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Seabuckthorn as a novel prebiotic source improves probiotic viability in yogurt

Aynur Gunenc [a], Christina Khoury [a], Candace Legault [a], Hannah Mirrashed [a], Jenny Rijke [b], Farah Hosseinian [a, c, *]

[a] Food Science and Nutrition, Chemistry Department, Carleton University, Ottawa, Ontario, Canada
[b] Applied Human Nutrition, Guelph University, Guelph, Ontario, Canada
[c] Institute of Biochemistry, Carleton University, Ottawa, Ontario, Canada

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ABSTRACT

It was aimed to i) investigate if seabuckthorn whole fruit (S), and seabuckthorn purified mucilage (SP) addition into yogurt enhance bacterial viability, by measuring total bacterial counts of different yogurt trials on selective media, pH and total titratable acidity (TTA) during 28 day cold storage at 4 °C, as well as ii) measure antioxidant activities of microwave extracted seabuckthorn crude mucilage (SC) and SP using oxygen radical absorbance capacity (ORAC) and DPPH scavenging activity. After 21 days of cold storage, yogurts with S and SP maintained higher viable bacteria counts in both of probiotics, *Lactobacillus acidophilus* (9.3 log cfu/mL) and *Bifidobacterium lactis* (9.2 log cfu/mL), higher TTA (0.8%), and lower pH (5.0) compared to the controls ($P < 0.05$). SC exhibited strong antioxidant activity with an ORAC value of 138.9 μmol Trolox equivalents/100 g, and a %DPPH scavenging activity value of 37.0%. Results of this study suggest S may serve as a new prebiotic source for functional foods and nutraceutical applications.

1. Introduction

Seabuckthorn (S), *Hippophae rhamnoides* is a thorny, deciduous shrub, native to Europe and Asia (Upadhyay, Kumar, & Gupta, 2010). S has non-digestible oligosaccharides (NDOs) like prebiotics having the ability to enhance the activity of health promoting bacteria found in the human digestive system (Roberfroid, 2007). Prebiotics are substances such as carbohydrate polymers and sugars that improve host health by stimulating the growth and activity of health promoting bacteria found in the human digestive system (Santos, San Mauro, & Diaz, 2006). Probiotics are defined as live microorganisms, beneficially affect the health of the host by improving the properties of the indigenous microflora (Champagne & Gardner, 2008; Schrezenmeir & de Vrese, 2001).

To the best of our knowledge, there is no report on employing S, and S-purified mucilage (SP) into yogurt as a source of prebiotic. Hence, the objectives were to i) investigate if the addition of S and SP into yogurt enhance bacterial viability through measuring total bacterial counts of different yogurt trials on selective media, pH and total titratable acidity (TTA%) during 28 days of cold storage at 4 °C, and ii) measure antioxidant activities of microwave extracted S-crude mucilage (SC) and SP using ORAC and DPPH scavenging activity.

2. Materials and Methods

2.1. Materials

Ethanol, methanol, and HCL were purchased from Caledon Laboratories LTC (Georgetown, ON, Canada). Trolox, mono- and di-basic potassium phosphate, fluorescein, rutin, AAPH, DPPH, and phenolphthalein were from Sigma (Oakville, ON, Canada). Sodium carbonate, sodium propionate was from Church and Dwight Canada Corp (Mississauga, ON, Canada). Protease (*Bacillus licheniform*, EC 232-560-9) and α-amylase (*Bacillus licheniformis*, EC 232-752-2) were from Sigma–Aldrich (St. Louis, Missouri, USA). Peptone, yeast extract powder, sodium acetate anhydrous, ammonium citrate, magnesium sulfate were from BioShop® Canada Inc. (Burlington, ON). *Lactobacillus delbrueckii* subsp. *bulgaricus* (B-548; USDA) and *Streptococcus salivarius* subsp. *thermophilus* (14485; ATCC), *Lactobacillus acidophilus* (B-4495; USDA) and *Bifidobacterium lactis*

* Corresponding author. Food Science and Nutrition, Chemistry Department, Carleton University, Ottawa, Ontario, Canada.
E-mail address: farah.hosseinian@carleton.ca (F. Hosseinian).

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(41405; USDA) were purchased from Oxoid Ltd. (Basingstoke, UK).

2.2. Sample preparations

2.2.1. S samples

Horticulture Research Center (Laval University, Quebec, Canada) has provided seabuckthorns as whole fruits upon arrival at −20 °C.

2.2.2. SC and SP extractions

SC: S was crushed using a juice processor (Black & Decker, WI, USA) and stored in Ziploc bags in the freezer at −20 °C prior to analysis. The samples (5 g) and 50 mL distilled water were added into a quartz vessel and placed into the CEM STAR System 2 microwave digestion system (CEM STAR System 2- Microwave, NC, USA) at 90 °C for 30 min (Liazid, Palma, Brigui, & Barroso, 2007). The mixture was cooled and then centrifuged at 4000 rpm for 20 min (Thermo Sorval, Nepean, ON, Canada) at room temperature (23 °C). The supernatant was collected and stored at −20 °C in the freezer until further analysis, while some extract was run through further purification. All analysis was made in triplicate.

SP: SC were treated with α-amylase and protease (20 μL/100 mL) and stirred at 37 °C for 24 h to eliminate proteins and starch molecules. The mixture was centrifuged at 4000 rpm for 20 min. The supernatant was heated at 95 °C for 5 min to inactivate the enzymes, cooled to room temperature and re-centrifuged at 6000× *g* for 20 min. Then, it was dialyzed against double distilled water for 48 h and replaced with fresh distilled water every 6 h to separate polysaccharides and other materials with a molecular weight cut-off of 3500 Da (Spectra/Por, Rancho Dominguez, CA, USA).

2.3. Prebiotic activity

The best concentration of S and SP to be added for each yogurt trial without resulting in syneresis was preliminary determined as 2% (Agil & Hosseinian, 2012). Therefore, 2% of S and SP was added to 50 mL pasteurized milk and incubated at 42 °C until completion of fermentation.

2.3.1. Microbial cultures

Starter cultures (*Lactobacillus bulgaricus* and *Streptococcus thermophiles*) and probiotics (*L. acidophilus* and *B. lactis)* were employed and each strain was grown in 10 mL sterile aliquots of corresponding broth liquid media (MRS or M17), and incubated at 37 °C for 48 h. The cultures were diluted with sterilized milk to obtain a concentration of approximately 6.5 log cell/mL. Then, 0.5 mL of each yogurt starter culture and 1.0 mL of required probiotic(s) was inoculated according to the experimental design (Table 1).

Table 1
Experimental design for evaluating S and SP mucilage addition effects on microbial viability in different yogurt trials.

Yogurt trials*	Fruit	Sample coding
Y	–	Y
Y + Pro1	–	Y+1
Y + Pro2	–	Y+2
Y + Pro1&2	–	Y+1+2
YS	+	S
YS + Pro1	+	S+1
YS + Pro2	+	S+2
YS + Pro1&2	+	S+1+2
YSP	+	SP
YSP + Pro1	+	SP+1
YSP + Pro2	+	SP+2
YSP + Pro1&2	+	SP+1+2

*S = seabuckthorn; SP = seabuckthorn purified mucilage; (−) = without S or SP; (+) = with S or SP; Y = yogurt with only starter cultures of *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus salivarius* subsp. *thermophilus*; Pro1 = probiotics of *Lactobacillus acidophilus*; Pro2 = *Bifidobacterium lactis.*

2.3.2. Yogurt trials

Whole milk was heated at 85 °C for 15 min, and cooled to 42 °C in a water bath. 50 mL portions was transferred to test tubes and inoculated with each starter cultures, probiotics, 2% S, and 2% SP. Twelve yogurt trials were divided in three groups (2% S homogenate, 2% SP, and controls). Tubes were placed in an incubator (42 °C) and terminated once pH reached ~ pH 5.0 and stored at 4 °C (Espírito Santo et al., 2010). All yogurt treatments were carried out in triplicate.

2.3.3. Bacterial counts

Bacterial enumerations were performed during 4 week storage; *Streptococcus thermophilus* (M17, aerobic, 24 h at 37 °C) (Santos et al., 2006), *L. bulgaricus* (MRS, aerobic, 72 h, 42 °C) (Goncalves, Freitas, Nero, & Carvalho, 2009), *L. acidophilus* (T-MRS, aerobic, 48 h, 37 °C) and *B. lactis* (LP-MRS, anaerobic, 72 h, 42 °C using a BBL GasPak™ System (Basingstoke, Hampshire, England) (Vinderola & Reinheimer, 1999). Colonies were counted as the log of colony forming units per microliter of sample using the following equation:

$$\mathrm{Log}\frac{\mathrm{CFU}}{\mathrm{mL}} = \frac{1000\ \mu\mathrm{L} \times \frac{\mathrm{CFU}}{\mathrm{plate}}}{10\ \mu\mathrm{L}} \times \text{dilution factor}$$

2.3.4. pH and TTA

pH (Denver Instrument) and TTA were determined (Espírito Santo et al., 2010) during 4 week storage. The amount of acid produced during fermentation was expressed as TTA% (Behrad, Yusof, Goh, & Baba, 2009).

2.4. Antioxidant activity

2.4.1. ORAC

Antioxidant activity of SC and SP was determined by ORAC assay (FLx800™ BioTek Instruments) (Hosseinian et al., 2007; Huang, Ou, Hampsch-Woodill, Flanagan, & Prior, 2002) and was expressed as μmol Trolox equivalents per 100 g of whole S.

2.4.2. DPPH scavenging activity assay

200 μL of SC and SP was mixed with 3.8 mL of DPPH (60 μM). The absorbance (UV–Visible Spectra-Max Plus384) of the mixture was measured at 515 nm at 60 min. DPPH was calculated as (Li, Hydamaka, Lowry, & Beta, 2009):

$$\%\mathrm{DPPH} = \left(1 - \left[\frac{A_{\mathrm{sample}}}{A_{\mathrm{control\ t=0}}}\right]\right) \times 100$$

2.5. Statistical analyses

All experiments were conducted in triplicates. A two-way analysis of variance (ANOVA) was used by SAS (Statistical Analysis System, 9th Version, SAS Institute Inc., Cary, NC) to compare the mean differences between groups. Duncan's Multiple Range test was used when significant ($P < 0.05$) mean comparison was performed.

3. Results and discussion

3.1. Probiotic activity

3.1.1. Starter culture enumerations

Both of starter culture counts (log cfu mL^{-1}) in all yogurt trials are shown in Table 2. On day 1, *S. thermophilus* and *L. bulgaricus* counts ranged from 7.18 to 8.16 log cfu mL^{-1} and 5.33 to 7.82 log cfu mL^{-1} amongst all treatments, respectively. S and S+1 yogurts showed significantly ($P < 0.05$) higher *S. thermophiles* counts than corresponding controls while there was no difference in comparison to SP yogurts. Control yogurts showed significantly lower *L. bulgaricus* counts ($P < 0.05$) compared to S and SP yogurts.

On day 7, *S. thermophilus* counts in SP yogurts were significantly higher ($P < 0.05$) than the Y and S yogurts. S yogurt had the highest ($P < 0.05$) *L. bulgaricus* counts, with an increase of over 1 log cfu mL^{-1}.

On day 14, S and SP yogurts exhibited higher *S. thermophilus* counts than Y, Y+1, Y+2, and Y+1+2 yogurts. Also, the highest *L. bulgaricus* count was recorded on day 14 of cold storage.

On day 21, all S and SP treatments showed significantly higher counts of *S. thermophilus* ($P < 0.05$) than the controls (Y, Y+1, Y+2, and Y+1+2). All S yogurts showed significantly higher *L. bulgaricus* ($P < 0.05$) count in comparison to the controls by more than 1 log cfu mL^{-1}

On day 28 *Streptococcus thermiphulis* counts were higher in S and SP yogurts than the controls. S addition no longer showed any effect on *L. bulgaricus* growth. Interestingly, almost all SP yogurts presented a lower growth in comparison to the controls. Suggesting that after 28 days of cold storage SP addition may have a negative impact on *L. bulgaricus*.

Overall, *S. thermophilus* count was better compared to those of *L. bulgaricus* which was parallel to the literature (Dave & Shah, 1997; Gunenc, Fang, & Hosseinian, 2015). Therefore, S and SP addition may have benefit of increasing the growth of starter cultures. After 28 days, *L. bulgaricus* seemed to be negatively impacted by the presence of SP compared to the control. This observation may be explained by the fact that counts of *L. bulgaricus* increase initially followed by a sharp decline during the latter stages of a storage period (Dave & Shah, 1997). In the present study, counts were initially low, followed by maximum increase at day 14 and slightly decreasing by day 28. This may be explained by *L. bulgaricus* requirements for simple sugars, like sucrose and lactose for growth (Vinderola, Costa, Regenhardt, & Reinheimer, 2002). Seabuckthorn polysaccharide extract contains more complex sugars, which may not have been easily digested by the starter culture. In addition, the presence of probiotics and *S. thermophilus* in different treatments and depletion of nutrients would be expected by the end of the cold storage. Furthermore, the growth of all probiotics (Fig. 1) in the presence of S and SP flourished and in some cases grew more with SP presence. Therefore, increased microbial activity of the probiotics and lack of simple sugars in the SP extract may have negative effect on *L. bulgaricus* growth. Therefore, the addition of SP may indirectly aid probiotic viability by antagonizing the growth of *L. bulgaricus* (Vasiljevic & Shah, 2008).

3.1.2. Probiotics enumerations

The viable microbial counts (log cfu mL^{-1}) of *L. acidophilus* and *B. lactis* in yogurt treatments during 28 days of cold storage were shown in Fig. 1A and B, respectively. On day 1, the presence of S and SP resulted in more growth ($P < 0.05$) of *L. acidophilus* in S+1 and SP+1 as well as S+1+2 and SP+1+2 yogurts. For *B. lactis*, both S and SP addition increased ($P < 0.05$) the growth in S+2 and SP+2 yogurts compared to the controls of Y+2 and Y+1+2 (Fig. 1B).

On day 7, the presence of S and SP revealed significantly higher ($P < 0.05$) growth of *L. acidophilus* in S+1 and SP+1 as well as in S+1+2 and SP+1+2 yogurts. *B. lactis* counts were significantly higher ($P < 0.05$) in S+2 and SP+2 as well as in S+1+2 and SP+1+2 yogurts in comparison to the controls (Y+2 and Y+1+2).

On day 14, *L. acidophilus* counts were significantly higher ($P < 0.05$) in S, SP S+1+2, and SP+1+2 yogurts in comparison to the respective controls. Also SP addition contained the highest amount of *L. acidophilus*: 9.1 log cfu mL^{-1} in SP+1 and 9.0 log cfu mL^{-1} in SP+1+2. *B. lactis* in S and SP yogurts was significantly higher ($P < 0.05$) than the controls (Y+2 and Y+1+2).

On day 21, S+1, S+1+2, and SP+1+2 yogurts showed significantly higher *L. acidophilus* growth ($P < 0.05$) than the controls (Y+1 and Y+1+2). Also, S and SP yogurts had higher ($P < 0.05$) *B. lactis* growth in comparison to the respective controls (Y+2 and Y+1+2). Interestingly, the yogurts containing SP (SP+2 and SP+1+2) had the highest amount ($P < 0.05$) of viable *B. lactis*: 8.9 log cfu mL^{-1} in the third treatment and 9.2 log cfu mL^{-1} in the fourth treatment, which paralleled results observed of *L. acidophilus* (Fig. 1A and B).

On day 28, only SP+1 yogurt exhibited higher ($P < 0.05$) *L. acidophilus* counts in comparison to the control Y+1. No differences ($P < 0.05$) in *B. lactis* counts between Y+2, S+2, and SP+2 yogurts were observed. However, SP+1+2 yogurt showed significantly higher ($P < 0.05$) counts of *B. lactis*.

Based on two-way ANOVA analysis, all control yogurts containing *L. acidophilus* were significantly lower than both S and SP added yogurts during the 28 days of cold storage. Yogurts containing the polysaccharide extract exhibited the highest counts of *L. acidophilus* in SP+1 and SP+1+2 yogurts. Both of S and SP additions had a positive effect, because they have substrates such as oligosaccharides; stimulating the growth of probiotic bacteria. Oligosaccharides are water-soluble low molecular carbohydrates and fermented by various strains of bifidobacteria and lactobacilli,

Table 2
S. thermophilus and *L. bulgaricus* counts (log cfu/mL) in yogurts with/without S and SP mucilage during 28 days cold storage at 4 °C.

S. thermophilus	Day 1	Day 7	Day 14	Day 21	Day 28
Y	7.83^{abc}	7.96^{cde}	8.29^{c}	8.55^{c}	8.72^{bc}
Y+1	7.68^{bc}	7.84^{cde}	8.46^{bc}	8.19^{d}	8.62^{bc}
Y+2	7.53^{cd}	7.49^{e}	7.85^{d}	8.64^{bc}	8.40^{cd}
Y+1+2	7.18^{d}	7.64^{de}	8.31^{c}	8.64^{bc}	8.12^{d}
S	7.68^{bc}	7.86^{cde}	9.18^{a}	9.25^{a}	9.04^{ab}
S+1	8.16^{a}	8.19^{abcd}	8.77^{ab}	8.94^{ab}	8.88^{ab}
S+2	7.76^{abc}	7.83^{cde}	8.95^{a}	9.17^{a}	8.87^{ab}
S+1+2	7.82^{abc}	8.14^{bcd}	8.85^{ab}	9.07^{a}	9.15^{a}
SP	7.77^{abc}	8.73^{a}	9.13^{a}	9.13^{a}	8.91^{ab}
SP+1	8.03^{ab}	8.20^{abcd}	9.00^{a}	9.22^{a}	8.87^{ab}
SP+2	7.94^{abc}	8.39^{abc}	9.05^{a}	9.16^{a}	8.75^{ab}
SP+1+2	7.66^{bc}	8.67^{ab}	9.15^{a}	9.10^{a}	8.67^{bc}
L. bulgaricus					
Y	5.96^{b}	7.27^{de}	8.04^{d}	7.68^{c}	8.32^{ab}
Y+1	5.33^{b}	7.25^{de}	9.14^{ab}	7.62^{c}	8.50^{a}
Y+2	6.01^{b}	7.22^{de}	8.95^{abc}	7.94^{c}	8.21^{ab}
Y+1+2	5.64^{b}	7.37^{cde}	9.18^{ab}	8.08^{bc}	7.80^{bc}
S	7.02^{a}	7.39^{cde}	9.27^{a}	9.16^{a}	8.76^{a}
S+1	7.31^{a}	7.65^{abcd}	9.15^{ab}	8.98^{a}	8.55^{a}
S+2	7.17^{a}	7.16^{e}	9.28^{a}	9.23^{a}	8.55^{a}
S+1+2	7.26^{a}	7.54^{bcde}	8.73^{abcd}	9.27^{a}	8.24^{ab}
SP	7.82^{a}	8.03^{a}	8.25^{cd}	7.63^{c}	7.10^{d}
SP+1	7.76^{a}	7.96^{ab}	8.75^{abcd}	8.83^{ab}	7.80^{bc}
SP+2	7.66^{a}	7.55^{bcde}	8.65^{abcd}	8.47^{abc}	7.51^{cd}
SP+1+2	7.46^{a}	7.73^{abc}	8.38^{bcd}	7.89^{c}	7.49^{cd}

*S = seabuckthorn; SP = seabuckthorn purified mucilage; Y = yogurt with only starter cultures of *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus salivarius* subsp. *thermophilus*; 1 = Probiotic1(*Lactobacillus acidophilus*); 2 = Probiotic 2 (*Bifidobacterium lactis*); Different letters in columns in the same day are significantly different ($P < 0.05$) in Duncan's multiple range tests.

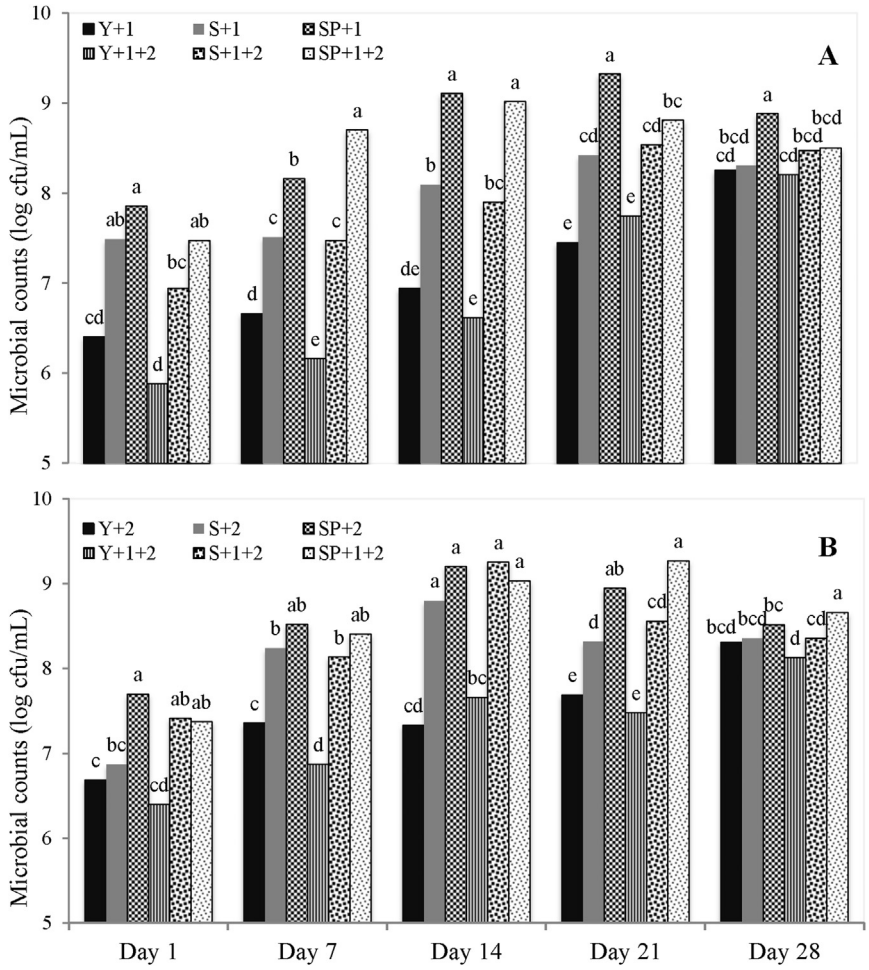

Fig. 1. A) Probiotic 1(*Lactobacillus acidophilus*) counts in control (Y+1, Y+1+2), S (S+1, S+1+2) and SP (SP+1,SP+1+2) yogurts over 28 days of cold storage; B) Probiotic 2-(*Bifidobacterium lactis*) counts in control (Y+2, Y+1+2), S (S+2, S+1+2), and SP (SP+2, SP+1+2) yogurts over 28 days of cold storage. Means with different letters are significantly ($P < 0.05$) different within the same day.

because they serve as a food source and S has a mixture of hydrophobic materials from the fruit and seeds, while SP contained a more potent amount of the NDOs (Mussatto & Mancilha, 2007) Therefore, SP might have a stronger effect on *L. acidophilus* than S since the higher level of polysaccharide substrates selectively stimulating microbial growth (Agil & Hosseinian, 2012). However, on day 28, *L. acidophilus* counts in SP+1+2 yogurt was 8.4 log cfu mL^{-1} and showed no difference compared to the control. Indicating that the presence of a second probiotic may have an effect on *L. acidophilus* counts due to an accumulation of cellular waste and nutrient depletion (Vasiljevic & Shah, 2008).

B. lactis showed a slightly different growth pattern from that of *L. acidophilus*. It was found that all control yogurts containing *B. lactis* were significantly lower than both S and SP addition throughout the 28 days of cold storage. SP yogurts exhibited the highest counts of *B. lactis* in SP+2 and SP+1+2 yogurts. S contains oligosaccharides and essential nutrients that promote viability and growth of microbes. In particular, Bifidobacteria utilizes amino acids such as valine, glycine, and histidine to support its growth (Vasiljevic & Shah, 2008). S has high quantities of valine, glycine, and histidine, with levels of 21.8 mg/100 g, 16.7 mg/100 g, and 13.7 mg/100 g, respectively (Beveridge, Li, Oomah, & Smith, 1999). Probiotic organisms have weak proteolytic activity, therefore they require these free amino acids to increase their numbers (Vasiljevic & Shah, 2008).

3.1.3. pH and TTA

On day 1, pH varied from 6.10 to 6.56 amongst all yogurt treatments. S and SP yogurts did not show a significant difference in pH ($P < 0.05$) compared to corresponding controls (Table 3). TTA data ranged from 0.1 to 0.22 mg lactic acid g^{-1}, where TTA data showed no significant differences between S and SP yogurts.

On day 7, S yogurts showed no difference ($P < 0.05$) in pH compared to the respective controls. The pH of SP yogurts was lower ($P < 0.05$) than those of the controls (Y, Y+2, and Y+1+2) with the exception of SP+1. TTA was significantly higher ($P < 0.05$) in the yogurts containing SP when compared to the controls without extracts and the yogurts containing S. From the S yogurts, only S+2 yogurt had higher ($P < 0.05$) TTA in comparison to the control (Y+2).

After 14 days, all S and SP yogurts exhibited significantly lower pH ($P < 0.05$) in comparison to the controls. Similarly, TTA data for all S and SP yogurt showed higher levels in comparison to their respective controls.

On day 21, pH ranged from 6.3 to 5.29 amongst the different treatments and it was observed that all S and SP yogurts had significantly lower pH ($P < 0.05$) compared to their respective controls. In addition, only S, S+1, S+2, and S+1+2 yogurts had higher levels ($P < 0.05$) of TTA in comparison to their respective controls, however SP showed no significant differences when compared to their controls.

Table 3
pH and TTA values of yogurts with/without S and SP mucilage during 28 days cold storage at 4 °C.

pH	Day 1	Day 7	Day 14	Day 21	Day 28
Y	6.56^{a}	6.47^{a}	6.41^{a}	6.31^{a}	6.04^{a}
Y+1	6.10^{a}	6.02^{bcde}	6.30^{ab}	5.90^{b}	5.71^{b}
Y+2	6.43^{a}	6.36^{ab}	6.16^{b}	6.31^{a}	6.10^{a}
Y+1+2	6.50^{a}	6.47^{a}	6.23^{b}	6.48^{a}	6.21^{a}
S	6.35^{a}	6.11^{abcd}	5.73^{de}	5.43^{c}	5.13^{c}
S+1	6.36^{a}	6.22^{abc}	5.86^{cd}	5.36^{c}	5.11^{c}
S+2	6.31^{a}	6.10^{abcd}	5.73^{de}	5.29^{c}	5.01^{c}
S+1+2	6.39^{a}	6.28^{abc}	5.91^{c}	5.44^{c}	5.21^{c}
SP	6.51^{a}	5.72^{de}	5.65^{ef}	5.41^{c}	5.16^{c}
SP+1	6.48^{a}	5.89^{cde}	5.62^{ef}	5.29^{c}	5.25^{c}
SP+2	6.24^{a}	5.69^{e}	5.56^{f}	5.33^{c}	5.16^{c}
SP+1+2	6.41^{a}	5.75^{de}	5.59^{ef}	5.31^{c}	5.12^{c}
TTA%					
Y	0.10^{b}	0.17^{d}	0.21^{c}	0.38^{bc}	0.39^{g}
Y+1	0.11^{b}	0.17^{d}	0.22^{c}	0.37^{bcd}	0.38^{g}
Y+2	0.13^{b}	0.17^{d}	0.24^{c}	0.37^{bc}	0.39^{g}
Y+1+2	0.11^{b}	0.16^{d}	0.23^{c}	0.36^{bcd}	0.42^{g}
S	0.21^{a}	0.21^{cd}	0.33^{b}	0.71^{a}	0.74^{b}
S+1	0.22^{a}	0.22^{cd}	0.35^{b}	0.68^{a}	0.87^{a}
S+2	0.22^{a}	0.24^{bc}	0.35^{b}	0.65^{a}	0.71^{bc}
S+1+2	0.19^{b}	0.21^{cd}	0.31^{b}	0.63^{a}	0.69^{cd}
SP	0.18^{a}	0.26^{bc}	0.33^{b}	0.41^{cd}	0.57^{de}
SP+1	0.21^{a}	0.35^{a}	0.42^{a}	0.32^{d}	0.53^{ef}
SP+2	0.20^{a}	0.30^{ab}	0.38^{ab}	0.36^{bcd}	0.53^{ef}
SP+1+2	0.21^{a}	0.33^{a}	0.35^{b}	0.44^{b}	0.51^{f}

*S = seabuckthorn; SP = seabuckthorn purified mucilage; Y = yogurt with only starter cultures of *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus salivarius* subsp. *thermophilus*; 1 = Probiotic 1(*Lactobacillus acidophilus*); 2 = Probiotic 2 (*Bifidobacterium lactis*); Different letters in columns in the same day are significantly different (P < 0.05) in Duncan's multiple range tests.

After 28 days, all S and SP yogurts exhibited significantly lower pH (P < 0.05) in comparison to the respective controls. All S yogurts had higher TTA levels in comparison to the respective controls and to their equivalent SP treatments.

Fermentation was allowed to reach a range between 6.2 and 4.9 following a U.S. patent for preparing cultured dairy products (Lundstedt & Corbin, 1983). A pH of approximately six was recommended as the most active form of the enzyme β-galactosidase (β-gal), found in the bacteria *S. thermophilus* and *L. bulgaricus*, which is responsible for digesting lactose in yogurt (Martini, Kukielka, & Savaiano, 1991). This enzyme helps break down the lactose, which makes it easier for lactase-deficient people to consume yogurt. After 28 days, all S and SP samples exhibited lower pH compared to the control which coincided with the TTA results. By this time S and SP treatments showed a pH drop of ~1.2 and 1.5 respectively, while those of the controls only dropped ~0.4 units from day 1 readings. Suggesting that in the presence of S, bacteria are potentially more active, thus producing more lactic acid and consequently decreasing pH.

The TTA test was used to identify and quantify the amount of lactic acid present in the yogurt samples. Lactic acid was targeted, because it is the most prevalent acid produced by probiotic bacteria, while other acids such as butyric and propionic acids are produced in lesser amounts (Agil & Hosseinian, 2012). It was observed that all yogurt treatments showed a general trend of increasing TTA levels indicating an increasing amount of bacteria activity, thus a result of growing number of bacteria (Gunenc et al., 2015). The TTA readings for both S and SP were significantly higher than the respective controls (P < 0.05) and suggesting that in the presence of S, bacteria produce more lactic acid, which confirm the pH results. Other studies have reported similar findings of increased probiotic viability in yogurts with increased TTA levels and corresponding lower pH levels (Agil & Hosseinian, 2012; Espírito Santo et al., 2010).

3.2. Antioxidant activity

3.2.1. ORAC

SC had higher antioxidant activity (P < 0.05) compared to SP with ORAC values of 138.95 ± 0.99 μmol TE/100 g of fruit and 29.22 ± 3.08 μmol TE/100 g of fruit respectively (Table 4). As expected, SC showed a much higher antioxidant activity than SP. SC contained water soluble materials including proteins, flavonoids, and polysaccharides. S contains high levels of ascorbic acid; ranging from 360 to 2500 mg/100 g of berries compared to 35–56 mg/100 ml of orange juice as well as flavonoids or quercitin bound to carbohydrates such as glucose, fructose, and xylose (Bal, Meda, Naik, & Satya, 2011). These compounds are most certainly responsible for the antioxidant activity exhibited in SC extracts. Decreased antioxidant activity was observed in SP extracts, because of the purification step of dialysis. Compounds that were greater than 3500 Da were eliminated from the extraction. In addition, the enzymes α-amylase and protease broke down proteins and carbohydrates conjugated to phenolic acids that are responsible for scavenging free radicals. Interestingly, polysaccharides in the purified solution showed some antioxidant activity, which may indicate that phenolic acids may be bound, such as gallic acid, a type of phenolic acid (Arimboor, Kumar, & Arumughan, 2008).

3.2.2. DPPH

The DPPH radical (60 μM) scavenging activity assay was used as an additional method to confirm antioxidant trends of seabuckthorn extracts (1 g/10 mL). SC clearly showed higher antioxidant activity (P < 0.05) than SP, confirming ORAC results (Table 4). After 60 min, SC showed 37% DPPH scavenging activity, while SP showed 12%. These results are comparable with the literature that found DPPH radical (60 μM) scavenging activity after 60 min was 29.97% for seabuckthorn (1 g/15 mL) (Li et al., 2009). The antioxidant activity of this berry may be attributed to the high content of flavonoids (mainly isorhamnetin, quercetin glycosides, and kaempferol), which can be found in the amount of 100–1000 mg/100 g fruit (Uruakpa & Utioh, 2012). In another study, the anthocyanin composition and antioxidant activity of various berries were measured. The % DPPH scavenging activity of raspberry and strawberry was 46% and 25% respectively (Ogawa et al., 2008). Our findings were in close range with those above mentioned studies.

For accurate comparison among the measured antioxidant capacity of samples varies with the assay method used, pH and time of reaction and it is a function of the array of individual antioxidants present in the sample. Therefore, accurate comparison among fruit samples require that reaction times be standardized and more than one assay should be used to describe the total antioxidant activity of fruit samples (Ozgen, Reese, Tulio, Scheerens, & Miller, 2006).

4. Conclusion

This study indicates that S addition may selectively enhance probiotic counts in yogurt. S yogurts displayed decreased pH and

Table 4
ORAC[a] and %DPPH[b] values of SC[c] and SP.[d]

Sample	ORAC (μmol TE/100 g)	%DPPH
SC	138.95 ± 0.99[a*]	37 ± 0.85[a]
SP	29.22 ± 3.08[b]	12 ± 0.98[b]

*Different letters in columns are significantly different (P < 0.05) in Duncan's multiple range tests.
[a] ORAC = Oxygen radical absorbance capacity values was calculated as μmole Trolox Equivalent (TE)/100 g of sample.
[b] %DPPH = 2,2-diphenyl-1-picryhydrazyl radical.
[c] SC = seabuckthorn crude mucilage.
[d] SP = seabuckthorn purified mucilage.

increased TTA after 28 days of cold storage as well as SC showed significant antioxidant activity which is attributed to flavonoids present in solution. Further studies are needed like animal models and human clinical trials to determine the reputable health-related properties of S and additionally its use in yogurt. This study is novel in the field, because S has never been studied in a yogurt medium. S can potentially be a valuable addition in dairy products due to the promotion of probiotic bacteria. This study suggests that whole fruit or crude extract have potential to make/produce probiotic yogurt with antioxidant activity. Optimization for production of yogurt in larger scale can be done for future studies.

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