USING OF STEVIA AS NON-CALORIC SUGAR SUBSTITUTES ON VIABILITY OF PROBIOTIC BACTERIA LACTOBACILLUS CASEI

1TULAY OZCAN, 2LUFIYE YILMAZ-ERSAN, 3ARZU AKPINAR-BAYIZIT, 4BERRAK DELIKANLI-KIYAK

1,2,3 Uludag University, Department of Food Engineering, 16059, Gorukle, Bursa/Turkey
E-mail: tulayozcan@uludag.edu.tr, luftyey@uludag.edu.tr, abayizit@uludag.edu.tr, berrakdelikanli@gmail.com

Abstract- The increased incidences of obesity and related health issues have resulted in an increased production and consumption of foods made with non-nutritive sweeteners without the risk of consuming additional calories contributed by normal sugar-based products. In this study, growing of probiotic bacteria L. casei on the different substrates such as glucose, inulin and 0.25, 0.50% stevia and viability were investigated. L. casei counts were stimulated by stevia the highest counts in basal medium and reconstituted fermented milk samples as much as glucose and inulin. Viable cell counts were higher than 8 log10 cfu g-1 during fermentation for recommended therapeutic effect of food matrix on microbial survival rates. Stevia seems to be a better substrate and able to utilize for probiotic bacteria strains.

Keywords- Stevia, Sugar substitutes, Lactobacillus casei, Fermented Milk

I. INTRODUCTION

Since high consumption of sugar has been associated with chronic diseases, low intake of this macronutrient is strongly recommended, however, non-nutritive sweeteners used in foods might increase the risk of obesity and metabolic disorders. A major challenge for food industry is the reduction of the sugar content in processed foods, which implies the reformulation of the products with sweeteners while maintaining their popularity and appealing characters. It is important to note that such alterations should not have significant changes in the sensory characteristics of the product [1]-[2].

Recently, research has focused on artificially sweetened beverages and dairy products [3-6]. Much attention has been placed on stevioside, a sweet glycoside extracted from the plant Stevia rebaudiana Bertoni. This plant belong to the family of Asteraceae and is native to South America. Nevertheless, it is widely cultivated for its sweet leaves in many countries like China, Malaysia, Singapore, South Korea, Taiwan and Thailand, and named as stevia, candy leaf, sweet leaf, sugar leaf or honey leaf. Due to the high sweetness and potential therapeutic properties of the leaves, S. rebaudiana has attracted economic and scientific interest. Use of these sweetening compounds has increased dramatically due to the health concerns related to sucrose usage, such as dental caries, obesity and diabetes [7-9].

High content of sweet diterpenes, called as steviol glycosides (about 4-20% in dry-leaf matter), are the group of natural sweeteners with low-calorie that have been extracted from stevia. The diterpenes, have been identified as stevioside, steviolbioside, rebaudioside A, B, C, D, E, F and dulcoside. The leaves of wild stevia plants have been stated to contain 0.3% dulcoside, 0.6% rebaudioside C, 3.8% rebaudioside A and 9.1% stevioside [10-13].

Stevia being a natural, sweet-tasting calorie-free botanical is a new promising renewable raw food stuff in the world market. Stevia is 250-300 times sweeter than sugar, and thus, have been applied as a saccharose-substitute or as an alternative to artificial sweeteners [11]-[14]. Stevia is reported to exert beneficial effects on human health, including anti-hypertensive, anti-hyperglycemic, anti-inflammatory, anti-tumoral, anti-diarrhoeal, diuretic, non-cartogenic, and immunomodulatory effects [15-17].

Stevia leaves are rich in nutrients, containing substantial amounts of protein, fibre, lipids, essential oils, free sugars, oligosaccharides, ascorbic acid, calcium, phosphorous, magnesium, and iron. Nonetheless, the leaves also contain flavonoids, alkaloids, chlorophylls, carotenoids, tannins, phenolic acids, chlorogenic acids, austroinulin, nilacin, rebaud oxides, gibberellic acid, indole-3-acetonitrile, apigenin, quercetin, isoquercitrin, luteolin, moicene, kaempferol, stigmasterol, xanthophyllus, umbeliferone, chlorogenic acid, caffeic acid, dicaffeoylquinic acid [9].

Stevia sweeteners, steviol glycosides or crude leaf extracts are currently in use in several industrial foods, such as flavored ice-tea, fruit/vegetable juices, sports drinks, flavored milk, and yogurt [18-21].

Stevioside is hydrolyzed in the gastrointestinal tract to yield steviol and glucose [22]. Furthermore, all steviol glycosides are metabolized to steviol via glycosidase activity of the human intestinal microflora (eg, Bacteroides sp.), and might exert prebiotic properties [23-26].

Commercial interest in functional foods containing probiotic strains has consistently increased due to the awareness of gut health. Probiotics are live microorganisms which confer beneficial effects for host health when they are consumed in appropriate quantities [27]. A great deal of attention was paid to...
sucrese-sweetened beverages in the field of diet-disease relations. For probiotic functional foods the demand has increased notably for light or diet foods with added natural or artificial sweeteners [28-30]. However, little is known about the effect of different sweeteners on the microorganisms present.

Thus, the objective of this research was to compare the growth-promoting and prebiotic effects of stevia on Lactobacillus casei by assessment of survival.

II. DETAILS EXPERIMENTAL

2.1. Materials and Procedures

Growth Media

To study the ability of L. casei (DSMZ No 20011, Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany) to utilise sugar-free MRS broth was used as a basal growth medium (Table 1). The pH was adjusted to 6.2 and the medium was sterilized at 121°C for 15 min. Stock solutions (10%) of glucose, inulin, stevia (Mayasan A.S., Istanbul) were prepared in ion-exchanged water and filter-sterilized by using 0.45 μm pore size membrane filters (Millipore). Sterile substrate solutions were added into basal MRS (DE Man, Rogosa, Sharpe) broth-medium to obtain final carbohydrate concentrations of 0.5% for glucose, inulin and 0.25-0.5% stevia.

Activation of Cultures

Bacteria were routinely cultured using MRS broth at 37°C, under anaerobic conditions (jars with AnaeroGen Gas Packs, Oxoid, Basingstoke, UK). Stock cultures were transferred into glycerol broths (50% glycerol in MRS) and stored in cryo-vials under oxygen-free nitrogen at -80°C.

A strain of L. casei was activated from stock cultures (-80°C) for overnight incubation at 37°C in 50 mL MRS broth [31]. 0.45 μL of inoculum of L. casei were added to 15 mL tubes containing 10 mL of basal medium (negative control) and containing glucose and inulin (positive control, 0.50%), and stevia (0.25 and 0.50%). Fermentation was carried out in an incubator for at 37°C for 48 h. pH, OD and viable cell counts were determined throughout 48 h. All incubations were performed under anaerobic conditions.

Preparation of Fermented Milk

Stevia was added at 0.25 and 0.50% level based on the reconstituted skim milk (10.70%). Both milks were then thermally treated at 90°C for 10 min in water bath. The heat treated milks were transferred to 15 mL sterile flasks, cooled in ice bath to 37°C. The milk was inoculated with L. casei at 0.45 μL, and then, incubated anaerobically at 37°C for 24 hours. Fermented milk samples were analyzed for pH and viable cell counts.

Table 1: Composition of carbohydrate-free MRS broth

<table>
<thead>
<tr>
<th>Carbohydrate-free MRS broth</th>
<th>g/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yeast extract</td>
<td>5.00</td>
</tr>
<tr>
<td>Casein peptone</td>
<td>10.00</td>
</tr>
<tr>
<td>Meat extract</td>
<td>10.00</td>
</tr>
<tr>
<td>Tween 80</td>
<td>1.00</td>
</tr>
<tr>
<td>Di-potassium hydrogen phosphate</td>
<td>2.00</td>
</tr>
<tr>
<td>Sodium acetate</td>
<td>5.00</td>
</tr>
<tr>
<td>Amonium citrate</td>
<td>2.00</td>
</tr>
<tr>
<td>Magnesium sulphate</td>
<td>0.20</td>
</tr>
<tr>
<td>Manganese sulphate</td>
<td>0.05</td>
</tr>
</tbody>
</table>

Growth and Viable Cell Count Determination

The growth of L. casei was quantified by measuring the optical density (OD) at 600 nm using a spectrophotometer (Shimadzu UV 1800, Kyoto, Japan) against basal media. The absorbency was recorded for the basal medium inoculated with L. casei (initial absorbance, on zero hour) and during fermentation (on 12, 24, 36 and 48th hour). pH of the basal media and fermented milk was determined by direct measurement with a digital pH meter (pH 315i/SET; WTW, Germany) at room temperature (25°C). For the total viable count (TVC) 1 mL of sample was diluted in 9 mL distilled water and then serial dilutions were prepared (10⁻¹-10⁻⁷). L. casei colonies were counted on MRS agar after anaerobic incubation of 72 h at 37°C. The average counts of typical colonies were expressed as colony forming units (cfu) per mL [32]. All measurement were performed in triplicate.

The viability of the probiotic cultures on basal medium and fermented milk system were determined during fermentation. Survival tests were initiated by growing L. casei anaerobically on MRS agar at 37°C. Viability proportion index (VPI) of probiotic microorganisms was calculated according to the equation given as follows [33]:

\[
VPI = \frac{\text{probiotic viability at the end of fermentation} (\log_{10}\text{cfu g}^{-1})}{\text{initial probiotic viability} (\log_{10}\text{cfu g}^{-1})}
\]

Statistical analysis

The Statistical Analysis System ANOVA and Duncan’s Multiple Range Test (DMRT) at P <0.05,0.01 were used to determine the mean differences.

III. RESULTS AND DISCUSSION

3.1. Counts of probiotic bacteria in basal medium and MRS agar containing Stevia

The effect of stevia (0.25 and 0.50%) supplementation to basal medium and fermented milk on the growth of L. casei was found to be significant (p<0.01) (Fig. 1a,b, 2, Table 2, 3). Optical density (OD) is commonly used to measure the growth of the microbial population, thus, the survival of L. casei on
different carbon sources was associated with OD values. OD values for 0.25 and 0.50% stevia supplementation were similar, however, were lower than inulin in basal medium. The highest OD was observed on glucose throughout 48 h (Fig. 1a). The lowest pH value and the highest OD values were determined at the 36th of the fermentation, due to microbial growth and reduction of pH by rapid acid production (Fig. 1a,b). However, increased acidity and oxidative pressure will decrease the viability of lactic acid bacteria during the storage period of dairy products. It has been reported that supplementation of prebiotics and utilization of microencapsulation technology could be applied to minimize these problems [34]-[35]. In this study, due to the carbohydrate source employed in the media variations in acidity and osmotic pressure were observed, and these changes might have contributed to the differentiations in microbial growth of L. casei during the storage period.

Carbohydrates are metabolized during the fermentation via intrinsic microorganisms. The pH decreases due to the organic acid production, and leads to sugar hydrolysis by low pH values. From the data presented in Fig. 1b, it was observed that the rate of sugar hydrolysis was faster in basal medium containing 0.50% stevia than 0.25%, thus displaying similar decrease in pH values of inulin and glucose. Furthermore, in medium containing 0.50% stevia the microbial population and VPI were higher than 0.25% stevia in accordance to lower pH. It could be stated that stevia stimulated the growth of L. casei as glucose and inulin.

All media showed high cell counts and VPI profile for L. casei during fermentation, which were above 7 log_{10} cfu g^{-1}. The numbers of L. casei in the stevia-supplemented samples were similar to the positive controls of inulin and glucose (Table 2). However, there were no significant differences in VPI values throughout fermentation. The steviol glycosides are hydrolyzed by bacteria in the gastrointestinal tract to steviol and glucose, and increase in growth of intestinal microbiota is observed due to their potential prebiotic effect [22]- [24]. Stevia, either at 0.25 or 0.50%, was found to support the growth of L. casei during incubation in basal medium, indicating that L. casei was able to metabolize the stevia as high as glucose, a readily available carbon source for bacterial metabolism.

The composition and metabolic activity of the gut microbiota can be influenced by the nature of the diet, host species, and both in the numbers of organisms present, their distribution along the gastrointestinal tract and their metabolic potential [24]. Wingard et al. [36] showed that the glycosidic sweeteners stevioside and rebaudioside A are degraded to the diterpenoid aglycone steviol by rat intestinal microflora in vitro and degraded to steviol by microbial action in the mammalian lower bowel. Hutapea et al. [37] reported that steviol, the final microbial metabolite of stevioside and rebaudioside A, remained unchanged during 72 h incubation with human microflora, indicating that bacterial enzymes are not able to cleave the steviol structure.

3.2. Survival of probiotic bacteria in fermented milks containing Stevia

Lactobacilli are extensively used in industrial functional dairy food production. The growth of Lactobacilli is affected by media formulation and fermentation conditions, such as pH and temperature [38]. In the present study, for fermented milks, inclusion of stevia at 0.25 and 0.50% concentrations stimulated the growth of L. casei. The pH of all samples decreased during 24 h-incubation (Fig. 2). Fermented milk quality is known to be strongly influenced both by the employed probiotic culture and the prebiotic source [39]. It has been recommended that to achieve beneficial probiotic effects in the host, lactic acid bacteria must be alive and reach the concentration of 10^7 to 10^8 cfu g^{-1} in dairy-related products; and should be within these viable count levels during their whole shelf-life, thus, cell viability is a critical factor for a successive probiotic product [40]. Several factors in the food matrix have been reported to affect the probiotic viability, including storage temperature, additives, acidity, hydrogen peroxide, oxygen content, sugar concentration (osmotic stress), water activity (aw), and metabolites [41-43].

Table 3 presents the microbial viability (log_{10} 10^7 cfu g^{-1}) of L. casei in fermented milk during fermentation. Counts of all microbial populations were over 8 log_{10} cfu g^{-1} in probiotic fermented milks supplemented with different carbon sources during fermentation (Table 3).

Stevioside, having a relatively high molecular weight (804.9), is unlikely to be absorbed in the intestine. In addition, gastric juice and digestive enzymes from animals and humans have failed to degrade stevioside. However, bacterial intestinal flora of humans have been reported to convert stevioside into its aglycon steviol [22]-[23]. Toxicological studies have shown that stevioside does not have mutagenic, teratogenic or carcinogenic effects. Likewise, allergic reactions have not been observed when it is used as a sweetener. Therefore, it is stated as 100% natural, zero-calorie, zero-carbohydrate, zero-glycemic index sweetener, making it an excellent choice for diabetics and a challenging substrate for probiotic foods [11]-[43]. Nevertheless, steviol has been shown to be toxic only at very high concentrations, much higher than is formed from the acceptable daily intake of steviol glycosides for humans. The ADI limit varying between 0 to 4 mg/kg body weight expressed as steviol equivalents was concurred by JECFA (The Joint FAO/WHO Expert Committee on Food Additives) and EFSA (European Food Safety Authority), mentioning that these levels do not pose a
risk of genetic damage following consumption of steviol glycosides [44-46].

CONCLUSION

Stevia is a suitable substitute for saccharose in dairy products. In this study, stevia was shown to enhance survival of L. casei in basal medium and fermented milk during fermentation. Results indicate that stevia, aside being a non-caloric sugar substitute for fermented milks, could be supplemented as potential prebiotic substrate. Furthermore, future studies needed to understand the prebiotic potential of stevia on fermentation and post acidification conditions with growth kinetics in different dairy matrices with other probiotic strains.

ACKNOWLEDGEMENT

This study was funded by the Scientific Research Project Unit of Uludag University (Project No. Z-2015/29).

REFERENCES


Fig. 1. Optical density (a) and pH (b) values of basal medium

Different capital letters denote significant differences (P<0.01) between different times of fermentation

Different lowercase letters denote significant differences (P<0.01) between different treatments

Table 2. Changes of microbial population and viability proportion index (VPI) of L. casei in a basal medium (log cfu g⁻¹)

<table>
<thead>
<tr>
<th>Basal Medium</th>
<th>0 h</th>
<th>24 h</th>
<th>48 h</th>
<th>V₀⁺</th>
<th>V₄⁺</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>7.71dB</td>
<td>8.00aA</td>
<td>8.11dB</td>
<td>1.04bA</td>
<td>1.05bA</td>
</tr>
<tr>
<td>Glucose</td>
<td>9.00aA</td>
<td>8.60aA</td>
<td>8.84aA</td>
<td>0.96aA</td>
<td>0.98aA</td>
</tr>
<tr>
<td>Stevia 0.50%</td>
<td>8.00cA</td>
<td>8.17aA</td>
<td>8.30bC</td>
<td>1.02bA</td>
<td>1.04aA</td>
</tr>
<tr>
<td>Stevia 0.25%</td>
<td>7.60dC</td>
<td>8.77aA</td>
<td>8.47bB</td>
<td>1.15aA</td>
<td>1.11aA</td>
</tr>
</tbody>
</table>

Different capital letters denote significant differences (P<0.01) between different times of fermentation

Different lowercase letters denote significant differences (P<0.01) between different treatments

Fig. 2. pH values of fermented milk

Different capital letters denote significant differences (P<0.01) between different times of fermentation
Using of Stevia as Non-Caloric Sugar Substitutes on Viability of Probiotic Bacteria Lactobacillus Casei

**Table 3. Viability proportion index (VPI) of L. casei in fermented milk (log_{10} cfu g^{-1})**

<table>
<thead>
<tr>
<th>Fermented Milk</th>
<th>0. h</th>
<th>12. h</th>
<th>24. h</th>
<th>V_{12}</th>
<th>V_{24}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stevia 0.50%</td>
<td>8.00^{cC}</td>
<td>9.09^{bB}</td>
<td>9.37^{aA}</td>
<td>1.14^{bA}</td>
<td>1.17^{aA}</td>
</tr>
<tr>
<td>Stevia 0.25%</td>
<td>8.83^{dD}</td>
<td>9.25^{aA}</td>
<td>9.48^{aA}</td>
<td>1.05^{aA}</td>
<td>1.07^{aA}</td>
</tr>
</tbody>
</table>

\(^a,b\) Different lowercase letters denote significant differences (P<0.01) between different treatments

\(^A,B\) Different capital letters denote significant differences (P<0.01) between different times of fermentation

\(^a,b\) Different lowercase letters denote significant differences (P<0.01) between different treatments

★★★