi.dey@gmail.com; Tel: +91-674-2725466; Fax: +91-674-2725732

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Abstract: The main objective of this paper was to evaluate the efficiency of probiotic strain, *Lactobacillus rhamnosus* GG (ATCC 53103) as a protective culture when present in a food system. A non-fermented sea buckthorn-based beverage was developed. To meet the required criteria for probiotic beverage (viable count of 8 log CFU mL^{-1}), the acidic juice had to be supplemented with whey protein concentrate (WPC). The obtained beverage had a shelf life of two weeks. Furthermore, the inhibitory potential of *Lactobacillus*-fortified-WPC-supplemented juice matrix was evaluated against *E. coli* (ATCC 25922) which is a major agent responsible for food contamination and shelf spoilage. Results indicated that the fortification of beverage with *L. rhamnosus* GG appeared to create an effective hurdle for multiplication of *E. coli* in the sea buckthorn-WPC system.

Keywords: sea buckthorn; probiotics; protective culture; *L. rhamnosus* GG; *E. coli*

1. Introduction

We are in the age of health fanaticism where functional foods and beverages have gained tremendous popularity. In the past few years, berry-based drinks infused with antioxidants seems to be one of the most dynamic sector of the beverage world. One such wonder berry is the sea buckthorn (*Hippophae rhamnoides*) belonging to the *Elaeagnaceae* family distributed over Europe, Asia, and Northern America. These berries have high contents of ascorbic acid, phenolic compounds, tocopherols and tocotrienols, carotenoids, and sterols [1]. The predominant flavonols are glycosides of isorhamnetin, quercetin, and kaempferol among others [2]. However, in such fruit based beverages, shelf stable product development is a challenge, since chemical preservatives are usually avoided as it does not go with the 'fresh' image. While the majority of consumers today prefer consumption of minimally processed, low, or no chemical preservative added food, even with the most recent technological advancements, issues related to food safety and security still remain to be completely resolved. Irrespective of the presence of modern safety measures like HACCP, the number of cases of food borne illness and poisoning seems to be increasing. Under these circumstances, use of food grade lactic acid bacteria (LAB) cultures as protective cultures offers a strategy for biopreservation. LAB, especially probiotics belonging to *Lactobacillus* genus, are uniquely equipped with potential for production of bacteriocins, organic acids, and hydrogen peroxide to exert biopreservative effects. One must point out here that an alternative strategy of direct addition of bacteriocin in foods does exist. For example, Choi and Beuchat in 1994, demonstrated anti-*lysterial* activity of bacteriocin produced by *P. acidolactii* M on Kimchi [3]. However, there are several factors which limit the efficacy of bacteriocin, such as their inactivation due to proteolytic enzymes, or due to binding with proteins and lipids of food matrix. It has also been reported that bacteriocins have varying degrees of sensitivities towards different strains of *Listeria* sp.

On the other hand, effective application of several protective cultures in sous vide products, sauerkraut, and in cheese and in cured pork has been recorded in past [4–9]. Apart from these, several commercial protective cultures are also available for example, nisin-producing BS-10® (*L. lactis* ssp, lactis) from Chr Hansen, BIO-PROFIT™ (*L. rhamnosus* LC 705) from BioGaia (Sweden), Bovamine Meat Culture™ effective against *E. coli* and *Salmonella* in meat, and HOLDBAC™ (*L. plantarum*, *L. rhamnosus*, *L. sakei*, *L. paracasei*, *P. freundenreichii* ssp *shermanii*) against *Listeria* from DANISCO (Denmark) [10].

Thus, controlling food safety risks through LAB seems to be a feasible approach. Moreso because of the underlying synergy between protective and probiotic potentials of *Lactobacillus* sp. In the past decade, several clinical studies have strongly established the reputation of *Lactobacillus* sp. as an effective biotherapeutic agent [11–13]. The unique application of *Lactobacillus* sp. as both bio control and as a bio therapeutic agent increases the consumer appeal of the novel functional foods. From this perspective, it seems a realistic possibility that a probiotic *Lactobacillus* strain may also be beneficial as a protective culture. However, the efficacy of each LAB strain to be used as probiotic and/or protective culture will differ and needs standardization for different food systems as the food matrices also play an equally important role in both the applications.

Keeping this in view, here we report on the development of an unfermented sea buckthorn-based probiotic beverage using *L. rhamnosus* GG (ATCC 53103). The probiotic strain in the juice matrix was also evaluated for its efficacy as a protective culture against the non-pathogenic, spoilage causing *E. coli* (ATCC 25922) strain. This strain has a history of being used as a surrogate for the pathogenic, enterohemorrhagic *E. coli* O157:H7 by several authors, while others have also used this as a representative spoilage organism [14–17].

2. Materials and Methods

2.1. Preparation of Cultures

L. rhamnosus GG (ATCC 53103) was reactivated by subculturing in MRS broth (HiMedia, Mumbai, Maharashtra, India) overnight at 37 °C. For high density harvest, cells were grown in MRS broth with 10% seed inoculum and incubated at 37 °C for 36 h. The cells were harvested by centrifugation at 6500 rpm for 15 min. The pellets were washed twice with 0.1 M phosphate buffer saline (HiMedia) and approximately 8 log CFU mL^{-1} of probiotics was added into the juice samples.

For the second part of our study, 5 mL of the overnight culture of *E. coli* (ATCC 25922) was inoculated in 100 mL of nutrient broth and incubated at 37 °C for 24 h. The cells were harvested by centrifugation at 6500 rpm for 15 min. The pellets were washed twice with 0.1 M phosphate buffer saline and added to the juice samples so as to maintain an initial concentration of approximately 6 log CFU mL^{-1}.

2.2. Preparation of Juice Matrix

Sea buckthorn berries were collected from Kaza, District Spiti, Himachal Pradesh, India. The juice extraction was followed according to the protocol by Bump with some modifications [18]. Freshly extracted juice was allowed to stand for 48 h at 4 °C, which led to the formation of a bilayer. The upper liquid layer was filtered and used further. The extracted juice was pasteurized at 85 °C for 30 min and stored at 4 °C. The juice was inoculated with 8 log CFU mL^{-1} of *L. rhamnosus GG*, stored at 4 °C for two weeks and evaluated for viable counts by serial dilution and spread plate method.

2.3. Development of Unfermented Sea Buckthorn Beverage Fortified with L. rhamnosus *GG*

The aliquots of sea buckthorn juice of initial pH 2.8 were adjusted to different pH by using tri-sodium citrate. Juice samples adjusted to different pH were further supplemented with different ingredients (whey protein concentrate (WPC), soy protein isolate (SPI) & skim milk (SM)). WPC-sea buckthorn beverage was prepared as per Sireswar et al. [19]. WPC was supplemented at the concentration of 4% w/v in juice having pH 4.5, SPI (4% w/v) in the juice having pH 3.0 and SM

(2% w/v) in juice having pH 2.8. All juice samples were pasteurized at 85 °C for 30 min and stored at 4 °C prior to use. Each supplemented sea buckthorn juice (100 mL) was inoculated with 8 log CFU mL^{-1} of *L. rhamnosus* GG, separately in air tight bottles in aseptic conditions and stored at 4 °C (cold storage) for a period of two weeks. The viability of the probiotic strain from the respective juice matrices were assessed for colony counts during two weeks of cold storage.

2.4. Evaluation of L. rhamnosus as Protective Culture

The method described by Millette et al. was followed with some modifications [20]. A known volume of WPC supplemented sea buckthorn juice (pH 4.5), was fortified with 8 log CFU mL^{-1} of *L. rhamnosus* GG. This WPC-sea buckhorn beverage was individually inoculated with 6 log CFU mL^{-1} of *E. coli* and incubated at 4, 10, and 37 °C for 14 days. Aliquots of the sample were taken on alternate days to enumerate *E. coli* and *L. rhamnosus* GG. At each time interval, the sample was serially diluted and plated on MacConkey agar (HiMedia) for *E. coli* and incubated at 37 °C for 24 h and on MRS agar plates for *L. rhamnosus* GG and incubated at 37 °C for 72 h. To confirm the results, a control experiment was performed to evaluate the potential of WPC-supplemented sea buckthorn juice (devoid of the probiotic), in the suppression of *E. coli*.

2.5. Statistical Analysis

All data were presented as the average of triplicate experiments with standard deviation. Results were statistically interpreted with two-way ANOVA using GraphPad Prism (v5.0, GraphPad Software, San Diego, CA, USA) and Origin (v8.5, OriginLab Corporation, Northampton, MA, USA).

3. Results and Discussion

The ability of spoilage organisms to adapt to the adverse environments and develop, for instance, acid tolerance, has practical importance in terms of food associated outbreaks. Under such circumstances, probiotic-fortified foods with reduced pH, having undissociated organic acids and fruit phenolics may be efficient in suppression of such spoilage/pathogenic organisms. Very recently, Siroli et al. reported that *L. plantarum* CIT3 effectively interfered with the multiplication of *L. monocytogenes* in sliced apples and lamb's lettuce [21]. Maragkoudakis and colleagues in 2009, demonstrated the application of *Enterococcus faecium* PCO71 and *L. fermentum* ACA-DC179 as protective culture in chicken meat against *L. monocytogenes* and *S. enteriditis*, respectively [22]. Similarly, there have been recent reports of inhibition of *L. monocytogenes* by a protective strain of *Lactococcus piscium* CNCM-I-4031 in cooked shrimp [23,24].

Our group has worked extensively on evaluating the inactivation potential of *L. rhamnosus* fortified sea buckthorn matrices on several enteropathogens like *Salmonella enteritidis*, enteropathogenic *E. coli*, *Shigella dysenteriae*, and *Shigella flexneri* [19]. However, there are practically no reports available on evaluation of *L. rhamnosus* as a protective culture against non-pathogenic, spoilage causing *E. coli* in a sea buckthorn matrix. Here we attempted to evaluate whether there is an enhanced degree of protection from *E. coli* due to the synergy between *L. rhamnosus* GG and fruit phenolics in the food matrix.

3.1. Development of Unfermented Sea Buckthorn Beverage Fortified with L. rhamnosus GG

Initial studies showed that probiotic strain *L. rhamnosus* GG was not able to survive in the unsupplemented juice matrix (Figure 1). The reason for this drastic decrease in the cell count could be due to the presence of high content of ascorbic acid (180–370 mg per 100 g) and other organic acids which brought down the juice pH to 2.8 [1]. Champagne et al. reported that adequate supplementation of the fruit juices with WPC, SPI, and SM enhances probiotic survival [25]. Owing to the inherent nature of WPC, which forms an aggregated gel structure thereby protecting the probiotic strain, it has been used in several applications [26,27].

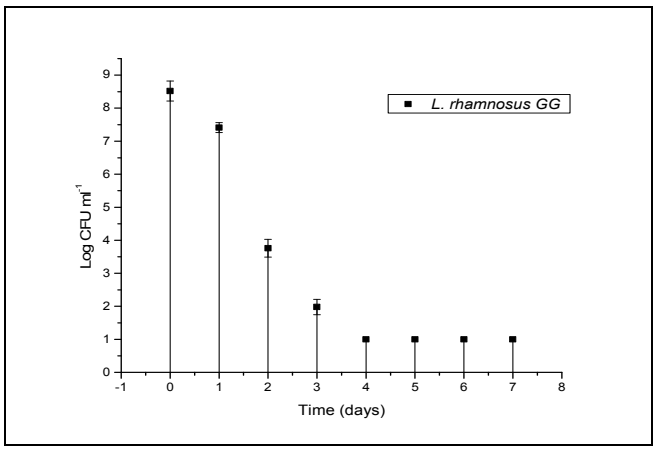

Figure 1. Viability of *L. rhamnosus* GG in unsupplemented sea buckthorn juice. The data presented are means of triplicate experiments ± standard deviation.

In this study, the sea buckthorn matrix was supplemented with WPC, SPI, and SM, individually to assess its efficiency as a matrix to support probiotic survival. However, on pasteurization of juice samples supplemented with the above ingredients, protein coagulation was observed. To reduce or eliminate the coagulation and precipitation, tri-sodium citrate was used as a chelating and stabilizing agent, as has been applied earlier by Pathomrungsiyounggul et al. for soy milk [28]. Additionally, dextrose (200 g L^{-1}) was added to increase the stability of the matrix since dextrose stabilizes against denaturation by increasing the hydration of the protein molecule and enhancing the water structure in its immediate surroundings [29].

Each of the probiotic beverages prepared by supplementing WPC (4% w/v), SPI (4% w/v), and SM (2% w/v) in sea buckthorn juice were adjusted to pH 4.5, 3.0, 2.8, respectively and pasteurized at 85 °C for 30 min. After pasteurization, juice matrices were fortified with *L. rhamnosus* GG at 8 log CFU mL^{-1} and stored at 4 °C.

3.2. *Viability of* L. rhamnosus *GG in Beverage*

Retainment of cell viability and shelf life is of primary significance in probiotic product development. The initial bacterial population of *L. rhamnosus* GG in sea buckthorn juice containing WPC (with pH adjusted to 4.5) was 8.56 log CFU mL^{-1}, which was maintained at 8.32 log CFU mL^{-1} after two weeks of cold storage. However, lower values of 4.46 log CFU mL^{-1} and 2.43 log CFU mL^{-1} were recorded in samples with SPI and SM, respectively, during two weeks of cold storage (Figure 2). *L. rhamnosus* GG showed better adaptability to WPC supplementation compared to SPI and SM. It needs to be pointed out that low pH in these samples may also be contributing to the decrease in cell viability. The initial pH of juice with SPI and SM was 3.0 and 2.8, respectively and could not be adjusted higher because of observed coagulation of the juice matrix during supplementation of SPI and SM.

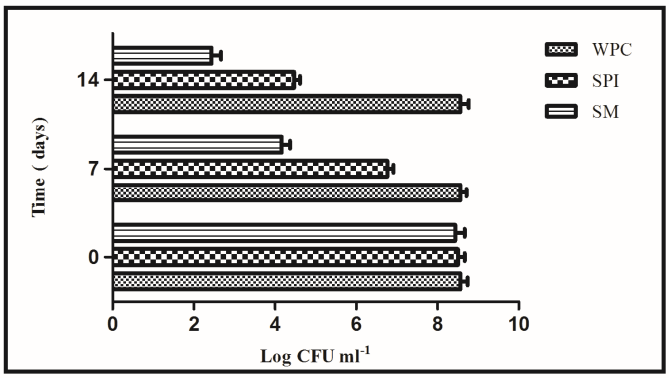

Figure 2. Viability of *L. rhamnosus* GG in WPC, SPI, and SM supplemented sea buckthorn juice during cold storage. The data presented are means of triplicate experiments ± standard deviation.

3.3. *Evaluation of* L. rhamnosus *as Protective Culture*

The property of LAB to inhibit target bacteria when they have reached their maximum level is usually described as the Jameson effect [30]. In our study, we observed that co-incubation of 6 log CFU mL^{-1} of *E. coli* with WPC-sea buckthorn beverage (containing 8 log CFU mL^{-1} of *L. rhamnosus* GG) at 4, 10, and 37 °C for two weeks, resulting in its complete elimination but at different time intervals (Figure 3).

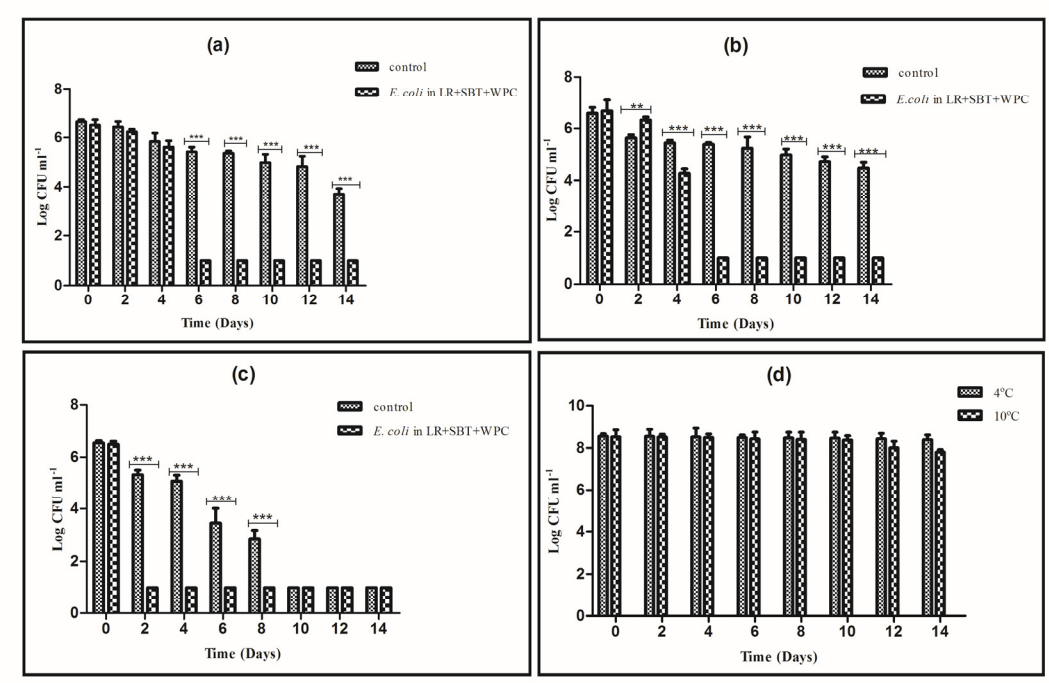

Figure 3. Anti-*E. coli* activity of *L. rhamnosus* GG fortified-WPC-supplemented sea buckthorn juice (LR + SBT + WPC) at (**a**) 4 °C, (**b**) 10 °C, (**c**) 37 °C, (**d**) viability of *L. rhamnosus* GG at 4 °C and 10 °C upon co-incubation. The data presented are means of triplicate experiments ± standard deviation. The variation of cell viability is found to be statistically significant at level $p \leq 0.001$ between juice samples during different time intervals (days).

Figure 3a,b depicts the suppression of *E. coli* by the probiotic fortified juice matrices stored at 4 °C and 10 °C. At both temperatures, complete elimination of the *E. coli* was observed within six days of co-incubation. Similar antibacterial results were obtained with *Lactobacillus* sp. in cheese matrix

as reported by Aljewicz and Grażyna [31]. Meanwhile, Figure 3c represents *E. coli* inhibition by the *L. rhamnosus* fortified juice matrix stored at 37 °C. There was a rapid decrease in the CFU count of *E. coli* from 6.4 log CFU mL^{-1} to below detection level (corresponding to CFU obtained below 1 log CFU mL^{-1}) within 24 h. This can be attributed to the production of organic acids or other antimicrobial compounds such as bacteriocins. Fayol-Messaoudi, has also implicated the non-lactic substance produced by *L. rhamnosus* GG, for its strong inhibitory activity against pathogens [32].

A control for each set of experiments at all three temperatures, was carried out with WPC-supplemented sea buckthorn juice (without *L. rhamnosus* GG). It was observed that the juice devoid of probiotics supported *E. coli* growth during the entire two weeks of cold storage at 4 °C and 10 °C. However, at 37 °C, *E. coli* could survive in the WPC supplemented sea buckthorn juice for up to eight days after which there was a decline in CFU which may have been due to the utilization of nutrients from the food system as a result of higher metabolic activity of *E. coli* at 37 °C. At all three temperatures there was a significant difference ($p < 0.001$) in survival of *E. coli* in probiotic fortified and unfortified juice matrices.

Figure 3d depicts the survival of *L. rhamnosus* GG in the juice matrix at 4 °C and 10 °C during two weeks of storage. The survival of *L. rhamnosus* in the juice was best maintained when stored at 4 °C. A stable count of 8 log CFU mL^{-1} was observed till the 14th day of cold storage. There was a reduction in viability of the probiotic in juice stored at 10 °C from 8.52 log CFU mL^{-1} to 7.98 log CFU mL^{-1}.

E. coli (ATCC 25922), being a common spoilage organism [16,17,33], and according to the studies by Mogna et al. [34] where they reported the highest antagonistic potential of *L. rhamnosus* GG against this strain, the current study assesses the potential of this probiotic strain as a protective agent in the WPC supplemented sea buckthorn beverage. The investigative probiotic, *L. rhamnosus* GG appeared to create an effective hurdle for multiplication of *E. coli* in the sea buckthorn-WPC system. Our work corroborates reports of other authors where they have suggested that fortification of Edam cheese with *Lactobacillus rhamnosus* LC705 significantly reduced *E. coli* counts [35,36]. Rolim et al. demonstrated inhibitory effects of *L. rhamnosus* incorporated in semi hard goat cheese (coalho cheese) against pathogens like *S. aureus*, *E. coli*, *L. monocytogenes*, and *S. enteritidis* [37]. Protein films added with cell free supernatants of *L. rhamnosus* also showed strong antimicrobial activity against *E. coli* along with other pathogens [38].

While the global probiotic market has advanced drastically over the years with a revenue collection of US$3,716.4 million in 2017 on sale of probiotic products, the Indian market still contributes less than 1% to the world market turnover in the probiotic industry [39,40]. However, according to the India Probiotic Market Forecast and Opportunities, 2019 (2014), probiotic market is projected to register a compound annual growth rate of 19.80% during 2014–2019 [41]. Dominated by mostly milk and fermented milk based products, there is an immense scope for the development of such non-conventional, non-fermented, novel fruit based probiotic formulations, which is expected to gain acceptance due to the emerging health consciousness among consumers, especially in India where there is a special need for women and pediatric nutrition. This is the first time *L. rhamnosus* GG has been evaluated as protective agent in a sea buckthorn-WPC matrix against a non-pathogenic strain, *E. coli* (ATCC 25922). Further evaluation of protective action of this beverage and the *L. rhamnosus* GG culture against other contaminating agents is in progress. Such exploratory studies increase the knowledge base and will help to improve the utilization of sea buckthorn fruit for novel product development.

4. Conclusions

The obtained results clearly indicate that the sea buckthorn matrix, when supplemented with protein and dextrose, may be susceptible to growth of spoilage organisms. However, the fortification with *L. rhamnosus* GG increased the safety against *E. coli.* Currently, the only sea buckthorn products available commercially in India, are in the form of juices like 'Lehberry'. Our work lays a foundation for development of a protein-supplemented, probiotic-fortified, ready to drink beverage which will add value to the available product line and increase future marketability.

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Author Contributions: Gargi Dey conceived and designed the experiments; Srijita Sireswar, Kinjoll Dey, and Arkasish Kundu performed the experiments; Srijita Sireswar analyzed the data; Gargi Dey and Srijita Sireswar both contributed to writing the manuscript.

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