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Formulation and Evaluation of Herbal Drink Probioticated Using Enterococcus durans Isolated From Curd

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Abstract - *The present study was performed to formulate and evaluate nutritional enriched probiotic herbal beverages using selected herbs. Hemides musindicus (Nannari roots), Ocimum tenuiflorum (Basil), and Aloe vera were selected for study, and it was formulated into herbal juice in three different flavors by incorporating lemon, pineapple, and ginger in the combination of various trials. The best formulation was selected based on Nine points Hedronic scale method. Lactic acid bacteria were isolated from different probiotic sources, and potential bacteria were identified through screening them using a different range of pH, bile, salt, and temperature. Potential strains were molecularly characterized and found to be Enterococcus durans. Hence, using this potential isolate, a probiotic herbal drink was formulated. The formulated drink was subjected to nutritional analysis and antioxidant assay, and it was found to be improved when compared to herbal drinks alone. A significant difference was noticed in antimicrobial activity between probiotic herbal drinks and herbal drinks alone.*

Keywords - *Herbal drink, Probiotic bacteria, Enterococcus durans, Nutritional analysis*

I. INTRODUCTION

Food and nourishment are core pillars of human health and development. However, due to modernization and transformation in lifestyle, some traditional food methods are being outdated. In the last few years, there has been an encouraging development in the field of herbal medicine. In many developing countries, a huge population depends on traditional practitioners in order to fulfill the needs of their health care. Evaluating medicinal plant products based on their therapeutic value lead to the discovery of new food products for treating various health disorders, which seems to need of the hour. This serves as a platform for the progress of new drugs from various plant sources [1].

Herbal extracts are chosen as safer and flavor enhancers without any side effects and used for culinary and medicinal purposes for ages [2]. Chemical compounds in herbal plants facilitate their effects on the human body through binding to receptor molecules present in the body. However, the synergistic combination of several active principles in some herbal preparations is responsible for their beneficial effects. Hence in the present study, popular herbs, namely, *Hemides musindicus* (Nannari roots), *Ocimum tenuiflorum* (Basil), and *Aloe vera,* will be utilized for the production of probioticated herbal drinks. These herbal varieties are easily available in Southern parts of India.

Nowadays, probiotics have become the buzzword of sorts and proved to have more health benefits through improving the microbial balance in the intestine, which concurrently enhance the immune system. Probiotics are live microbial food supplement which has several beneficially effects on the host through improving the intestinal microbial flora [3]. Most probiotic microorganisms are Lactic Acid Bacteria (LAB), such as *Lactobacillus acidophilus, Lactobacillus Plantarum, Lactobacillus casei, and Streptococcus lactis* [4]. Research has shown that the addition of probiotics to food provides several health benefits, including a reduction in the level of serum cholesterol, improved gastrointestinal function, enhanced immune system, and lower risk of colon cancer [5]. Probiotic product to be beneficial, it must contain at least 10^6cfu/g of viable bacteria[6]. The development of new food products turns out to be increasingly challenging since it has to be healthy and fulfill the consumer's expectancy. Probiotic products are commonly available in the form of fermented milk and yogurt. However, many individuals cannot consume these products because of their high cholesterol and lactose content. Fruit, vegetable, and Herbal based juices have been suggested as media for cultivating probiotics because of their beneficial nutrients [7]. Hence, in the present research, an attempt was made to formulate herbal health drinks using these traditional herbs. Since probiotics play a vital role in our life and these microorganisms have a symbiotic relationship with the host organisms and its combination in the herbal drink will definitely provide numerous benefits to people.

II. MATERIALS AND METHODS

A. Formulation of herbal drinks

Herbal leaves from *Ocimum tenuiflorum* (Basil), Lemongrass (*Cymbopogon citratus*), and herbal root of Nannari (*Hemidesmus indicus*) were procured from Erode district. The leaves and roots were washed well and blended in a pestle mortar. The thick paste was used to extract the juice with the help of distilled water and a muslin cloth. Fresh *Aloe vera* leaves were collected, and the gel was removed using a clean and sterile spoon. It was blended into juice using a food-grade blender. Above ingredients, the juice was then used in different ratios in the preparation of the herbal juice [8].

Ginger was sliced into small pieces and then blended very finely in a pestle mortar. The juice was extracted using distilled water. Lemon juice and pineapple juice were extracted using a household mixer and then used along with the pulp. Sugar syrup was made using 100 gms of sugar and 50 ml of water heated to 100°C. The herbal drink was formulated using the above ingredients in three different flavors of lemon, pineapple, and ginger with the addition of 50ml of sugar syrup (Table 1).

Table 1. Composition of herbs for the formulation of Herbal drink

Trial	Quantity of Herbal extract (ml)				Juice (ml)		
	Basil	Nannari	Lemon grass	*Aloe vera*	Lemon	Pineapple	Ginger
A	5	5	10	5	25	-	-
B	5	5	10	5	-	25	-
C	5	5	10	5	-	-	25
D	3	10	5	7	20		5
E	7	5	10	3	5		20
F	5	5	10	5	15	5	5

B. Sensory evaluation of herbal drinks:

The sensory evaluation was performed with the help of ten panels, and each panelist individually evaluated the herbal drink. The herbal drink quality was assessed as color, aroma, taste, infusion, and overall appearance. The rating for each characteristic was based on a Nine -point hedonic scale method [9].

C. Isolation and identification of potential probiotic strains

For isolation of Lactic acid bacteria, samples such as milk, yogurt, buttermilk, and curd samples were serially diluted and incubated anaerobically at 37°C for 2-3 days using De Man, Rogosa, and Sharpe agar (MRS agar). The plates were observed for individual colonies, and triplicates were maintained.

D. Biochemical analysis of LAB isolated from various probiotic sources

Biochemical tests such as Gram staining, Indole test, Catalase test, Casein hydrolysis test, motility, and carbohydrate fermentation test were performed to characterize the isolated Lactic acid bacteria.

E. Screening of potential isolates:

The potential strains for the probiotic action process were selected based on acid, bile, Nacl, and temperature tolerance assay [10,11]. To identify the acid-tolerant isolates, each parent strain of LAB bacteria (1%) was inoculated in MRS broth at 37°C for 24hrs. Cell pellets were collected from the above broth and were washed twice and resuspended into 10 ml of phosphate-buffered saline (PBS) with a pH of 3.5. The tubes were incubated at 37°C, and the viable organisms were counted after exposure to acidic conditions for 0, 1, 2, 3, and 4 hours on MRS agar incubated at 37°C for 48 hours [12].

The survival percentage was calculated as follows:

% survival = final (cfu/ml) / control (cfu/ml) × 100

Bile salt tolerance of the strains was investigated by a method illustrated by Gopal et al. (1996)[13]. MRS broth containing different levels (0.05%, 0.1%, 0.15%, and 0.3%) of bile salts (Oxgall) was used for the study. Freshly prepared cultures were inoculated (1%) into medium and incubated at 37°C for 24 h under anaerobic conditions. The ability of selected strains to grow in NaCl tolerance was assessed by inoculating in MRS broth supplemented with different concentrations of NaCl (1.5, 2, 2.5, 3, and 3.5%). Similarly, temperature tolerance was determined at 20°C, 30°C, 40°C, and 50°C through inoculating the strains in MRS broth for 24 hr. The growth was assessed by measuring the optical density at 560nm using a UV-VIS spectrophotometer.

F. Molecular characterization of selected strain:

The potential probiotic isolate obtained was subjected to molecular characterization to identify the specific strains through 16S rRNA technology.

G. Probiotication of herbal drinks

The potential probiotic cultures screened were used for the probiotic action of herbal drinks. A total of 2% inoculums of *Enterococcus durans* (Identified as a potential isolate)were added to the sterile herbal drink for the probiotic action process. The inoculated drink was incubated at 37° C for 72 h. Bacterial counts were measured periodically during incubation.

H. Proximate Analysis and Antioxidant Activity of Probiotic Herbal drink:

The number of carbohydrates present in the herbal drink was estimated by the Anthrone method [14]. The absorbance was measured at 620nm against the blank, and total sugar content

was expressed as g/100 ml of glucose. The total protein present was estimated by Lowry's method [15], and the Vitamin content present in the probioticated herbal squash was evaluated by the method outlined by Ranganna, 1999 [16]. The antioxidant activity of the sample was evaluated by DPPH assay.

I. Antibacterial activity

The antibacterial activity of probioticated Herbal juice was evaluated through a well diffusion method [17]. Two bacterial culture *Staphylococcus aureus and Escherichia coli* were selected for the present study and was obtained from Department of Biotechnology, K.S.Rangasamy College of Technology, Tiruchengode, Namakkal dist., The plates were incubated at 37^0C for 24 hours, and the zones of inhibition were measured.

J. Shelf life analysis

The probioticated herbal drink was prepared under sterile conditions, packed in an airtight container, and stored at 4°C for 4 weeks. Samples are taken at weekly intervals, and the viability of probiotic cultures in probioticated juice is determined and expressed as a colony-forming unit (CFU) using MRS media. The contamination was checked using nutrient media in the time interval of 7days for one month.

III. RESULTS AND DISCUSSION

A. Optimization of Herbal RTS beverage:

Lemon, Pineapple, and Ginger based herbal RTS beverage was prepared in six different combinations and subjected to sensory evaluation for optimization of the percentage herbs to be added in the drink. The results obtained from the sensory evaluation (Table 2) clearly indicate that formulation D with the combination of both lemon and ginger-based RTS gave a better score than compared to other formulations. Flavor and taste gave the score of 8 and 9, and overall acceptability was found to be 7.75. However, appearance and consistency have got a similar score of 7 with like moderately. Hence, formulation D was selected for further analysis. According to results found by Brito et al. (2004)[18] in a study of nannari syrup, the scores were obtained for color (1.5 to 3.5), for aroma (1.6 to 4.1), for turbidity (2 to 3.1).

Table 2. Sensory evaluation of formulated herbal squash

Trial	Criteria				
	Appearance	Flavor	Taste	Consistency	Overall Acceptability
A	6	6	7	6	6.25
B	5	5	6	6	5.5
C	6	6	7	7	6.5
D	7	8	9	7	7.75
E	5	6	7	7	6.25
F	7	6	7	7	6.75

B. Isolation and biochemical characterization Lactic acid bacteria:

The population density of LAB isolated from various probiotic samples, along with their designation, is mentioned in Table 3. Among the total of 72 colonies, the desired four glistening colonies representing each source were picked up and subcultured in MRS media to get pure culture. Four strains were designated as CO1, Y02, M01, and B03 with respective to their source of isolation, such as curd, yogurt, milk, and buttermilk. Gram staining results showed purple-colored rod-shaped, short, and medium-chain, which indicates the presence of gram-positive and typical characteristics of *Lactobacillus* spp. Another biochemical tests such as indole, catalase, MR-VP test showed negative results, and all the strains were found to be non-motile. Casein hydrolysis test showed positive results, and a zone of clearance was observed. Hence the above basic biochemical analysis confirmed the presence of Lactic acid bacteria. The results of the carbohydrate utilization test revealed that all the isolates were able to ferment glucose, fructose, lactose, and sucrose.

Table 3. Biochemical analysis of Lactic acid bacteria isolated from different probiotic sources

S.No	Biochemical analysis	Strains isolated*			
		C01	Y02	M01	B03
1	Gram staining	+	+	+	+
2	Indole test	_	_	_	_
3	Catalase test	_	_	_	_
4	Casein hydrolysis test	+	+	+	+
5	Motility	_	_	_	_
6	Methyl Red (MR) test	_	_	_	_
7	VogesProskauer (VP) test	_	_	_	_
8	Glucose fermentation	+	+	+	+
9	Fructose fermentation	+	+	+	+
10	Lactose fermentation	+	+	+	+
11	Sucrose fermentation	+	+	+	+

*C01 isolated from curd, Y02 isolated from Yogurt, M01 Isolated from Milk and isolated from Buttermilk

C. Screening of potential probiotic isolates:

The results of acid tolerance of LAB at pH 3.5 for the exposure time of 1hr, 2hr, and 3 hr were illustrated in Fig. 1. Among the four strains, CO1 isolated from milk showed higher tolerance to acidic pH. The survival percentage was 35.29%, and it was followed by BO3 with 18.75% of survival. M01 and YO2 gave a survival percentage rate of 23.5 and 66.67% initially at one hr incubation at the acid condition. However, after three hours of incubation, both the cultures were unable to proliferate. The study conducted by Brizuela et al. (2001)[19] revealed that *Lactobacillus* cultures were able to tolerate the acidic pH 3. These results highly correlate with the present study, whereasstrainCO1 was able to tolerate the high pH and proliferate in MRS agar.

The survival rate of lactic acid bacteria at different concentrations of Oxgall was clearly illustrated in Fig. 2. Initially, at the concentration of 0.05%, no significant difference was observed between the isolates. Conversely, a vast significant difference was observed between the isolates at an increased concentration of bile salt at 0.3%. CO1 strain isolated from curd showed the highest survival rate of 40%, followed by BO3 isolate of 12.5%. The isolates MO1 and YO2 were weakly tolerant and could not able to survive the 0.3% Oxgall. The growth of lactic acid bacteria in different concentrations of NaCl is illustrated in Fig. 3. The results revealed that, as the concentration of NaCl increases, the growth of lactic acid bacteria gradually decreases. However, the CO1 isolate obtained from curd showed the highest growth rate compared to other isolates. Similarly, temperature tolerance was also favorable to CO1 isolate, followed by Y02 (Fig. 4). Hence from the experiment, CO1 was found to be highly potential and selected for further studies. According to the study conducted by Yuliana et al. (2010) [20], revealed that *L. acidophilus* was able to tolerate the temperature of 5°C.

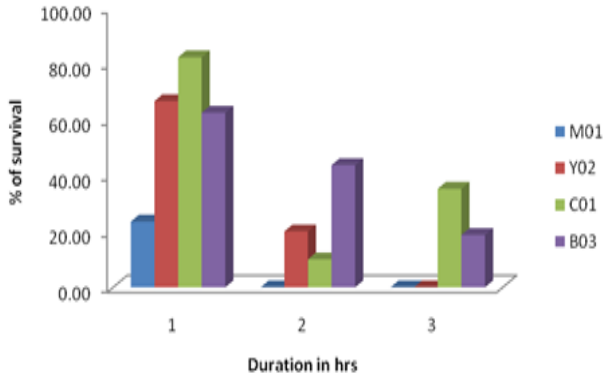

Fig. 1 Survival rate of lactic acid bacterial strains in acidic condition (3.5 pH)

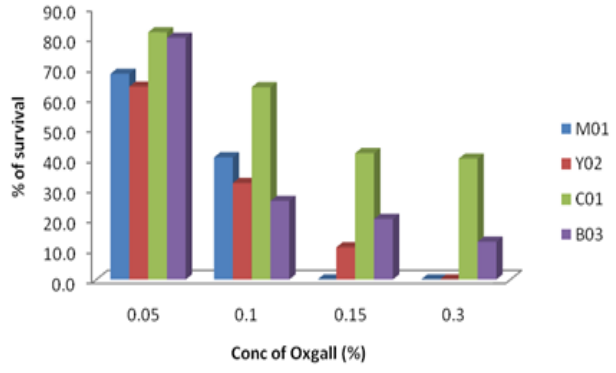

Fig. 2. The survival rate of lactic acid bacterial strains the indifferent concentration of Bile salts

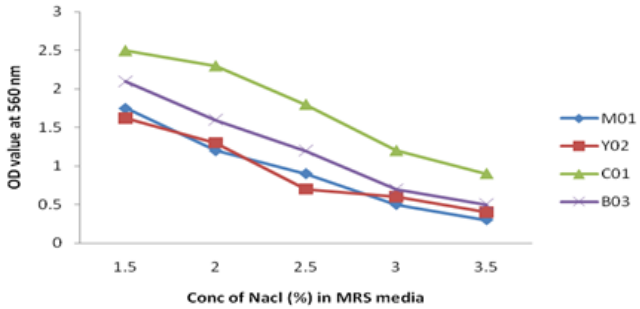

Fig. 3. Growth of lactic acid bacterial strains in different concentrations of NaCl

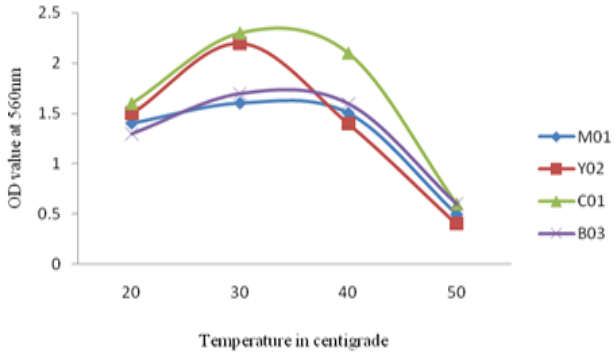

Fig. 4. Growth of lactic acid bacterial strains in a different range of temperatures

D. Molecular characterization of CO1 isolate:

CO1 isolate was subjected to molecular characterization, and it was identified as *Enterococcousdurans*. The identified bacteria sequence was deposited at NCBI, Maryland, USA. Hence the accession number of isolated strain was KX129817.

E. Growth of Probiotic bacteria in Herbal juice:

E.durans isolate was used as probiotic bacteria to develop probiotic herbal drinks. The culture was inoculated into the sterile herbal juice at various concentrations of 1%, 2%, and 3%. Growth was monitored through OD value, CFU, and pH, which were illustrated in Figures 7, 8, and 9, respectively. Initial OD values were found to be almost similar for all the concentrations. Growth was found to increase significantly after 72hrs of incubation in a 3% concentration of inoculum. After 96 hrs, the OD value has decreased gradually. It reveals the occurrence of stationary phase followed by decline phase of growth. Hence the result clearly focuses that, 3% concentration of *E.durans*inoculum was able to proliferate in herbal juice. The number of colonies obtained after serially diluted probiotic juice was highly correlated with OD values.

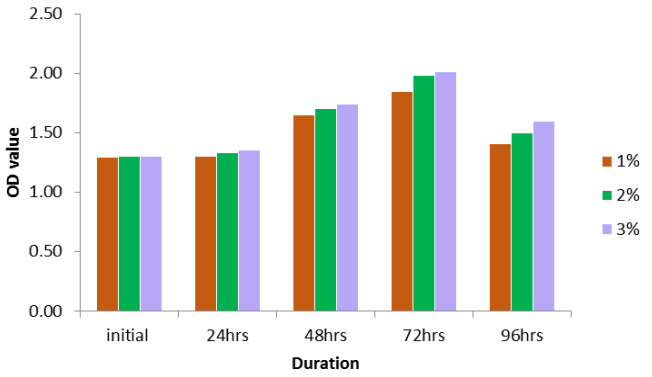

Fig. 5 OD Value of *E.durans*inoculated in the herbal drink.

***F. Nutritional evaluation of Probiotic Herbal drink*:**

Total sugar content present in the samples were discussed in fig. 10. Among the different herbs used, lemongrass was found to have the least sugar content with 0.414 mg/ml. However, no significant difference was observed between the samples. The probiotic herbal drink was reported to have 0.435mg/ml of total sugar, and whereas the Herbal drink was reported to have 0.429mg/ml of total sugar content. Vitamin C content of Probiotic herbal drink was found to be 2.04mg/ml. This was found to be higher than herbal drinks with a significant difference.

G. Antioxidant activity of probiotic herbal drink

The results of ferric reducing ability (FRAP) were illustrated in Fig. 3.10. The development of intense blue color reveals the reduction of ferrous compounds, which correlate with the antioxidant present in the samples. The R^2 value of probiotic herbal juice was found to be 0.979 and which was higher than herbal juice and a standard sample of 0.946 and 0.947, respectively. However, no significant difference was observed with all the samples. According to Ravi et al. (2012)[21], the study was conducted.

Table 4 Nutritional evaluation of probiotic herbal drink and herbal drink alone

S.No	Parameters	Probiotic herbal drink	Herbal drink alone
1.	Total sugar content (mg/ml)	0.44±1.37	0.43±1.65
2.	Protein content (mg/ml)	0.54± 0.73	0.52± 0.65
3.	Vitamin C (mg/ml)	2.04±0.56	1.89±0.76

Values represent average ± SD

H. Scavenging of Hydrogen peroxide activity for Probiotic Herbal juice:

The percentage of inhibition of Hydrogen peroxide activity was evaluated to determine the Antioxidant of Probiotic herbal juice and Herbal juice, and it was illustrated in Fig. 8. The results reveal that probiotic herbal juice exhibit a higher percentage of inhibition at a different level of concentration. A significant difference was observed at an initial concentration of 20µg/ml. However, no significant difference was observed for other concentrations for both probiotic herbal juice and herbal juice. Hence, the present study clearly illustrates that antioxidant activity was found to be improved due to the probiotic action process.

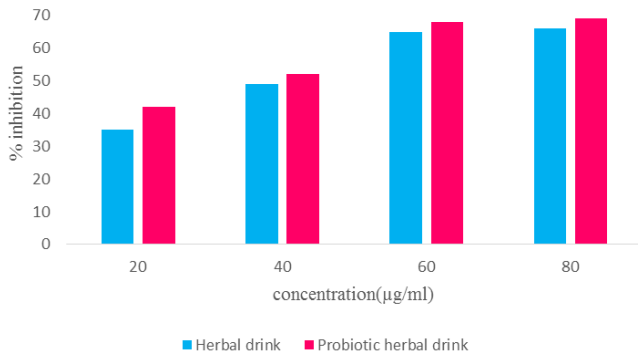

Fig. 8 Scavenging of Hydrogen peroxide activity for Probiotic Herbal juice and Herbal juice

I. Antibacterial activity of Probiotic Herbal Drink and Herbal juice

Antibacterial activity of probiotic herbal juice and Herbal juice against *Staphylococcus aureus* and *Escherichia col* was described in Table 5. The zone of inhibition was measured at two different concentrations. Probiotic herbal juice gave 2.10cm and 2.26cm zone for 50µl and 100µl respectively for *S. aureus*, and zone of inhibition had slightly increased when probiotic herbal juice was evaluated against *E.coli*. Hence, the above results clearly pictured that probiotic herbal juice poses a significant amount of antibacterial compounds when compared to Herbal juice and standard ampicillin disc.

Table 5 Antibacterial activity in Probiotic herbal drink

Bacterial strains	Zone of inhibition (cm)				
	Control (Ampicillin)	Herbal juice		Probiotic Herbal juice	
		50µl	100 µl	50 µl	100 µl
Staphylococcus aureus	1.20 ±0.05	1.70 ±0.1	1.80 ±0.10	2.10 ±0.10	2.26 ±0.05
Escherichia coli	1.16 ±0.05	1.86 ±0.05	2.10 ±0.10	2.13 ±0.05	2.53 ±0.05

IV. CONCLUSION

An attempt was taken to formulate and evaluated probiotic herbal drinks in different flavors. The formulated herbal drink was found to be acceptable, with an overall score of 7.75 in the sensory evaluation test. It was further probioticated using potential probiotic bacteria isolated for curd. Probiotic bacteria showed higher resistance to lower pH, bile, Nacl, and temperature, and it was molecularly characterized as E.durans. The probiotic herbal drink was subjected to nutritional assay and antioxidant studies, which gave outstanding results. Shelf life analysis gave positive results, and hence, herbal drinks fortified with potential probiotic isolates will infer the nutraceutical benefits to the consumers through providing several health benefits.

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CONFLICT OF INTEREST

The authors declare that they have no Conflict of interest

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