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Research article

Short-chain fatty acids production by *Bifidobacterium species* in the presence of salep

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ABSTRACT

Background: Salep is obtained by grinding dried orchid tubers and used as a valuable ingredient in the food industry. Because of the glucomannan content of salep, it is thought to have prebiotic potential. However, there is little information in studies concerning the fermentation characteristics and potential prebiotic properties of salep. The objective of this study was to investigate the effect of salep on bifidobacterial growth by measuring the highest optical density (OD), calculating the specific growth rates, and determining the production of lactic acid and short-chain fatty acids (acetic, propionic, and butyric acid) as a result of bacterial fermentation.
Result: The OD and pH values obtained in this study showed that salep was utilized as a source of assimilable carbon and energy by the *Bifidobacterium* species (BS). All *Bifidobacterium* strains produced lactic, acetic, propionic, and butyric acid, indicating that salep is readily fermented by these bacteria. Salep at 1%(w/v) showed a similar effect on bifidobacterial growth as that promoted by 1% (w/v) glucose used as a traditional carbon source.
Conclusions: Bifidobacterium species can develop in media containing salep as well as in glucose and exhibit the potential to be used as new sources of prebiotics.
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1. Introduction

In recent years, there has been increasing evidence implicating short-chain fatty acids (SCFAs) as critical contributors to human health. Short-chain fatty acids are defined by The International Union of Pure and Applied Chemistry (IUPAC) as "carboxylic acids containing aliphatic tails less than 6 carbon atoms." Common SCFAs are formic acid (C1: methanoic), acetic acid (C2: ethanoic), propionic acid (C3: propanoic), isobutyric acid (C4: 2-methylpropanoic), butyric acid (C4: butanoic), isovaleric acid (C5: 3-methylbutanoic), valeric acid (C5: pentanoic), and 2-methylbutanoic acid (C5). Acetic, propionic, and butyric acids are the major end metabolites produced as a result of the intestinal microbial fermentation of predominantly nondigestible dietary carbohydrates, simple sugars, sugar alcohols, unabsorbed or undigested proteins, and endogenous substrates [1,2,3,4,5,6].

Short-chain fatty acids exert multiple positive effects to maintain human wellbeing. The positive metabolic health effects of SCFAs are i) an essential factor in maintaining gut integrity, ii) a reduction in the luminal pH, iii) inhibiting putrefactive and pathogenic bacteria, iv) protecting the integrity of intestinal epithelial cells from mechanical, chemical, and microbial damage, v) increasing mineral bioavailability, vi) supplying energy to the intestinal mucosa, vii) stimulating the host's immune system, viii) decreasing the risk of infectious intestinal disease, and ix) having an anti-inflammatory and an antitumorigenic role [2,7,8,9,10,11,12,13,14].

The production of SCFAs may be modulated by a number of factors, including the numbers and types of microbiota present in dietary intake, substrate source, and gut transit time. Probiotics, prebiotics, and a combination of the two (synbiotic) in particular, play a most important role in the formation of SCFAs. Probiotics are live microorganisms that, when administered in adequate amounts, confer a health benefit on the host (FAO/WHO 2014). Prebiotics are substrates resistant to hydrolysis and digestion in the stomach and small intestine, and they stimulate the growth of potentially probiotic bacteria such as *Bifidobacterium* and *Lactobacilli* species, thus they exert health-promoting effects. *Lactobacilli* can produce end products, such as pyruvate, by the fermentation of carbohydrates during the glycolytic metabolic pathway, while *Bifidobacteria* can produce mainly

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acetate and formate by using the fermentation pathway. Because of the beneficial health effects of probiotics, prebiotics, and their metabolites, they are used as food supplements or the SCFA producer, and *Bifidobacteria*/*Lactobacilli* are added into solution, such as in yogurt. In addition, researchers are interested in the development of commercially available prebiotics and their metabolites in *in vitro* and *in vivo* media [3,15,16,17,18,19,20].

Prebiotic substances are present naturally in fruits, vegetables, plant roots, and seeds such as artichoke, chicory root, raw banana, yam, garlic, onion, leek, wheat bran, and asparagus. Commercial prebiotics like inulin, fructooligosaccharides, oligofructose, and (trans-) galactooligosaccharides (TOS or GOS) were derived through biochemical and/or enzymatic techniques from these foods. *Orchidaceae* commonly known as the orchid family are mostly cultivated as beautiful flowers having floricultural and commercial use as well as medicinal importance. These flowers have an estimated 800 genera including nearly 25,000 species. Salep, a Turkish word (Greek "salapi"/Arabic word "sahlab"), is obtained from the dried roots or tubers of the Orchidaceae [21,22,23,24,25,26]. Ophrys, Orchis, Himantoglossum, Serapias, Ana-camptis, Compreria, Barlia, Dactylorhiza, Aceras, and Neotinea naturally grown in Turkey have been used for the production of salep. Salep has been used traditionally as a therapeutic ingredient to treat diarrhea, tuberculosis, Parkinson's disease, cancer, fever, and enhance sexual activity. It is also used industrially as a thickening and flavoring agent, as an emulsifier and stabilizer in ice cream production, confectionery, and beverages [27,28,29,30,31,32]. Although the components of salep vary according to the season of collection and orchid species, generally it contains mucilage (48%), moisture (12%), sugar (1%), starch (3%), nitrogenous substance (5%), ash (2%), and glucomannan (16–60%). Glucomannan, a water-soluble polysaccharide, is highly fermented by bacteria in the colon; thus, foods containing glucomannan have been classified as "emerging prebiotics" owing to their potential for this type of application [29,33,34,35,36,37]. However, salep may be fermented by probiotic microorganisms probably because of its glucomannan content (from 16% to 60%). Usta and Yilmaz-Ersan [38] reported that the ability of salep to support bifidobacterial growth has been demonstrated.

In recent years, probiotics, prebiotics, and synbiotics are the best documented substances with the potential to generate SCFAs. In fact, this ability to produce SCFAs by both probiotic and prebiotic means is analyzed under fecal microbiota in a model system of the human colon. To date, there are limited experimental studies *in vitro* that have quantified the production of SCFAs specifically related to the use of prebiotics [39,40,41,42,43,44,45,46]. The objectives of this research were to study the effect of salep on the growth kinetics of four *Bifidobacterium* species (BS), their ability to produce lactic acid, and SCFAs: acetic, propionic, and butyric acids, and the effect of a synbiotic of salep and probiotic bacteria *in vitro*.

2. Materials and methods

2.1. Bacterial strains

The bacteria used in this study are listed in Table 1. These strains were obtained from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany) and activated at 37°C using an anerobic atmosphere generation system (Anaerocult A, Merck, Darmstadt, Germany).

Table 1
Bifidobacterium species used in this study.

Microorganisms	Strain
Bifidobacterium longum subsp. *infantis*	DSM 20288
Bifidobacterium animalis subsp. *lactis*	DSM 10140
Bifidobacterium longum subsp. *longum*	DSM 20219
Bifidobacterium bifidum	DSM 20239

2.2. Preparation of the salep solution

Salep from orchids grown in different regions of Turkey was used in this study. It was obtained from Kadem Sahlepcilik (Istanbul, Turkey). Salep contains 88.72% dry matter, 4.55% ash, 6.71% protein, 1.90% carbohydrate, and 6.75% dietary fiber. Stock solutions of salep (10% w/v) were prepared by modifying them according to the method suggested by Kaplan and Hutkins [47]. The salep solution was sterilized using a Millipore-Stericup-GP 0.45 μm filtration system.

2.3. Growth conditions

Tryptone Peptone Yeast Extract (TPY) was used in this study as the basal medium containing peptone (5.00 g L^{-1}), yeast extract (2.50 g L^{-1}), glucose (5.00 g L^{-1}), Tween 80 (1.00 g L^{-1}), $K_2HPO_4 \cdot 3H_2O$ (2.00 g L^{-1}), $MgCl_2$ (0.50 g L^{-1}), $ZnSO_4 \cdot 7H_2O$ (0.20 g L^{-1}), $CaCl_2$ (0.15 g L^{-1}), $FeCl_3 \cdot 6H_2O$ (0.003 g L^{-1}), and L-cysteine HCl (0.50 g L^{-1}). The medium was sterilized at 121°C for 15 min. Then, 1% and 2% (w/v) sterile salep solutions were added into the basal TPY medium to obtain the final concentrations. TPY without carbohydrates was used as the negative control, while TPY with 1% (w/v) glucose served as the positive control. An overnight culture of 2% (v/v) *Bifidobacterium* spp. was added to the basal media.

2.4. Measurement of pH

The pH of each sample was determined during fermentation using a pH-meter (pH 315i/SET; WTW, Germany).

2.5. Growth measurement

The optical density (OD) or cell density of the bacteria was determined at a wavelength of 600 nm (OD_{600}) with a spectrophotometer (Shimadzu UV 1800, Kyoto, Japan) during fermentation. The corresponding sterile TPY solutions without bacteria were used as blanks for the absorbance measurements.

2.6. Growth rate

The growth rate of each microorganism was calculated using the following equation [48]:

$$\text{Growth rate} : \frac{\text{OD of latest fermentation time} - \text{OD of previous fermentation time}}{\text{OD of previous fermentation time}}$$

2.7. Lactic acid and SCFA analyses

To evaluate the efficiency of the fermentation of salep by the *Bifidobacterium* spp., high performance liquid chromatography (HPLC) was performed. Lactic, acetic, propionic, and butyric acids can be detected in the growth medium and quantified by HPLC (Shimadzu marka LC-20 AD, Japan). The HPLC equipment consisted of a Transgenomics ORH-801 column and a refractive index detector (Shimadzu, Kyoto, Japan) connected to a recorder. Samples were filtered through a 0.45-μm syringe filter before injecting into the HPLC column. The injection volume was 20 μL. The mobile phase used was 0.0025 N H_2SO_4 at a flow rate of 0.6 mL min^{-1} at 65°C. Calibration curves including a broad concentration range were analyzed for lactic, acetic, propionic, and butyric acids. For each acid, validation parameters such as the limit of quantification, limit of detection, and

Table 2
Changes in maximum OD and final pH values between substrates.

Strains	OD_{max}				Final culture pH			
	Control	Glucose (1%)	Salep (1%)	Salep (2%)	Control	Glucose (1%)	Salep (1%)	Salep (2%)
B. bifidum	0.118^{cC}	1.049^{bC}	1.152^{aB}	0.657^{bD}	5.93^{aA}	4.60^{cA}	5.23^{bA}	4.62^{cA}
B. infantis	0.331^{cA}	1.630^{aB}	1.230^{bA}	1.049^{bA}	5.45^{aC}	3.52^{cD}	4.32^{bD}	4.23^{bB}
B. lactis	0.088^{dD}	1.836^{aA}	1.129^{bC}	0.748^{cC}	5.75^{aB}	4.08^{dC}	4.69^{bC}	4.27^{cB}
B. longum	0.258^{cB}	1.067^{aC}	1.083^{aD}	0.998^{bB}	5.94^{aA}	4.29^{dB}	5.04^{bB}	4.61^{cA}

Significance level, significant at $p < 0.01$ (**), different lower-case letter indicates different substrates; different uppercase letter indicates different strains.

linearity were determined according to FDA documents [49]. Peak retention times describing pure standard of each acid were determined. For the correct identification of peaks, standard mixture acid solution was also added to each sample extract. For repeatability of the method, relative standard deviation values for the responses of the retention times obtained from standard solution mixtures containing different levels of acid were calculated.

2.8. Statistical analyses

Statistical analyses were performed with Minitab 17.0 statistic package for Windows, and the data were compared using variance of analysis (ANOVA). The LSD Multiple Range test was used for multiple comparisons. The difference was used to determine the effect of the substrate type, strain type, and fermentation on the lactic acid and short-chain fatty acid contents. Different letters were used to label values with statistically significant differences. The hierarchical cluster analysis, which highlights intuitively similar relationships between any one sample and the entire data set, was performed following an unweighted pair group method with an arithmetic average based on a dissimilarity matrix using JMP 7 software.

3. Results and discussion

When fermentation substrates such as prebiotics are consumed by both animals and humans, the acidity in the colon decreases depending on the structure and dose of the prebiotics. Lower pH values result in a decrease in the growth of pathogenic bacteria and an increase in the growth of beneficial microbiota (*e.g.*, *Bifidobacteria* and *Lactobacilli*) [8]. Thus, pH is a critical value to assess the effectiveness of candidate prebiotics in *in vitro* and *in vivo* models. To determine the fermentability of salep by some *Bifidobacteria*, their maximum OD and final pH values obtained from this study during the growth of these strains on different substrates at 37°C for 48 h, are shown in Table 2. The results are presented as the mean value of the BS and the mean value of each substrate. For all parameters, significant differences were detected ($p < 0.01$). It was determined that glucose, which is metabolized quickly by probiotics as a nonprebiotic simple carbon source, had the lowest pH value, while media containing 2% salep had the second lowest value. Of the four species when compared with the media containing salep, *B. infantis* grew the fastest and recorded the lowest pH value. The sample with *B. lactis* in the media that contained 2% salep had the next lowest pH value. According to the results of this study, a decrease in pH values was found due to salep being metabolized by the BS. These results are in agreement with those reported by Kaplan and Hutkins [47], Tzortzis et al. [50], and Mumcu and Temiz [51]. As shown in Table 2, *B. bifidum* and *B. longum* in media containing 1% salep had a higher cell density value than the other substrates. Regarding the strains, the highest value of OD was recorded for *B. lactis*, followed by the *B. infantis* in the media with glucose. The OD values of BS grown in media with 1% salep were similar to those of glucose; thus, it could be said that salep is used by

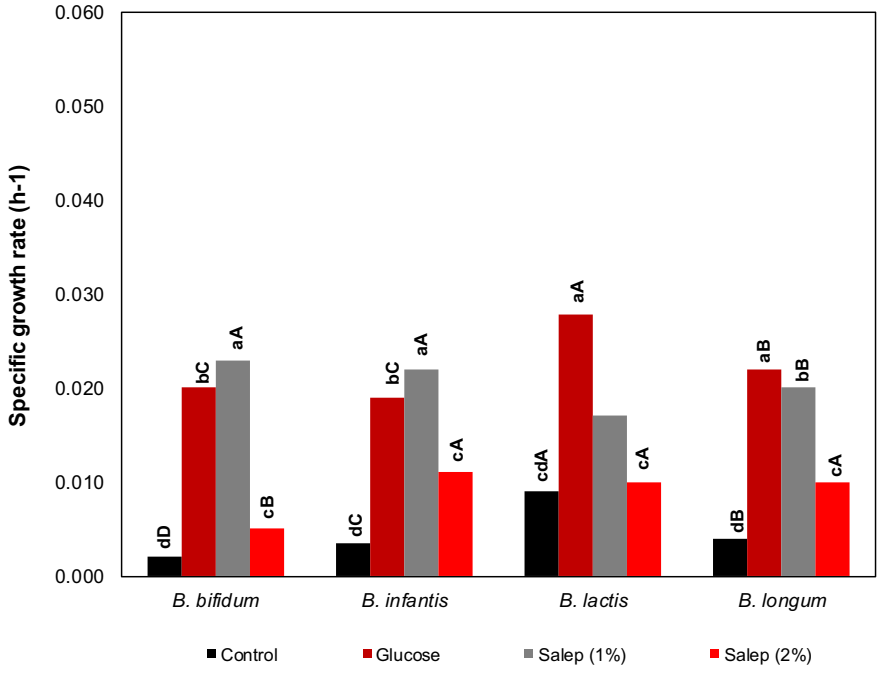

Fig. 1. Specific growth rates (h^{-1}) of *Bifidobacterium* species. Significance level, significant at $p < 0.01$ (**), different lowercase letters indicate different substrates; different uppercase letters indicate different strains.

Table 3
Lactic, acetic, propionic, and butyric acid production by *Bifidobacterium* species in different substrates during 48 h fermentation.

	N	Lactic acid	Acetic acid	Propionic acid	Butyric acid
Substrate type (ST)					
Control	24	0.399^{dA}	0.355^{dB}	0.039^{cD}	0.144^{bC}
Glucose (1%)	24	0.877^{aA}	0.698^{aB}	0.087^{aD}	0.170^{aC}
Salep (1%)	24	0.520^{bB}	0.535^{bA}	0.043^{bD}	0.114^{cC}
Salep (2%)	24	0.450^{cB}	0.527^{cA}	0.044^{bD}	0.052^{dC}
Bifidobacterium species (BS)					
B. bifidum	24	0.499^{cA}	0.308^{cB}	0.095^{aD}	0.129^{bC}
B. lactis	24	0.319^{dB}	0.744^{bA}	0.044^{cD}	0.095^{dC}
B. infantis	24	0.862^{aA}	0.818^{aB}	0.046^{bD}	0.121^{cC}
B. longum	24	0.567^{bA}	0.245^{dB}	0.028^{dD}	0.134^{aC}
Fermentation time (FT; hours)					
0	32	0.109^{cB}	0.278^{cA}	0.035^{cD}	0.061^{cC}
24	32	0.559^{bA}	0.516^{bB}	0.042^{bD}	0.152^{aC}
48	32	1.016^{aA}	0.792^{aB}	0.083^{aD}	0.147^{bC}
ANOVA					
Substrate type (ST)		**	**	**	**
Bifidobacterium species (BS)		**	**	**	**
Fermentation time (FT)		**	**	**	**
ST × BS		**	**	**	**
ST × FT		**	**	**	**
BS × FT		**	**	**	**
ST × BS × FT		**	**	**	**

ST = substrate type; BS = Bifidobacterium species; FT = fermentation time; ST × BS = interaction between substrate type and Bifidobacterium species; ST × FT = interaction between substrate type and fermentation time; BS × FT = interaction between Bifidobacterium species and fermentation time; ST × BS × FT = interaction among substrate type, Bifidobacterium species and fermentation time; Significance level, significant at $P < 0.01$(**), different lower case on the same column indicate significant differences; different uppercase letter on the same line indicate significant differences.

BS as a source of carbon and energy. The cell density values determined in the study were similar to Lee et al. [52], Holt et al. [53], Maischberger et al. [54], and Yang et al. [55].

The calculated specific growth rate in bacterial populations with the substrates tested are presented in Fig. 1. The highest specific growth rates were recorded with *B. lactis* grown in media containing glucose, followed by *B. bifidum*, and then *B. infantis* in media containing 1% salep. The negative control, containing no additional carbohydrate, had minimal specific growth rates. From the data presented in Fig. 1, salep is used by BS as a carbon source to promote growth.

The amounts of lactic, acetic, propionic, and butyric acid are shown in Table 3. The results are presented as the mean value of each fermentation time (FT), regardless of the substrate type (ST) and BS. For all parameters at all times, a significant interaction was detected ($p < 0.01$). There were significant differences ($p < 0.01$) within the level of acid contents for substrates and species. Regarding the fermentation time, higher values were recorded for lactic and acetic acid compared to propionic and butyric acid. The tested acids increased significantly ($p < 0.01$) during the 48 h fermentation as a result of the bifidobacterial enzymes. Lactic acid produced by *Lactobacilli, Bifidobacteria, Enterococci, Streptococci,* and *Eubacterium* in the gastrointestinal tract is a major organic acid in the fermentation process of prebiotics. As it is further metabolized to acetate or butyrate and propionate, respectively, by cross-feeding species – particularly with the butyrate-producing bacteria – it does not substantially accumulate in the colonic lumen. According to the results of this study, lactic acid was the most abundantly produced metabolite for all the tested substrates and strains. The concentration of lactic acid ranged from 0.319 g L^{-1} for *B. lactis* to 0.877 g L^{-1} for media with 1% glucose, depending on the strain and type of substrate. *B. infantis* produced the highest lactic acid among the tested strains (Table 3). Barczynska et al. [56] reported that the amount of lactic acid was determined to be 109.3 mg 100 mL^{-1} for *B. bifidum* Bb12 and 108.8 mg 100 mL^{-1} for *B. animalis* DN–173 010 in the broth containing the tartaric acid-dextrin. Acetic acid, a precursor for lipogenesis and cholesterol synthesis, stimulates anti-inflammatory responses and reduces the appetite because of its interaction with the central nervous system [2,57]. It is normally produced by *Lactobacilli* and *Bifidobacteria* [58]. Using the bifidus pathway *Bifidobacteria* produce more ATP from carbohydrates than homo and heterofermentative pathways. Theoretically an acetate: lactate ratio of 1.5:1 mol from 1 mol of glucose can be produced by the bifidus pathway. But researchers reported that such acetate: lactate ratios are impossible in practice, as pyruvic acid is converted into formic acid and ethanol rather than into lactic acid by some *Bifidobacteria* [59]. The acetic acid concentrations depended on the strain and substrate type. It ranged from 0.245 g L^{-1} for *B. longum* to 0.818 g L^{-1} for *B. infantis*. Generally,

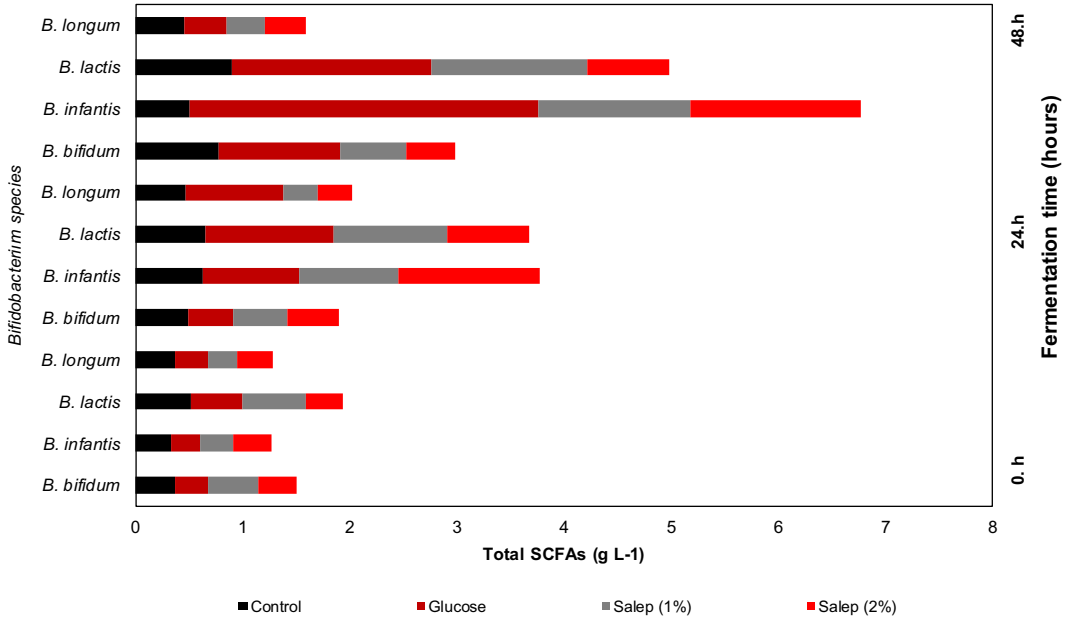

Fig. 2. Total short-chain fatty acid production by *Bifidobacterium* species in different substrates during a 48-hour fermentation.

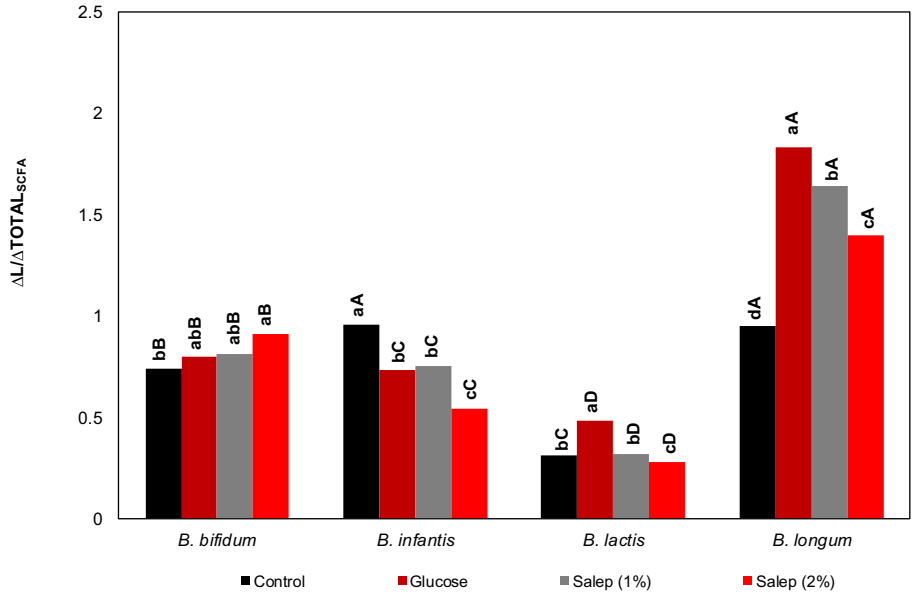

Fig. 3. $\Delta L/\Delta TOTAL_{SCFA}$ of *Bifidobacterium* species. Significance level, significant at $p < 0.01$(**), different lowercase letters indicate different substrates; different uppercase letters indicate different strains.

during the whole fermentation progress, samples – including salep powder – exhibited a lower acetic acid value than glucose. Acetic acid content was determined to be 19.1 mg 100 mL^{-1} for *B. bifidum* Bb12 and 16.9 mg 100 mL^{-1} for *B. animalis* DN–173 010 [56]. Propionic acid produced from fermentable substrates has anticholesterol and anticarcinogenic health effects [60,61]. Propionic acid contents ranged from 0.028 g L^{-1} for *B. longum* to 0.095 g L^{-1} for *B. bifidum*. For substrate types, the maximum propionic acid value was observed in media with glucose. Barczynska et al. [56] reported that a propionic acid content of 4.5 mg 100 mL^{-1} for *B. bifidum* Bb12 and 4.6 mg 100 mL^{-1} for *B. animalis* DN–173 010 was found in the broth containing the tartaric acid-dextrin as the only source of carbon after a 24-hour incubation. The production of butyrate, an important SCFA, is the main energy source for epithelial cells. It can reduce inflammation, carcinogenesis, oxidative stress, and improves the intestinal barrier function and colonic health [13,62,63,64]. For the tested BS, the maximum butyric acid value was observed for *B. longum*, while media with glucose had a higher butyric acid value than others. SCFA values obtained in this study were similar to the results reported by Vulevic et al. [65] using sucrose, guar gum, FOS and TOS, Haddadin [66] using olive leaf extract, and Khaleel and Haddadin [67] using Hawthorn leaf extracts.

SCFAs are produced by different bacterial species possessing specific enzymes through the glycolytic pathway. As every bacterial species has its own characteristic profile of SCFA products, these are often used in species identification. *Bifidobacteria* are unable to make use of the usual glycolytic pathway or the hexose monophosphate shunt pathway due to a lack of aldolase and glucose-6-phosphate NADP + oxidoreductase. *Bifidobacteria* produce mainly acetic and lactic acids by using the pentose phosphate pathway in the presence of fructose-6-phosphate phosphoketolase (F6PPK), while *Lactobacilli* can produce pyruvate by the fermentation of carbohydrates through the glycolytic pathway and also by the phosphoketolase pathway under heterofermenting conditions [68,69,70]. In addition, SCFA production is affected by several factors, including type and number of microorganisms present in the colon, the source and chemical

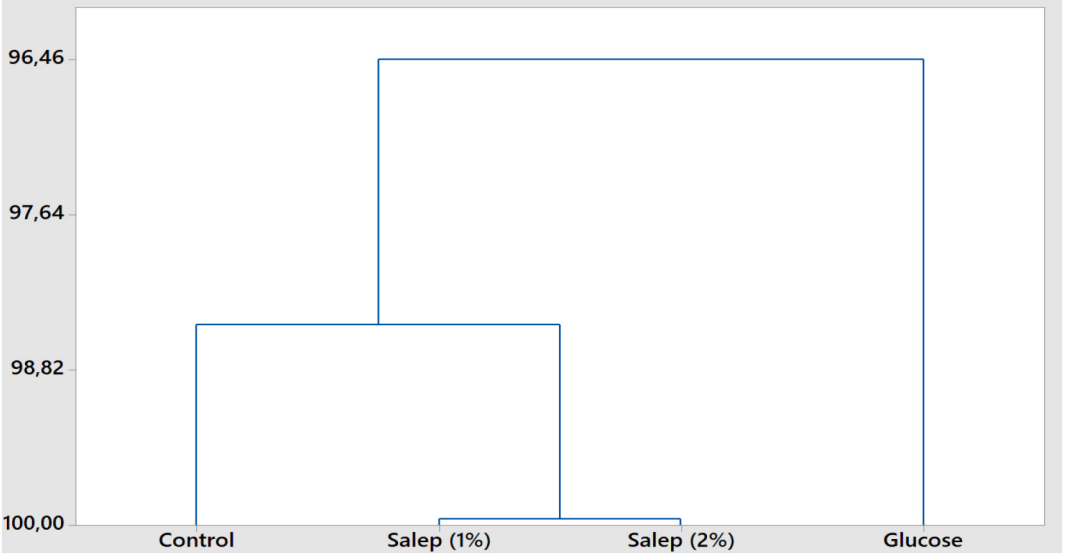

Fig. 4. Cluster analysis of substrates used.

structure of substrates, and the gut transit time [71]. Several studies have shown that some prebiotic components such as oligofructose and inulin are associated with a higher production of SCFA either *in vitro* or *in vivo* [35,72,73,74,75,76,77,78]. The way in which total SCFAs increase is an important parameter to evaluate the fermentation capacity of microorganisms used in candidate prebiotic components. Fig. 2 presents the variations in total SCFA values produced by the BS samples during fermentation. There were significant differences in total SCFA values among the strains depending on the ST used. At the beginning of the fermentation process, the total SCFA values produced by strains varied from 1.27 g L^{-1} for *B. infantis* to 1.94 g L^{-1} for *B. lactis*; and thereafter, increased gradually throughout the fermentation period. The largest increase in total SCFAs was observed for *B. infantis* and *B. lactis*, respectively, because they have higher metabolic activity than the others.

Vulevic et al. [65] stated that the prebiotic effect is associated with lactic acid-producing microorganisms such as *Bifidobacterium* and *Lactobacilli* species. To provide a qualitative as well as quantitative assessment of each substrate tested, the ratio of lactic acid production compared to the total SCFA production is calculated. The $\Delta L/\Delta TOTAL_{SCFA}$ of BS is presented in Fig. 3. For *B. bifidum*, the highest ratio is produced by the substrate containing 2% salep. For *B. lactis* and *B. longum*, the highest ratios were produced by the substrate with glucose and 1% salep, respectively. *B. longum* samples had the highest $\Delta L/\Delta TOTAL_{SCFA}$.

Hierarchical clustering was performed to determine if there was a relationship for the different substrates between the growth of BS and their ability to produce metabolites (Fig. 4). Cluster analysis was tested using the values of the growth parameters OD, pH, lactic, acetic, propionic, and butyric acid values, the total SCFA amounts and the $\Delta L/\Delta TOTAL_{SCFA}$ ratios obtained in this study. According to this analysis, the glucose and different salep concentrations demonstrate some differences on bifidobacterial growth. The salep samples (1% and 2%) clustered together, while samples containing glucose formed a separate cluster. The sample with 1% salep is closer to glucose – namely the positive control – than the others.

4. Conclusions

Salep has been used in the food industry because of its techno-functional properties such as a thickening, flavoring agent, emulsifier, and stabilizer; it also has therapeutic importance in the prevention and treatment of diseases. In this study, to determine the effect of salep on the growth of probiotic bacteria such as BS as well as its prebiotic potential was aimed. The results of this study demonstrated that salep exhibited the potential to be used as a new source of prebiotics, increasing the OD values of bifidobacterial cells, decreasing pH values, and forming metabolic products (lactate, acetate, propionate, and butyrate). However, these results only represent the *in vitro* situation. More sophisticated studies conducting a human volunteer trial using a large number of volunteers under more controlled conditions, and in gut models using fecal microbiota, are required to be able to describe salep as a new prebiotic food. Thus, there is a need for comparative studies on the prebiotic properties of salep using a standard protocol.

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Conflict of interest

We declare that there are no conflicts of interest.

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