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In vitro Evaluation of Antidiabetic Properties of Probiotic Lactic Acid Bacteria Isolated from Herbal Extracts (*Asava, Aristha, Sarko, Swarasa*)

Research Article

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Abstract

Probiotic bacteria have immense potential to be used as biotherapeutic agents. They are alternatives to many allopathic medicines, antibiotics that can be used for the treatment of diabetes without causing any side effects. Generally, Lactic acid bacteria have been isolated from human and animal origin to be used as therapeutic or nutraceutical agents. However, there is lack of information about the role of natural herbs associated LAB to be used as biotherapeutic agent for the treatment of diabetes. Diabetes is a common disease that affects globally to major population worldwide. The impact of this disease is greater however, complete cure of diabetes s is not available till date. Thus many reseraches is going on this direction to fi d the better cure in a natural way. In the present study isolation of Lactic acid bacteria was carried out from the herbal extracts like *Asava, Aristha, Sarko and Swarsa*. About 29 isolates were screened for probiotic potential. On confirmation, the isolates were subjected to evaluate for anti-diabetic properties. The results revealed that out of 29 isolates about 8 isolates were found to have antidiabetic properties. From the result, it can be said that, this opens new dimension in the field of medicines for the treatment of diabetes and related ailments with biotherapeutic agents.

Keywords: Lactic acid bacteria, Probiotics, Herbal extract, Antidiabetic potential.

Introduction

Probiotics have beneficial effects on the healthy body. It keeps microbial balance in the gastrointestinal tract. According to previous scientific reports, probiotics have anticancer effects, cholesterol lowering effects and anti-allergic. It also improves immune response of host, treats irritable bowel syndrome and improves intestinal inflammation (1). These microbial probiotic strains have been collected directly from the gastrointestinal tract or other sources such as from faeces or milk. The most common group of microorganism used for the preparation of probiotics is the lactic acid bacteria (LAB), which are natural flora of gastrointestinal tract of human. Thus, they are considered to be safe according to Food and Drug Administration (FDA) (2). Causes behind diabetes mellitus are deficiency of insulin, hyperglycemic condition, metabolic dysfunction of sugar, fats and proteins (3). A world wide survey held between 1980-2014 revealed increase in diabetes from 108 million to 422 million. According to report of 2012, 2.2 million deaths had been occurred before age of 70 due to hyperglycemia .in blood. Similarly, in 2016 approximately 1.6 million deaths were caused by diabetes (4). Other health problems faced by diabetic patients are kidney failure, heart attacks, amputation of the lower limb, blindness (5). Moreover, Type -2 diabetes may also cause due to formation of reactive oxygen species (ROS) that leads to malfunctioning of cells (6,7). According to the report of World Health Organization (WHO), around 70-80% of the world's population relies on non-conventional medicine mainly from herbal sources. Demand of medicines prepared from herbal sources is gradually increasing in developing countries (8). Therefore, the use of natural, safe and economic alternatives like the use of probiotics from herbal sources can be used to cope with these issues.

In India, herbal biomedicine has been practiced for thousands of years under the science of Ayurveda. The term Ayurveda is derived from the words "Ayur" means life and "Veda" means knowledge. These herbal remedies are diverse, including herbal teas, infusions, decoctions, tinctures, capsules, powders, infused oils, salves, creams and lotions, etc., as well as arishta (fermented decoctions) and asavas (fermented infusions). As recently reported, asava and arishta are very popular formulations of Ayurvedic medicine.

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These asava and arishta preparations are also called medicinal wines because they involve a fermentation process to produce alcohol (9). These medicinal preparations have many advantages such as better shelf life, effective separation of drugs from herbal preparations and better therapeutic properties (10).

According to the literature survey, it has been realized that, no systemic approach has been made to evaluate antidiabetic properties of probiotic organisms isolated from the herbal extracts. Thus, to bridge the gap of existing knowledge, the present study involves in vitro evaluation of antidiabetic potential of probiotic isolates from herbal extract viz., *Asava, Aristha, Sarko, Swarasa.*

Materials and Methods

Sampling and Enumeration of Microorganisms

The samples of herbal extracts viz. *ASAVA* (Kumari Asava no-1), A*RISHTA* (*Dashamoolarist), SARKO* (Apple cider vinegar), *SWARASA* (Amla) were collected from Dhanvantri clinic, Vyara, Dist-Tapi, Gujarat, India.

For isolation, about 1ml of each sample was enriched in a De Man, Rogosa, and Sharpe (MRS) broth and incubated under aerobic condition at 37°C for 24h. The enriched broth samples were diluted using phosphate buffer saline (PBS) and plated on a nutrient agar medium, and then incubated under aerobic condition at 37°C for 24h. The morphologically discrete colonies were further sub -cultured on to MRS agar plates. The viable cultures were stored in nutrient agar slants at 4°C. The stock culture was maintained at -20°C in 40% glycerol stock for further analysis.

Biochemical characterization of bacteria

The bacteria were identified by biochemical tests (Gram's staining, phenotypic characteristics, citrate utilizing assay, osmotic stress (sodium chloride:3%and 5%) resistance, and sugar fermentation ability of isolates by assimilation of different sugar supplements (11,12). The growth of the isolates at different pH levels were also tested.

Survival under *in-vitro* gastrointestinal conditions

Acid tolerance

Initial screening of isolates was carried out for acid tolerance. For the same isolates were exposed to pH 2. The isolates were grown on sterile nutrient agar plate (pH was adjusted as aforesaid). The cell suspension was then plated on nutrient agar and incubated at 37°C, After, incubation plate with more than 57% growth were considered for the further study (13).

Bile salt tolerance

The medium with varying concentrations of bile salt (0.3% and 0.5%) was inoculated with each selected bacterial culture and incubated at 37°C for 24h, then 0.1mL inoculum was transferred to nutrient agar by pour plate method and incubated at 37°C 24h. The growth of lactic acid bacteria (LAB) cultures on agar plates indicates that isolate as bile salt tolerant (13).

Auto aggregation and coaggregation

Auto aggregation assay was performed according to Del Re et al. 2000 modification (14). Isolates were grown for 24 h at 37°C in nutrient broth. The cells were harvested by centrifugation at 5000 rpm for 15 min, washed twice and resuspended in their culture supernatant fluid or in phosphate buffer saline (PBS) to give viable counts of approximately 10 CFU ml^{-1}. Cell suspensions (4mL) were mixed by vortexing for 10s and auto aggregation was determined during 5h of incubation at room temperature. Every hour 0.1mL of the upper suspension was transferred to another tube with 3.9mL of PBS and the absorbance (*A)* was measured at 600nm.

The auto aggregation percentage is expressed as:

$$1\text{-}(At|Ao) \times 100$$

where *A,* represents the absorbance at time *t*=1,2,3,4 or 5 h and *A*o the absorbance at *t*=0.

The method for preparing the cell suspension for coaggregation was the same as that for auto aggregation assay. Equal volume (2mL) of each cell suspension were mixed together in pairs by vortexing for 10 s. The absorbance (*A*) at 600 nm of the suspension were measured after mixing and after 5 h of incubation at room temperature. Samples were taken in the same way as in the auto aggregation assay.

Coaggregation was calculated using equation of Handley et al., 1987 in percentage as follow (15):

$$\text{Percentage of Coaggregation} = \frac{[(Ax+Ay)/2]-A(x+y)}{Ax+Ay/2} X\ 100$$

Where, x and y represent each of the two strains in the control tubes, (x+y) represents mixture of the two strains

Safety Evaluation

DNase activity

The isolates were streaked onto a deoxyribonuclease (DNase) agar medium to test for production of the DNase enzyme. The plates were then incubated at 37°C for the and observed for the zone of DNase activity. A clear pinkish zone around the colonies was consider as positive DNase activity (16).

Evaluation of *in vitro* antidiabetic activities

The α-amylase and α-glucosidase inhibitory activities of the isolated strains were assessed using culture supernatant (CS)**.** To prepare CS, each strain was cultured in nutrient broth and incubated at 37°C for 15h. After incubation, culture was centrifuge at 800×g for 15min.

Alpha (α)-amylase inhibitory activity

α-amylase inhibitory activity of the strains was evaluated as described by Chen et al. (17). About 250µL of culture supernatant (CS) was added to 250µL of α-amylase solution (0.5mg/mL) and pre-incubated at 25°C for 10min. The reaction mixture was then incubated with 250µL of starch solution (1% w/v in 0.02M sodium phosphate buffer) and incubated at 25°C for

10min. Next, the reaction was terminated with the addition of 50µL of DNS colour reagent (96 mM DNS and 5.31M sodium potassium tartrate in 2M sodium hydroxide solution). The reaction mixture was then boiled for 5min, allowed to cool, and diluted four-fold with water. The absorbance was measured at 540nm. The inhibition was calculated as follows:

$$\text{Inhibition (\%)} = (A-B)/A \times 100$$

Where, A is the absorbance of the control and B is the absorbance of the sample.

α-Glucosidase inhibitory activity

α-Glucosidase inhibitory activity of the strains was measured according to the methods described by Chen et al. (17). About25 µL of culture supernatant (CS) was added to a reaction mixture containing 150µL of 0.01M PBS (pH 7.0) and 75µL of 0.02M PNPG solution, and pre-incubated at 37°C for 10min. The reaction was initiated with the addition of 50 µL α-glucosidase (0.17units/mL) and the sample was incubated at 37 °C for 10 min. Next, the reaction was terminated with the addition of 1mL of 0.1M Na2CO3. The amount of p-nitrophenol released was determined by measuring the absorbance at 40 nm. The inhibition was calculated as follows:

$$\text{Inhibition (\%)} = [\ 1 - (C - D)\ (A - B)] \times 100$$

where A is the absorbance with α-glucosidase but without sample, B is the absorbance without α-glucosidase and sample, C is the absorbance with α-glucosidase and sample, and D is the absorbance without α-glucosidase but with the sample.

Results and Discussion

Isolation of lactic acid bacteria (LAB) was carried out on sterile MRS (De Man, Rogosa and Sharpe agar) solid medium. Total 29 isolates were obtained. These isolates were further subjected to physiological and biochemical tests for the identifications of isolates.

Preliminary characterisation of isolates

All the isolates grown within 24 h at 37°C were selected. Majority of isolates shared common features of round shaped colonies having flat and convex type of elevation. The size was categorised as large and small. Most of colonies of an isolate showed entire and undulate margins. Consistency of all colonies were moist. Opacity of all isolates was found to be opaque. Isolates formed mainly pigmented colonies (yellow or pale yellow). From, the results of various physiological and biochemical tests, 17 isolates were selected. These isolates were of Gram-positive nature, gave catalase test negative, disable to reduce nitrate, unable to utilise Simmon's citrate, able to show growth in osmotic stress condition were confirmed as lactic acid bacteria (LAB). Lactic acid bacteria are a group of non-sporulating, Gram- Positive, aerobic, facultative aerobic and anaerobic, rods or cocci. They produce lactic acid (18). These confirmed LAB isolates were further subjected to assess probiotic potential through *in vitro* characterisation (Table 1).

Table 1: Preliminary characterisation of isolates by biochemical investigation

No	Isolates	Nitrate reduction assay	Citrate utilization	Catalase	Nutrient lactose broth	Nutrient glucose broth	Nutrient sucrose broth	Osmotic tolerance 3%	Osmotic tolerance 5%
1	Iso 1	+	-	-	+	+	-	+	-
2	Iso 2	+	-	-	+	-	-	++	+
3	Iso 3	-	-	-	+	+	-	+	-
4	Iso 4	-	-	+	+	+	-	+	-
5	Iso 5	-	-	-	+	+	-	+++	++
6	Iso 6	+	+	+	+	+	-	-	-
7	Iso 7	+	+	+	+	+	+	++	+
8	Iso 8	-	-	-	+	+	+	++	+
9	Iso 9	+	-	+	+	+	+	++	+
10	Iso 10	-	-	-	+	-	+	+	-
11	Iso 11	-	-	-	+	-	-	++	+
12	Iso 12	+	-	-	+	+	+	++	+
13	Iso 13	-	-	+	+	+	+	+	+
14	Iso 14	+	-	+	+	-	-	+	+
15	Iso 15	+	-	-	+	+	+	+	-
16	Iso 16	+	-	-	+	+	+	++	+
17	Iso 17	+	-	-	-	+	+	-	-
18	Iso 18	+	-	-	-	+	+	++	+
19	Iso 19	+	-	+	+	-	+	++	+
20	Iso 20	+	-	+	+	+	+	-	-
21	Iso 21	-	-	-	+	+	-	++	-
22	Iso 22	-	-	-	+	+	-	+	+
23	Iso 23	+	-	+	+	+	+	+	-

24	Iso 24	+	-	-	+	-	+	+	-
25	Iso 25	+	-	+	+	+	-	+	+
26	Iso 26	+	-	-	+	+	-	++	+
27	Iso 27	-	-	-	+	-	-	+	-
28	Iso28	+	-	+	+	+	-	+	+
29	Iso 29	-	-	+	+	+	-	+	+
Where, + indicates Test is Positive, – indicates Test is Negative									

In vitro characterisation of probiotic properties of lactic acid bacteria isolates

Selected 17 LAB isolates were subjected to in vitro characterisation in order to assess the probiotic potential of isolates. The biochemical investigation revealed that 17 isolates were showing high potential of probiotic potential Thus, isolates were exposed to simulated gastric intestinal environment with acidic pH 2, high bile salt concentration. Isolates were also assessed for its autoaggregation and coaggregation abilities.

When exposed to gastric environment of low pH 2 out of 17 isolates only 8 isolates were able to tolerate low acidic pH of gastric environment. With losing potential of probiotic properties. Thus for further studies only 8 isolates with good survivability were selected and rest 9 were omitted from the study.

Resistance to acid, bile, simulated gastric and intestinal conditions

Probiotic bacteria have to essentially prevail through the stress environment, acidic stomach, bile salts and colonise in the intestine. As, ingested LAB is exposed to gastric fluid, bile enzymes and subsequently attaches to the intestine (19, 20). The pH of stomach varies between pH 1.5 and 3.0.

Initial screening showed that the 17 isolates were tolerant to pH 2 (Table 2). As the acidic pH cause destruction to many microorganisms ingested, resistance to human gastric environment transit play major selection criteria for probiotic microorganisms. The gastric transit was observed from 0 to 90 min and then up to 180 min (21) (Table 3). *In vitro* bile salt tolerance helps to determine metabolic activity and colonisation of isolates in the small intestine. The survival of the isolates in the bile salts for 24 h at 37ºC is presented in Results of bile salt and acid tolerance are represented in (Table 4).

Table 2: Survival of the potential LAB isolates in the acidic condition

No.	Isolates	Acidic (2pH)
1	Iso 3	+++
2	Iso 5	+
3	Iso 8	+++
4	Iso 10	++++
5	Iso 11	++
6	Iso 21	+
7	Iso 22	+
8	Iso 27	++
Where, ++++ Excellent, ++ Good, +++ Moderate, + Poor		

Table 3: Tolerance of the LAB isolates to *in vitro* simulated gastric juice condition

No.	Isolates	Exposure time	Gastric tolerance pH 2	
			Viable cells (cfu/mL)	Survival rate %
1	Iso 3	0 h	5.17×10^2	100
		90 min	3.21×10^2	62.08
		180 min	1.88×10^2	36.636
2	Iso 5	0 h	8.4×10^2	100
		90 min	5.9×10^2	70.23
		180 min	3.2×10^2	38.09
3	Iso 8	0 h	8.75×10^2	100
		90 min	6.96×10^2	79.54
		180 min	5.32×10^2	59.77
4	Iso 10	0 h	8.45×10^2	100
		90 min	6.32×10^2	74.79
		180 min	4.65×10^2	55.02
5	Iso 11	0 h	6.29×10^2	100
		90 min	4.21×10^2	66.93
		180 min	1.28×10^2	20.34
6	Iso 21	0 h	4.12×10^2	100
		90 min	2.09×10^2	50.72
		180 min	1.09×10^2	24.51
7	Iso 22	0 h	2.5×10^2	100
		90 min	1.12×10^2	44.8
		180 min	0.4×10^2	16
8	Iso 27	0 h	8.21×10^2	100
		90 min	6.25×10^2	76.12
		180 min	5.76×10^2	70.15

Table 4: Survival of the selected LAB isolates under bile salt stress condition

No	Isolates	Viable cells (log CFU/mL)	
		Bile salt (%)	
			0.5%
1	Iso 3	9.53×10^4	4.52×10^4
2	Iso 5	49.14×10^4	38.21×10^4
3	Iso 8	$>100 \times 10^4$	$>100 \times 10^4$
4	Iso 10	63.26×10^4	18.06×10^4
5	Iso 11	13.54×10^4	6.045×10^4
6	Iso 21	33.68×10^4	21.64×10^4
7	Iso 22	6.03×10^4	3.51×10^4
8	Iso 27	39.26×10^4	41.18×10^4

Autoaggregation and coaggregation

Aggregation properties are important characteristic of bacterial strains that are used as probiotic (22). Aggregation plays an important feature in biofilm formation; However, coaggregation with pathogens may be considered as a positive

characteristic. Thus, aggregation is one of the steps required for the elimination of pathogenic or nondesirable organisms from gastrointestinal tract regions (23). The genes encoding for surface proteins could be the reason for binding of mucus, aggregation and intracelluar adhesion of the strains (24). *In vitro* evaluation of autoaggregation and ability to coaggregate with potential entric pathogen can be used for preliminary screening and selection of the best probiotic strain. The isolates were further subjected to aggregation and coaggregation assay, the results revealed that, all the selected isolates have great potential to get aggregate and coaggregate when exposed to selected pathogen (*S. typhi*) **(Table 5)**

Table 5 Autoaggregation and coaggregation abilities of Lab isolates

No.	Isolates	% autoaggregation (5 h)	% coaggregation (5 h)
1	Iso 3	30±0.55	10±0.88
2	Iso 5	25±0.25	10.5±0.45
3	Iso 8	3±0.57	10.5±0.56
4	Iso 10	4±0.78	15.76±0.59
5	Iso 11	14±0.45	6.38±0.23
6	Iso 21	8±0.67	25±0.46
7	Iso 22	8±0.90	8.6±0.54
8	Iso 27	50±0.34	13.68±0.78

Values expressed in Mean ± SD pooled from triplicates

Safety evaluation of LAB strains

DNase activity

The safety evaluation of the isolates was determined by their DNase activities, which prove the non-pathogenic status of LAB isolates. Safety evaluation through DNase test to check for the pathogenicity of bacteria producing DNase enzyme that may cause hydrolysis of the DNA molecules was performed. Therefore, the absence of DNase in antimicrobial strains tested was confirmed to support the safety of their use in fermentations (25,26). Out of 17 isolates, 8 isolates showed no DNase activities, which was confirmed by the "no zone" in the test plates that contain DNA**.** Thus, from the results it can be said that these 8 isolates are safe to utilise as probiotic organism and has great potential to be used directly or as a supplement.

Evaluation of *in vitro* antidiabetic activities

α-Amylase is the hydrolytic enzyme that cleaves α-1-4 glycosidic bond present in amylase and amylopectin. Ultimately yielding limit dextrin, maltose, and glucose**.** Digestion of starch starts right at mouth initiated by the salivary amylase which continues in the stomach, until the amylase is inactivated by the low pH of the gastric juice. Nevertheless about 50% of starch present in the food will be digested by this time (27). α-glucosidase cleaves glucose units from starch and glycogen.

Concerning the source of inhibition of digestive enzyme, specially of α-amylase and α-glucosidase different sources have been explored including the microbial source. While studying the inhibitory activity of α amylase by *Lactobacillus plantarum* strain isolated from Kimchi Kim et al., observed about 94% inhibition of the enzyme (28). The inhibition of α-amylase delay glucose absorption and reduce post-prandial blood glucose levels**.** α- glucosidase inhibitors reduce post-prandial hyperglycemia by interfering with the activity of carbohydrate digestive enzyme and dealing absorbance of glucose (29).

LAB helps to stimulate immune response by reducing/inhibiting the pathogens through several mechanisms. Previous studies reported Alpha-glucosidase inhibition activity (30), by bacteriocins production that have antagonistic potential against pathogenic bacteria (31). In this study, selected 8 isolates (Iso 3, Iso 5, Iso 8, Iso 10, Iso 11, Iso 21, iso 22, Iso 27) with probiotic potential were further subjected to α-amylase and α- glucosidase inhibition, in order, to assess the reduction of glucose level. The results revealed that all 8 isolates found to inhibit the porcine pancreatic α-amylase and α- glucosidase strongly. Thus, from the result it can be said that, isolates have antidiabetic properties (Table 6).

Table 6: Analysis of α-Amylase and α-Glucosidase activity of LAB isolates

No.	Isolates	Inhibition (%)	
		α-amylase	α-glucosidase
1	Iso 3	73.91±0.02	37.4±0.45
2	Iso 5	39.13±0.09	0.00±0.00
3	Iso 8	58.69±0.10	26.9±0.05
4	Iso 10	65.2±0.05	45.12±0.66
5	Iso 11	19.06±0.04	42.6±0.07
6	Iso 21	0.00±0.00	39.14±0.44
7	Iso 22	80.42±0.33	34.9±0.98
8	Iso 27	76.25±0.46	40.0±0.67

Values expressed in Mean ± SD pooled from triplicates

Conclusion

It is known that the diabetes is the biggest challenges for the medical sciences. A lot of research focuses on finding out the new generation medicines, that can be used for the treatment of diabetes. However, the allopathic medicine has lot of limitations with other associated difficulties. The present study was conducted to select probiotic strains for industrial applicability as a functional agent with antidiabetic properties. From the results of the present study, total 8 probiotic lactic acid bacteria strains isolated from the herbal extracts viz., Sample 1-ASAVA, Sample-2 ARISTA, Sample -3 SARKO, Sample-4 SWARASA. These isolates had a remarkably high inhibitory activity on α- glucosidase and α- amylase. Thus, the probiotic microorganism forms the fermented herbal extracts can be suggested as a feasible and reliable source of antidiabetic components. These isolates can be recommended to be used for the treatment of diabetes and related ailments. In addition, selected isolates also passed safety evaluation. However, further investigation is needed to be done in this area, that will open new dimension in

the field of ayurveda for the treatment of diabetes with the help of probiotic organisms.

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