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Research Article

AN *IN VITRO* COMPARATIVE ANALYSIS OF PROPERTIES OF PROBIOTIC BACTERIA PRESENT IN BEVERAGES

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ABSTRACT

Probiotics for human consumption including *Lactobacillus sp.* and *Acetobacter sp.* are of increasing interest due to the growing evidences of health advantages associated with them. The viability of these bacteria in food as well as during transit through the gastrointestinal tract is crucial for positive health effects. The present study comparatively analyzes the survivability and functional properties of commercial probiotic drinks in a simulated gastrointestinal environment. The sample drinks were divided into two sections viz.; Dairy and Non-dairy, both containing either *Lactobacillus acidophilus* or *Acetobacter xylinum* and estimated for properties including numbers, gram character, NaCl tolerance, bile salt resistance, optimal pH and production of organic acids. Viability and activity in the gastrointestinal environment was evaluated by subjecting the above to an *in vitro* digestive treatment using a combination of enzymes and digestive solutions. It was observed that amongst all the selected samples, *Lactobacillus acidophilus* present in the dairy beverages displayed the highest probiotic potential. These microorganisms displayed the greatest NaCl (1-9%) and bile salt tolerance (0.05-0.4%) along with maximum organic acid production (1.81%), pH resistance (2.0-7.5) as well as overall survival during *in vitro* gastric, bile and intestinal digestion ($p \leq 0.5$). Moreover, non-dairy liquids with *Acetobacter xylinum* manifested improved performance compared to *Lactobacillus* in this food environment. Therefore, the functionality of a probiotic product may not only be dependent on the type of microorganism but also the food medium. Knowledge of the above may enable the consumer in making a careful selection of a desirable food product to gain maximum health benefits.

Keywords: Dairy, gastrointestinal, non-dairy, probiotics, survival, tolerance, viability.

1. INTRODUCTION

Health awareness is on a constant rise amongst consumers. Natural, functional and convenient products that can deliver health benefits without compromising the taste continue to thrive in the beverage market. Probiotic drinks are becoming an increasingly popular option due to the evolving dietary habits and accelerating social perception towards healthy gut and positive functional attributes [1]. Owing to the marketing strategies of renowned commercial brands, consumers are now looking for healthy probiotic counterparts. Probiotics, primarily defined as living microorganisms with beneficial functioning, are capable of promoting and supporting the balance of the autochthonous microbial population of the gastrointestinal tract (GIT). Although these organisms may not be constantly present in the gut, yet they impose a positive health effect [2, 3]. A healthy gut has always been linked to a strong immune system. Probiotic beverages are healthy liquids that can help in digestion, weight loss, infectious diseases as well as allergic disorders [4, 5]. Globally, these drinks are being commercialized in many different forms and are generally either milk or fruit based [6]. While dairy based probiotic liquids are known for their traditional taste and aroma; non-dairy probiotics, especially those containing fruit juices are also being accepted due to their flavor and appearance [7]. The viability of the probiotics used in beverages of dairy and non-dairy origin is important for conferring suitable health benefits [8]. In order to impart positive outcomes, the probiotic bacteria should possess the ability to colonize the small intestine post resistance against the deleterious effects of gastric acidity, bile salts, elevated osmolarity and action of digestive enzymes. Moreover, sufficient numbers of these organisms are required in the viable state to bring about the expected outcomes [9]. Therefore, determination of the viability of these species in the GIT is essential for assessment of the effectiveness of the same inside the human body [10]. Probiotic bacteria that are delivered through food systems have to survive during their transit through the

upper GIT followed by their persistence in the host intestine to confer benefits [11]. The present study comparatively evaluates the survival of probiotic bacteria including *Lactobacillus acidophilus* and *Acetobacter xylinum* present in commercial dairy and non-dairy beverages in a simulated gastric and intestinal microenvironment. Properties including numbers, gram character, NaCl tolerance, bile salt tolerance, optimal pH and production of organic acid were estimated. In order to provide a realistic and predictive reproduction of human gastric and duodenal processing; the GIT was duplicated *in vitro* by using the major chemical components of the *in vivo* digestive juices. Hence, the beverages were tested for the probiotic activity and survival in simulated gastro-intestinal environment. Awareness of the same may help the consumer in adopting a cautious choice of an appropriate product to avail the desired health effects.

2. MATERIAL AND METHODS

2.1. Sample selection

The study was conducted on the probiotic beverages of both dairy and non-dairy origin owing to their popularity and usage within the world population. For the analysis, *Lactobacillus acidophilus* and *Acetobacter xylinum* in dairy drinks were coded as DL and DA, whereas the same species from non-dairy sources were coded as NDL and NDA respectively. Three samples of each category were selected for analysis based on random sampling method.

2.2. Extraction and identification of probiotic species from commercial drinks

The probiotic species were extracted from the beverages by serial dilution followed by inoculation into MRS (De Man, Rogosa, Sharpe) broth and Acetobacter broth for the growth of *Lactobacillus acidophilus* and *Acetobacter xylinum* respectively. These microorganisms were identified using gram staining; catalase-oxidase test and sugar metabolizing patterns [12].

2.3. Determination of NaCl tolerance and bile salt tolerance

Lactobacillus sp. and *Acetobacter sp*. from dairy and non-dairy sources were cultured in Nephelometric flasks followed by treatment with different concentrations of NaCl (ranging from 1-9%) in the growth medium for 48 hours at 37°C. The survival was estimated by measuring the Optical Density (O.D) at 560 nm using a Spectro-photometer (Hitachi UV-VIS Spectrophotometer U2900, India). In order to check bile salt tolerance, overnight MRS broth or Acetobacter broth cultures were inoculated with 0.05% to 0.4% bile salts followed by incubation for 48 hours at 37°C. The survival was estimated by measuring O.D at 560 nm [13].

2.4. Determination of optimum pH of growth and organic acid production

Determination of Optimum pH for growth was evaluated by estimating the survival of the probiotics isolated from the samples at different pH. The above microorganisms were grown in their respective growth mediums maintained at pH ranges from 2 to 6.5 for 48 hours at 37°C. The desired pH was obtained by addition of the required amounts of 1M HCl or 1M NaOH to the culture broths and monitoring the pH using a pH meter (Environmental and Scientific Instruments Co, India). The viability was calculated by measuring the O.D at 560nm. The amount of organic acid produced by the probiotics was measured by titration with reference to standard lactic acid at 24-72 hours post incubation [14].

2.5. Survival during *in vitro* gastrointestinal digestion

2.5.1. Determination of survival against in vitro-gastric digestion

Simulated gastric juice was formulated with glucose (3.5 g/liter), NaCl (2.05 g/ liter), KH_2PO_4 (0.60 g/ liter), $CaCl_2$ (0.11 g/ liter) and KCl (0.37 g/ liter), adjusted to pH 2.0 using 1 M HCl, and autoclaved at 121°C for 15 min. 25ml of the respective cultures mediums were inoculated with 1% (vol/vol) bacteria and incubated overnight (16 hours) in a Nephelometric flask. The cultures were subsequently centrifuged at 7,000 g at 4°C for 15min, washed once in an equal volume of cold 0.25% Ringer's solution, and subsequently re-centrifuged (7,000 g at 4°C for 15 min). Pellets were then re-suspended in an equal volume of simulated gastric juice at 37°C followed by incubation for 90 min with constant stirring. Samples were taken at 0, 10, 30, 60, and 90 min, serially diluted in maximum-recovery diluents, plated on MRS medium, and incubated at 37°C for 48 hours. The survival of each strain was evaluated by determination of the OD at 560nm and analyzed using ANOVA.

2.5.2. Determination of survival against in vitro bile digestion

Simulated bile solution was prepared by dissolving Oxgall (Difco laboratories, India) in distilled water. All solutions were sterilized at 121°C for 15 min. 9.0 ml simulated bile solution (0.5% or 2.0%) were added to overnight

cultures and vortexed for 20 sec for complete dispersion of the cells. Samples were taken immediately after mixing (0 h) to determine the viability of the culture. The mixtures were then incubated at 37°C with continuous shaking for 30-90 minutes. The survival was evaluated by determination of the O.D. at 560nm in a UV-VIS spectrophotometer.

2.5.3. Determination of survival against in vitro intestinal digestion

Pancreatic juice solution was prepared by using NaCl (125.0 mM), $CaCl_2$ (0.6 mM), $MgCl_2$ (0.3 mM), trypsin (11 U/mL), α-chymotrypsin (24 U/ml) and pancreatic lipase (590 U/mL). The cultures were then incubated at 37°C with periodic shaking for 30-90 minutes. The survival was calculated by determination of the O.D. 560nm.

2.6. Statistical analysis

Statistical analysis was performed by using the Data Analysis Software pack of Microsoft Excel (version 2010). Data was represented as mean of n≥3 individual experiments. The attributes of the data were further analyzed via the 'Regression Model' and ANOVA data analysis pack (Graph Pad, 7.04, California). $P \leq 0.05$ was considered as significant. F value was used to denote variance among groups. A large F value (>1) indicated significant differences between the groups.

3. RESULTS AND DISCUSSION

3.1. Extraction and identification of probiotic species from commercial drinks

The isolates were grown in DeMan, Rogosa and Sharpe (MRS) medium at pH 6.5 and Acetobacter broth at pH 7.4 respectively. All the isolates produced small, irregular and round shaped colonies with shiny whitish cream or brown colour, being morphologically similar to *Lactobacillus acidophilus* and *Acetobacter xylinum*. *Lactobacillus* was identified as gram positive, catalase as well as oxidase negative whereas *Acetobacter sp.* were gram negative, catalase positive and oxidase negative. Also, *Lactobacillus* and *Acetobacter* produced lactic acid and acetic acid respectively upon fermentation of sugars (Table 1).

Table 1: Identification of probiotic species

Parameter	*Lactobacillus acidophilus*	*Acetobacterxylinum*
Gram Character	Positive	Negative
Catalase test	Negative	Positive
Oxidase test	Negative	Negative
Acid Production	Lactic acid	Acetic acid

3.2. Determination of NaCl and bile salt tolerance

High salt tolerance is a desirable property for organisms to be used as probiotics and hence it is expected that the probiotic bacteria from either dairy or non-dairy sources provide constant affectivity even at salt concentrations as high as 9%. It is known that NaCl may inhibit growth of certain types of bacteria and hence the tolerance test was conducted to check the efficiency of the probiotic species present in various beverages. Isolated probiotic bacteria DL, DA, NDL and NDA grew well at 1% NaCl. Fig. 1A shows the maximum growth (O.D) of isolates DL (0.992) and NDA (0.984) at 1% NaCl. The survival of the bacteria was found to gradually decrease with an increase in NaCl concentration. However, *Lactobacillus* from dairy products was observed to display maximum viability compared to the other varieties even at high salt percentages as displayed by an absorbance of 0.62 and 0.59 at NaCl contents of 8% and 9% respectively (Fig. 1A). The probiotic bacteria in the food samples should also possess the capability to survive during physiological bile salt exposure. Therefore, bile salt tolerance was evaluated by subjecting the samples to 0.05-0.4% of bile concentration in order to mimic the human intestinal tract, which can possess a maximum of 0.3% bile. It was observed that although all isolates were able to survive at bile contents of 0.05%, 0.1%, 0.15%, 0.2%, 0.25%, 0.3%, 0.35% and 0.4%, yet most of them displayed hampered multiplication of cell numbers at increased concentrations. All microbes were seen to grow well in the presence of 0.05% bile salt. Indeed, maximum growth (OD) of DL and NDA was detected at 0.05% bile salt intensity. Moreover, the growth rate displayed a decrease with an increase in bile salt percentages. Results portrayed DL to have the highest probiotic potential followed by NDA ($P<0.05$) owing to their resistance against a range of bile salt environments along

with their capability of manifesting adequate growth even at bile concentrations of 0.3% (Fig. 1B). Therefore, *Lactobacillus sp*. in dairy beverage (DL) was found to perform best in terms of both NaCl and bile salt tolerance followed by NDA. The ANOVA table (Table 2) displayed the results to be statistically significant.

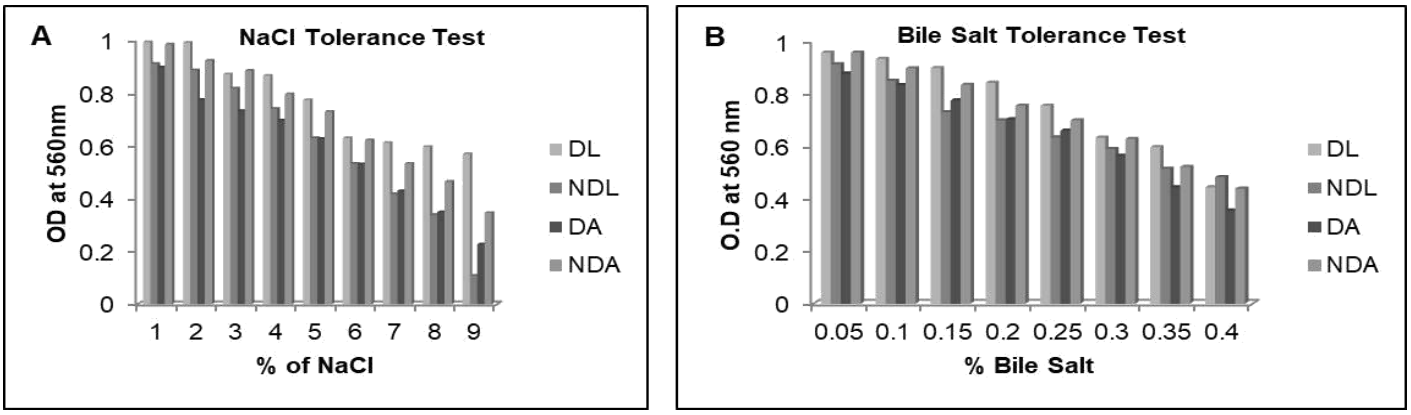

Fig. 1: NaCl Tolerance Test (A) and Bile Salt Tolerance Test (B)

3.3. Determination of optimum pH of growth and organic acid production

Probiotic bacteria are required to survive the extreme pH conditions in different regions of the gastrointestinal tract. Therefore, optimum pH was evaluated in the samples to analyze the suitability of the beverages. As shown in fig.2A, DL and NDA were able to grow in the pH ranges 2.0-6.0 and 2-5.5, as depicted by Optical Density values of 0.989 and 0.956 at pH 6.0 and 5.5 respectively (Fig. 2A). Although these microorganisms were able to grow at the above pH ranges, yet the optimum growth was observed between pH 4.5 and 5.5 when grown in MRS broth and Acetobacter broth at 37°C independently. The results displayed the growth rates of *Lactobacillus sp*. and *Acetobacter sp*. to manifest a decrease beyond the optimum pH. Furthermore, *Lactobacillus sp*. from dairy products showed an enhanced survival compared to *Acetobacter sp*. even under alkaline conditions. This may be attributed to a tolerance response induced in the bacteria when exposed to a range of H^+ concentration during growth. Probiotic microorganisms produce a variety of substances with antibacterial properties including organic acids, H_2O_2 and bacteriocins, that affect bacterial metabolism, toxin production as well as inhibition of pathogens. The organic acids produced by *Lactobacillus sp*. and *Acetobacter sp*. were found to be lactic acid and acetic acid respectively. The results indicated the organic acid production to increase with an enhancement in the incubation time (Fig. 2B). Highest acidity (1.81%) was observed after 72 hours of incubation at 37°C for probiotic *Lactobacillus sp*. isolated form dairy samples (DL). Hence, it can be concluded that DL exerted better probiotic activity by producing high amount of organic acid along with survival in a broader pH range compared to other samples. The data was found to be statistically significant as indicated in Table 2.

3.4. Survival during *in vitro* gastric, bile and intestinal digestion

In order to be render suitable health benefits, probiotics must survive transit through the stomach and colonize the intestine. The viability of the bacteria present in the beverages was found to gradually decrease with an increase in the time of exposure to simulated gastric juice (pH 2.0). However, DL and NDA portrayed improved tolerance to the hostile *in vitro* gastric conditions compared to the other samples as displayed by OD values of 0.99 and 0.96 post 90 minutes of simulated gastric digestion respectively (Fig. 3A). Moreover, DL showed the highest survival under these conditions throughout the time point assay compared to all the samples. *Lactobacillus sp*. is considered to be intrinsically resistant to acid. The acid tolerance of *Lactobacillus* may be attributed to the presence of a constant gradient between extracellular and cytoplasmic pH. Protection against acidic conditions is known to be mediated by F_0F_1-ATPase in these organisms. Furthermore, survival against bile concentrations produced in the human small intestines along with colonization and multiplication in large intestine is an important characteristic that should be associated with

dietary probiotic adjuncts. Interestingly, the results of our study show that *Lactobacillus acidophilus* present in the dairy environments performed better in terms of the above compared to those isolated from non-dairy beverages. Nonetheless, *Acetobacter xylinum* from the non-dairy samples (NDA) displayed a good survival compared to *Lactobacillus* (NDL) in this food medium. Interestingly, DL showed significantly high ($P<0.05$) survival in bile solutions even at 90 minutes, when compared to NDA, DA and NDL (Fig. 3B). Additionally, NDA portrayed better performance versus DA and NDL against bile acids. Furthermore, DL also manifested maximum survival in simulated intestinal environment compared to all other samples as displayed in Fig. 3C. DL showed an OD of 0.522 followed by NDA (O.D 0.489) post 30 minutes of digestion. Evidence suggests that commensal bacteria enhance intestinal epithelial homeostasis and barrier integrity. Indeed, probiotic bacteria regulate a number of host processes, including nutrition, development, and immune responses, that are relevant for both health and disease. From the comparative study using ANOVA table (Table 2), DL was observed to possess the highest survivability amongst all the studied samples, followed by NDA under the experimental gastrointestinal environment. The probiotic functionality of NDL and DA were found to be of a lower intensity compared to both DA and NDA.

Table 2: ANOVA Test for Analysis Parameters

Analysis parameters	F value	P-value
NaCl tolerance	39.275	0.0002
Bile salt tolerance	17.98	5.15E-06
pH	9.474	0.001
Production of organic acid	14.688	0.003
Survival during Gastric Digestion	30.854	0.001
Survival during Bile Digestion	39.275	0.0002
Survival during Intestinal Digestion	77.517	5.17E-05

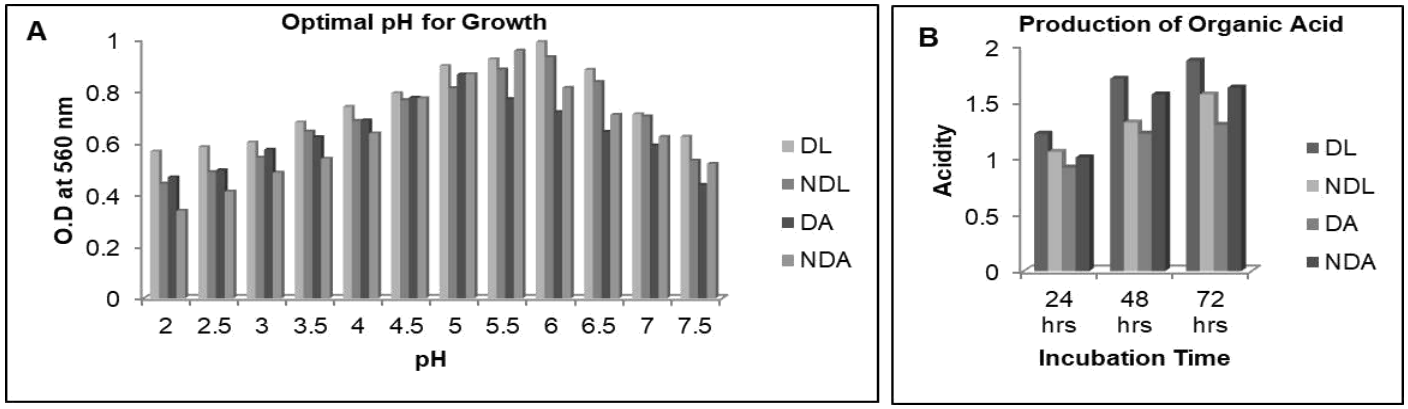

Fig. 2: Optimum pH for growth (A) and Production of Organic Acid (B)

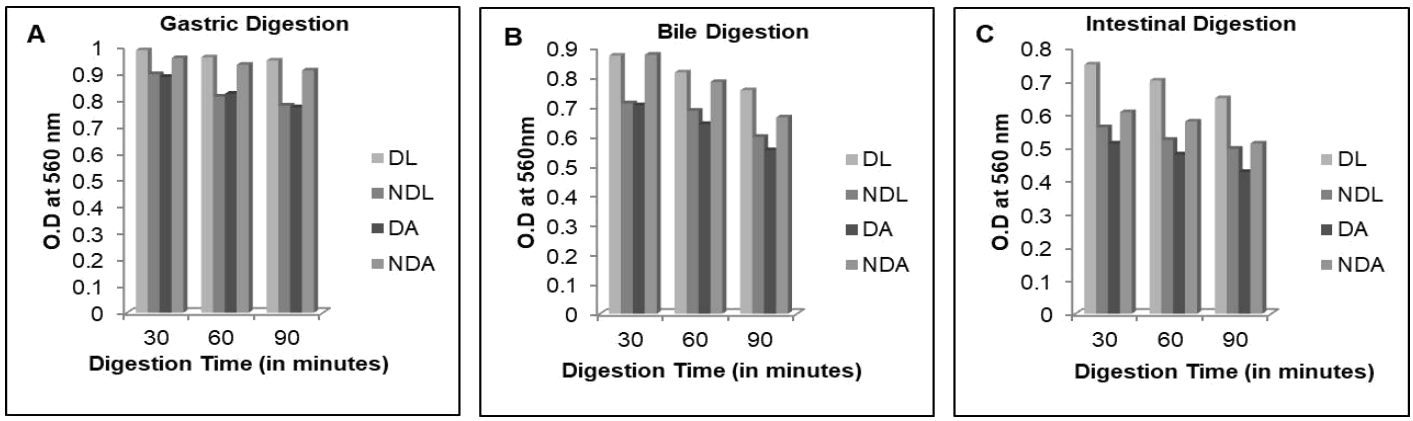

Fig. 3: Survival during Gastric Digestion (A), Bile Digestion (B), Intestinal Digestion (C)

4. CONCLUSION

The viability and effectiveness of probiotics is known to be dependent on its inherent properties and the carrier matrix. Additionally, bacterial strain identification is a key requirement for the above. The results of the analysis indicated that *Lactobacillus acidophilus* available in dairy probiotic beverages performed best in terms of survival and viability compared to all other samples. DL not only displayed high NaCl and bile salt tolerance but also showed maximum organic acid production, pH resistance as well as overall survival during *in vitro* gastric, bile and intestinal digestion as compared to DA, NDL and NDA. However, in the non-dairy beverages, *Acetobacter* (NDA) was found to display improved probiotic efficiency compared to *Lactobacillus* in the same food medium (NDL). Moreover, although NDA displayed a generally reduced survivability versus DL, the functionality of the former was observed to be more desirable in contrast to NDL and DA. Therefore, the qualities of different probiotic drinks vary and are dependent not only on the on the microorganism but also the medium used. Hence, thorough knowledge of the above may help the consumer in effectively selecting a probiotic product according to their requirements. A choice of probiotic products with *Lactobacillus sp* in cases of dairy beverages and *Acetobacter sp* in non-dairy drinks may deliver optimum health benefits.

5. ACKNOWLEDGEMENT

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Conflict of Interest

There is no conflict of interest in this manuscript.

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