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Colonic fermentation of polyphenolics from Sea buckthorn (*Hippophae rhamnoides*) berries: Assessment of effects on microbial diversity by Principal Component Analysis

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ABSTRACT

The present study investigates the stability of polyphenolic in Sea buckthorn berries juice (SBJ) during different phases of digestion and its effect on colonic microbial diversity. At each stage, the Total polyphenolic content (TPC), Total antioxidant activity (TAA) and polyphenolic profile was determined. A 1.64 and 2.20 folds increase in TPC with 4.88 and 9.61 folds increase in TAA were observed during gastric and small intestine digestion ($p < 0.05$) with the release of quercetin from food matrix. The digestion resulted in deformation of intact crystalline structure as indicated by scanning electron micrographs. The colonic fermentation resulted in an increase in quercetin, caffeic acid with decrease in rutin and chlorogenic acid after 36 h of fermentation ($p < 0.05$). The Shannon diversity index (H) of beneficial groups including Lactic acid bacteria (LAB), Bacteroides/Prevotella and Bifidobacteria was increased by 35%, 71% and 17%, respectively ($p < 0.05$). The PCA analysis indicated that the presence and digestion of polyphenolics promote the proliferation of Bacteroides/Prevotella group as well as Lactic acid bacteria and Bifidobacteria. The results suggest that SBJ is good source of prebiotic substrate in terms of the proliferation of beneficial gut microbiota.

1. Introduction

Polyphenols form a major proportion of the human diet as these are present in a broad range of commonly consumed berries, fruits, vegetables, and plant-derived products. The intake of polyphenol rich diets is reported to have beneficial effects by decreasing the risk of various chronic diseases, such as coronary heart disease, specific cancers, and neurodegenerative disorders (Cueva et al., 2017; Xie et al., 2017). Also, plant based polyphenols assert prebiotic properties which can enhance the gut ecology, leading to host health benefits (Cardona, Andrés-Lacueva, Tulipani, Tinahones, & Queipo-Ortuño, 2013). In view of the new perception of food products by consumers, increasing consumer awareness about the effect of diet on the incidence of risk of chronic diseases promotes the development and production of functional foods. However, the suggested health benefits of these polyphenolic rich foods depend on their bioavailability, which is measured as an amount of nutrients that are digested, absorbed and metabolized through regular metabolic pathways (Dueik & Bouchon, 2016; McGhie & Walton, 2007). Therefore, it is important to understand the metabolic profile of these food ingredients in terms of absorption, metabolism, and elimination from the body, in order to ascertain there *in vivo* actions.

Sea buckthorn (*Hippophae rhamnoides*), a plant of the Elaeagnaceae family has been reported to possess high nutraceutical and therapeutic values (Patil, 2017). SB berries grown in the trans-Himalayan regions of India, with an elevation of 3000–4000 m are being consumed locally for their health benefits. The polyphenolic rich berries and their products have been reported to inhibit the low-density lipoprotein (LDL) cholesterol oxidation and platelet aggregation, reduction of atopic dermatitis, immunomodulation, cytoprotective effects and protection from gastric ulcers (Gasparrini et al., 2017; Guo, Guo, Li, Fu, & Liu, 2017; Suryakumar & Gupta, 2011). The ripened berries of sea buckthorn are orange-red and are rich source of organic acids, polyphenols (gallic acid, catechin, epicatechin, *p*-coumaric acid, caeffic acid, ferulic acid, rutin, quercetin, resveratol, myricetin *etc.*), carbohydrates, carotenoids, proteins, minerals and fatty acids (Bal, Meda, Naik, & Satya, 2011; Bittová, Krejzová, Roblová, Kubáň, & Kubáň, 2014; Chauhan & Varshneya, 2012; Guo et al., 2017; Pop et al., 2014). Despite their potential biological activities, the effect of digestion on bioaccessibility of the active ingredients of SBJ and their effect on gut microbiota is not known more specifically certain beneficial bacterial species belonging to the genera Lactobacillus, Bacteroides/Prevotella and Bifidobacterium. These bacterial groups are considered beneficial

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microorganisms as these contribute to health benefits by inhibiting a wide range of pathogens, improvement of lactose digestion, reduction of serum cholesterol, stimulation of the immune system through cytokine stimulus, increased mucus secretion and reinforcement of intestinal epithelial cell tight junctions (Duggan, Gannon, & Walker, 2002; Gotteland et al., 2008; Vitali et al., 2010).

During gastrointestinal (GI) absorption, most of the easily digestible food components are generally metabolized or absorbed in the upper GI tract. Remaining complex carbohydrates like dietary fiber, oligosaccharides, arabinogalactan, cellulose, xylan, pectin and polyphenols remain indigestible in the upper GI tract and are utilized by gut microbiota in the lower gut (Hooper, Midtvedt, & Gordon, 2002; Kamiloglu et al., 2017). Among these ingredients, the bioaccessibility of polyphenols is reported to be influenced by factors such as chemical structure and food matrix. It has been estimated that 5–10% of the polyphenols are absorbed in the small intestine and remaining accumulates in the colon where the gut microbiota plays important role in the breakdown of large polyphenolic compounds into low molecular weight absorbable polyphenols for their beneficial effects (Tagliazucchi, Verzelloni, Bertolini, & Conte, 2009). Indeed, a range of these potential benefits of polyphenols have been demonstrated through *in vitro*, *ex vivo* and animal assays (Perez-Vizcaino & Duarte, 2010; Spencer, Vafeiadou, Williams, & Vauzour, 2012). However, human trials often are constrained by ethical considerations while animal model often gives observations that are not applicable in humans due to differences in microbial gut composition between those of humans and animals (Nguyen, Vieira-Silva, Liston, & Raes, 2015; Venema & van den Abbeele, 2013). On the other side *in vitro* studies offer many advantages such as simplicity, ease of application and low cost so they are preferred to *in vivo* studies (Yi, Akoh, Fischer, & Krewer, 2006).

This study aimed to assess the stability of polyphenolic compounds in the SBJ during an *in vitro* simulated gastric and small intestinal phase. Following the small intestine digestion, the impact of the digested fraction of SBJ on specific gut microbial communities was assessed under colonic batch-culture fermentation.

2. Material and methods

2.1. Sea buckthorn berries collection and processing

The sea buckthorn berries were collected from the Spiti region (North latitude 31°44′57″ & 33° 42′54″ and East longitude 76°56′29″ & 78°41′34″) of Himachal Pradesh and were washed with distilled water followed by treatment with potassium metabisulphite (1.5 g/kg). Berries juice was prepared as described by Bump (1989) and yield of juice was 1 L/2.5 kg berries. The heat treatment to 1 L of SBJ was given in closed contained (2 L) at 80 °C for 30 min to prevent spoilage of the juice and it was stored at 4 °C until further use.

2.2. In vitro gastric and small intestine digestion

The SBJ was subjected to initial HCl/pepsin digestion followed by small intestinal pancreatin/bile digestion (Attri, Singh, Singh, & Goel, 2017; Boyer, Brown, & Liu, 2005). A 50 mL of juice was diluted with 200 ml HCl (Merck, Mumbai, India) containing 8.0 g/L NaCl (Merck, Mumbai, India) and 1.2 g/L porcine pepsin (MP Biomedicals, California, USA) adjusted to pH of 1.2 for gastric phase digestion. The digestion was done for 1 h at 37 °C at an agitation of 120 rpm. The gastric phase (50 ml) treated samples were diluted with 80 ml phosphate buffer (0.1 M, pH 7.5) containing 0.175 g/L oxgall bile (MP Biomedicals, California, USA) and 1.1 g/L porcine pancreatin (Sigma-Aldrich, Missouri, USA) for small intestine phase digestion and was further incubated for 1 h at 37 °C at 120 rpm. Before and after the gastric and small intestinal digestion phases, the digested SBJ aliquots were stored at − 20 °C until further analysis. A blank was also prepared with same processing conditions without the SBJ. After the small intestinal pancreatic digestion, the digested sample was lyophilized and used as substrate for fermentation under colonic fermentation.

2.3. Scanning electron microscopy

The scanning electron microscopy (SEM) was performed with Field emission scanning electron microscope (FE, SEM, Quanta 200 FEG) for morphological characterization of lyophilized undigested, gastric and small intestine phase digested SBJ.

2.4. Colonic fermentation

2.4.1. Faecal sample preparation

Faecal samples were collected in sterile vials from five healthy individuals who have no history of antibiotic treatment three months prior to the study (Table S1). These stool samples were stored at − 20 °C, were pooled together and processed within 3 h of collection. A 10 g of the pooled faecal matter was homogenized in anaerobic sterile 100 mL phosphate buffer (0.1 M; pH 7.5) containing 0.1% sodium thioglycolate (Himedia, Mumbai, India) as a reducing agent. The faecal slurry obtained was used as inoculum for colonic fermentation.

2.4.2. Colonic batch fermentation

Batch culture fermentation was carried out in glass serum bottles with rubber top (Volume 150 mL) in triplicates. Briefly, 45 mL of carbohydrate free basal sterile medium previously described by Valdés-Varela, Ruas-Madiedo, and Gueimonde (2017), containing 2 g/L yeast extract (Himedia, Mumbai, India), 2 g/L peptone (Himedia, Mumbai, India), 0.1 g/L NaCl (Merck, Mumbai, India), 0.04 g/L K_2HPO_4 (Fisher Scientific, Waltham, MA, USA), 0.04 g/L KH_2PO_4 (Fisher Scientific, Waltham, MA, USA), 2 g/L $NaCO_3$ (Merck, Mumbai, India), 0.01 g/L $MgSO_4 \cdot 7H_2O$ (Fisher Scientific, Waltham, MA, USA), 0.01 g/L $CaCl_2 \cdot 6H_2O$ (Fisher Scientific, Waltham, MA, USA), 2 ml/L Tween 80 (Fisher Scientific, Waltham, MA, USA), 0.05 g/L haemin (Himedia, Mumbai, India), 10 mg/L vitamin K_1 (MP Biomedicals, California, USA), 0.5 g/L L-cysteine hydrochloride monohydrate (MP Biomedicals, California, USA), 0.5 g/L ox gall bile (MP Biomedicals, California, USA), 1 mg/L resazurin (Himedia, Mumbai, India) was dispensed into the serum bottles. The pH of the medium was adjusted to 7.0 and anaerobic conditions were maintained by flushing O_2 free N_2 gas. The serum bottles containing media were inoculated with 5 mL of faecal slurry (1:10 w/v). To check the effect of SBJ on gut microbiota, 250 mg lyophilized fraction of small intestine digested SBJ was added to media, whereas SBJ control containing 250 mg of lyophilized fraction of undigested juice. A serum bottle without any SBJ was used as experimental blank. In order to mimic the conditions of the large intestine serum bottles were fluxed with O_2 free N_2 gas for 15 min two times a day and were incubated at 37 °C for 72 h. For analysis of Total polyphenolic content, changes in gut microbial population and polyphenolics, 1 mL aliquot was drawn from each bottle at an interval of 12 h up to 72 h and the samples were stored at − 80 °C until analyzed.

2.5. Total polyphenol content (TPC) analysis

Total polyphenol content was determined using the Folin-Ciocalteu method (Singleton & Rossi, 1965). Briefly, a 250 µL sample of predigested, gastric, small intestinal phase and colonic fermented sea buckthorn were diluted with 15 mL distilled water. To this mixture 1.25 mL of Folin-Ciocalteu reagent (2N, Merck, Mumbai, India) and 3.75 mL sodium carbonate (20% w/v, Fisher Scientific, Waltham, MA, USA) were added and final volume was made up to 25 mL. After incubation at room temperature in dark for 2 h, the absorbance at 765 nm was measured. Total polyphenol contents were expressed as milligram of gallic acid equivalents per litre (mg GAE/L).

2.6. Total antioxidant activity (TAA)

The antioxidant activity was determined by the ABTS method as described by Pellegrini, Yang, and Rice-evans (1999). A 7 mM solution of ABTS (MP Biomedicals, California, USA) was prepared by mixing a stock solution with potassium per sulphate (2.45 mM; Fisher Scientific, Waltham, MA, USA) in an equal quantity and left to stand for 12–16 h at room temperature in the dark until reaching a stable oxidative state. The $ABTS^+$ solution was diluted with 80% ethanol to an absorbance of 0.80 ± 0.05 at 734 nm. For determination of antioxidant activity in the different samples, 100 µL samples of predigested, gastic, small intestinal phase and colonic fermented SBJ were added to 2.9 mL ABTS solution and were incubated at room temperature for 30 min in the dark. The absorbance was measured at 734 nm against 80% ethanol as blank. The calibration curve for ABTS was obtained using 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), (MP Biomedicals, California, USA), a water-soluble analog of α-tocopherol, as standard and antioxidant activity was expressed as Trolox equivalent antioxidant activity micro molar per litre (TEAC µM/L).

2.7. DNA isolation, PCR and DGGE analysis

DNA extraction was performed using the method as described in Mahajan, Nikitina, Litti, Nozhevnikova, and Goel (2016). To determine the diversity of gut microbiota, total Bacterial DNA amplicon as well as specific PCR amplicons from Lactic acid bacteria, Bacteroides/Prevotella and *Bifidobacteria* were amplified using specific primer sets (Table 1) and a nested PCR approach was used for DGGE analysis. In the first PCR round, group specific primer sets were used, and in the second PCR round, primers PRBA338fGC and P518r were used. DGGE was performed using the DCode Universal Mutation Detection system (Bio-Rad, Hercules, USA). The PCR fragments were loaded onto 6% (w/v) polyacrylamide gels in 1X TAE (20 mM Tris, 10 mM acetate, 0.5 mM EDTA, pH 7.4) (Bio-Rad, Hercules, USA). To separate the amplified DNA fragments, the polyacrylamide gels were made with denaturing gradients ranging from 20% to 45% (100% denaturant is defined as a mixture of 7 M urea and 40% deionized formamide). The electrophoresis was run for 16 h at 60 °C and 75 V. After running the gel, staining of the gel was done with ethidium bromide (Bio-Rad, Hercules, USA) solution. The DGGE patterns were analyzed with Quantity one software (Bio-Rad, Hercules, USA). The richness (S) of the bacterial community was determined from the number of bands in each lane. The diversity of the microbial community was calculated by the Shannon index of general diversity H (Shannon & Weaver, 1963). It was calculated on the basis of the peak intensity of DGGE bands from the different bacterial groups (16S rDNA bands). The equation for the Shannon index is: $H = -\Sigma(ni / N)\log(ni / N)$, where ni is the intensity of the band and N is the sum of all bands intensity. Similarity between microbiota compositions at different months was determined by Sorenson's pairwise similarity coefficient (Cs). Cs values were determined by the following equation: $Cs = (i{\cdot}j / T) * 100$ where i is the total number of lanes to be compared, j is the number of common DGGE band, T is the total number of DGGE band in all lanes to be compared. Two completely identical profiles give 100% similarity whereas completely different give 0% similarity (Schwiertz et al., 2003).

2.8. RP-HPLC profiling

The RP-HPLC analysis was carried out on a LaChrom HPLC system (Merck-Hitachi Darmstadt, Germany) using a reverse phase column (125 × 4 mm Purospher RP-18e, Merck, Darmstadt, Germany). All the reagents used for analysis were of HPLC grade. The temperature of the column oven was set to 25 °C. The analysis of quercetin and chlorogenic acid were performed at a flow rate of 0.6 mL/min using 2% v/v acetic acid (Merck, Mumbai, India) (solvent A) and methanol/acetonitrile (Merck, Mumbai, India) (40/15, v/v) mixture (solvent B) under the following gradient program as: 0–8 min (70% A and 30% B), 8–19 min (60% A and 40% B) and 19–30 min (50% A and 50% B) as previously described by Bittová et al. (2014). The analytes were detected at 270 nm and injection volume was 20 µL. The standards of rutin, quercetin, chlorogenic acid, caffeic acid and ferulic acid (1 mg/mL in 100% methanol) were used in the analysis.

2.9. Statistical analysis

Each experiment was performed in triplicate, and data are presented as mean values (n = 3). Comparison of results was done by ANOVA and Tukey's multiple comparison test ($p < 0.05$) with SPSS version 21.0 (SPSS Inc., Chicago, IL, USA). To test for similarities among polyphenolic compounds of SBJ at different stages of digestion and microbial community during colonic batch fermentation, Principal Component Analysis (PCA) was applied to the data set through multivariate exploratory techniques using XLSTAT software version 2017.03 (Addinsoft SARL, Paris, France).

3. Results and discussion

The study evaluates the SBJ under simulated *in vitro* digestion for the bioaccessibility of polyphenols and the impact of digested SBJ fraction on gut microbiota under colonic fermentation. The antioxidant activities of SBJ were assessed at each phase of digestion fermentation and microbial diversity as well as polyphenolic profile was analyzed after colonic fermentation.

3.1. Gastric and small intestine digestion of SBJ

The digestive stability of the polyphenolics in SBJ was investigated by *in vitro* model that simulate biochemical and physiochemical conditions like pH, temperature, digestive enzymes and time. The gastric and small intestine digestion of SBJ led to an increase in TPC content by

Table 1
Details of 16S rRNA targeted PCR primers and PCR reaction conditions.

Target	Primers	Product size (bp)	Reference
Total bacteria	PRBA338fGC[a]: 5′-CGCCCGGGGCGCGCCCCGGGCGGGCGGGGGCACGGGGGGACTCCTACGGGAGGCAGCAG-3′ P518r: 5′-ATTACCGCGGCTGCTGG-3′	236	Possemiers, Verthé, Uyttendaele, and Verstraete (2004)
Lactic acid bacteria	SGLAB0159F: 5′-GGAAACAGRTGCTAATACCG-3′ SGLAB0667R: 5′-CACCGCTACACATGGAG-3′	550	Heilig et al. (2002)
Bifidobacteria	Bif164-F: 5′-GGGTGGTAATGCCGGATG-3′ Bif662-R: 5′-CCACCGTTACACCGGGAA-3′	523	Mangin et al. (2006)
Bacteroides/ Prevotella	FDI: 5′-AGAGTTTGATCCTGGCTCAG-3′ rP2: 5′-ACGGCTACCTTGTTACGACTT-3′	1400	Kontula et al. (1998)

[a] GC-clamp allows the detection of sequence variation in the amplified DNA fragment.

Table 2
Total polyphenolic content (TPC), Total antioxidant activity (TAA), polyphenolic compounds of Sea buckthorn juice (SBJ) under simulated gastric and pancreatic digestion.

Treatments	TPC (GAE mg/L)	TAA (TEAC mM/L)	Polyphenolic compounds (μg/mg of digesta)			
			Rutin	Quercetin	Chlorogenic acid	Caffeic acid
Blank	$522.71^{a} \pm 42$	$6.23^{a} \pm 0.5$	$30.23^{a} \pm 1.4$	$11.64^{a} \pm 0.9$	$14.24^{a} \pm 0.8$	$2.28^{a} \pm 0.3$
Gastric	$854.52^{b} \pm 61$	$30.40^{b} \pm 0.9$	$34.47^{a} \pm 2.3$	$12.99^{a} \pm 0.7$	$14.81^{a} \pm 1.1$	$3.10^{a} \pm 0.6$
Small intestine	$1151.56^{c} \pm 58$	$59.86^{c} \pm 2.2$	$41.56^{b} \pm 1.9$	$19.76^{b} \pm 1.8$	$15.76^{b} \pm 0.7$	$5.22^{b} \pm 0.5$

a–c = Means in the column with same superscript letter are not significantly different as measured by 2 sided Tukey's – post-hoc range test between replications.

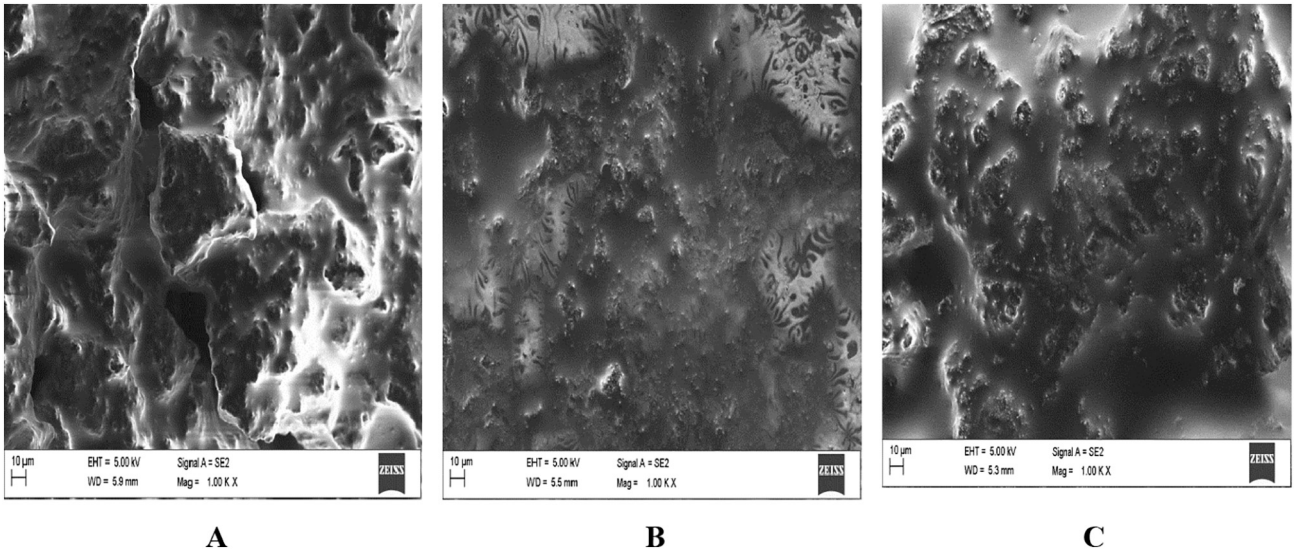

Fig. 1. Scanning electron microscopy of Sea buckthorn juice under different stages of digestion (under magnification 1000 ×).
A – control SBJ, B – SBJ after gastric juice, C – SBJ after small intestinal phase.

1.64 and 2.20 folds with 4.88 and 9.61 folds increase in TAA as Trolox Equivalent units (Table 2). This increase in TPC and TAA of the digested SBJ can be correlated with the release of other polyphenolic and antioxidant active compounds from the SBJ matrix due to digestive enzymes and physiochemical conditions. Additionally, it has been reported that the increase or higher antioxidant activity obtained after digestion is generally due to the biotransformation of polyphenol compounds to other phenolics in mild alkaline conditions (Gayoso et al., 2016). The effect of *in vitro* digestion processes on the stability and bioaccessibility of different polyphenols was evaluated. Among the polyphenols detected, higher amount of rutin, quercetin and caffeic acid was determined after small intestine digestion ($p < 0.05$). However, a small increase was observed after gastric phase digestion. The chlorogenic acid remained stable throughout the gastric and small intestine digestion, whereas an increase 1.70 folds was observed for quercetin ($p < 0.05$) at the small intestine phase of digestion (Table 2). The increase in quercetin, rutin and caffeic acid observed might have been due to the difference in pH between the stomach and small intestine and structural changes in a food matrix (Fig. 1). Olthof, Hollman, Buijsman, van Amelsvoort, and Katan (2003), reported that during digestion with digestive enzymes, the quercetin glycosides get transformed to quercetin. Previous study, also reported similar results where significant increase in quercetin was observed after gastric and small intestine phase digestion of onion extract (Hur, Lee, Kim, Chun, & Lee, 2013). Bermudez-soto, Tomas-Barberan, and Garcia-Conesa (2007), have reported stability of chlorogenic acid present in chokeberry (*Aronia melanocarpa*) during gastric phase and 24% increase after small intestinal phase. However, in our study, we did not observe any increase in chlorogenic acid after small intestinal phase. We therefore assumed that the increased quercetin, rutin and caffeic acid in gastric and small intestine phase digestion could be one of the reasons for the observed increase in the TPC and TAA, however, the presence and possible role of other phenolic compounds (not determined in this present study) such as ascorbic acid, phytosterols and carotenoids in this increase in TPC and TAA cannot be neglected (Bal et al., 2011).

The impact of digestive conditions on release of food ingredients was also assessed by microscopic observation of food matrix which clearly indicates that it may affect the level of binding between food constituents. The microscopic observations can be an important tool in studies on the characteristics of food matrix, providing information about the size characteristics and surface morphology. It can be used to characterize, morphologically, whether the food matrix is influenced by a digestion process. The SEM images (Fig. 1) indicate the microstructure of the food matrix obtained during different stages of digestion. Before digestion, the food matrix of SBJ was intact and in crystalline form which changed to irregular shapes with more intercellular spaces after gastric and pancreatic digestion. The digestion resulted in cell collapse with larger and more irregular shapes indicating that the release of food ingredients from compact structures. Similar results were observed by Yongliang, Haixiang, Ming, and Gongming (2014), who have also reported deformation of raw banana starch granules subjected gastric and small intestinal phase observed under SEM.

3.2. Colonic batch fermentation

In vitro colonic batch fermentation system can be considered as a simple model to simulate and study the metabolic capabilities of human colonic microbiota. Recently, several studies have been done to analyze the variations in polyphenolic profiles in foods subjected to colonic fermentation (Guergoletto, Costabile, Flores, Garcia, & Gibson, 2016; Zhou et al., 2016). Polyphenols are generally considered as xenobiotic whereby only a very small amount of total polyphenol intake is

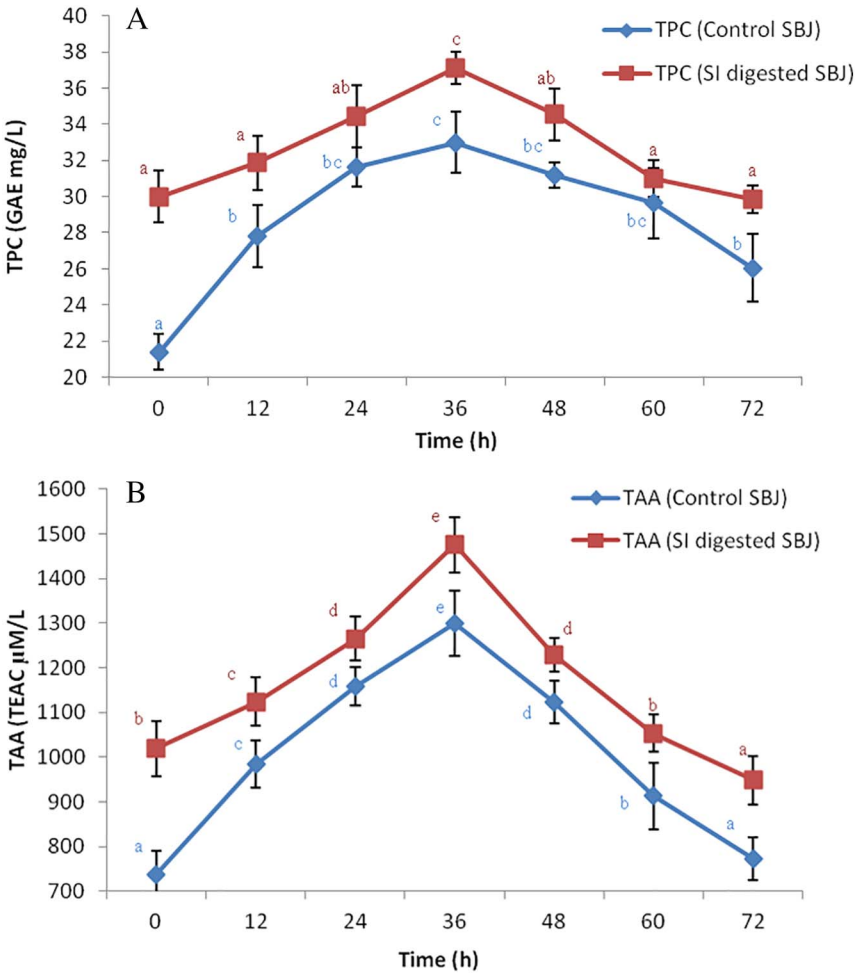

Fig. 2. a: TPC (GAE mg/L) of small intestine phase digested and control SBJ during batch fermentation model for 72 h.
a–c = Mean values in the graphs with same superscript letter on the individual series are not significantly different as measured by 2 sided Tukey's – post-hoc range test between replications.
b: TAA (TEAC mM/L) of small intestine phase digested and control SBJ during batch fermentation model for 72 h.
a–e = Mean values in the graphs with same superscript letter on the individual series are not significantly different as measured by 2 sided Tukey's – post-hoc range test between replications.

absorbed in the small intestine. The remaining polyphenols are reported to accumulate in the large intestinal where the colonic microbial community is responsible for the breakdown and biotranformation of the original polyphenolic compounds into a series of low molecular weight metabolites (Mahajan, Goel, & Attri, 2016). Therefore, the present study investigated the release of polyphenolic contents from SBJ under simulated *in vitro* batch fermentation by human colonic microbiota.

3.3. Impact of colonic fermentation on polyphenolic contents

The samples of SBJ as control and small intestine digested fraction were added to serum bottles containing basal media and faecal slurry and experiment was performed upto 3 days. The pattern of changes in TPC and TAA was determined for both control and experimental SBJ (Fig. 2a and b). In case of digested SBJ, an increase in TPC was observed up to 36 h, after which it gradually declined, however, for the control SBJ, a sharp increase in TPC was observed after 12 h which also further declined after 36 h indicating that during the initial stages of fermentation, the polyphenolics are extracted from the food matrix followed by their degradation under longer incubations. In order to follow up the bioaccessibility of polyphenolic compounds in SBJ during digestion, the polyphenolic profile of compounds was monitored by HPLC (Table 3, Fig. S1). From HPLC results, compared to control, the digested fraction of SBJ resulted in highest quercetin and caffeic acid level after 36 h which may have contributed to highest TPC at this point. This increase may be due to transformation of other polyphenolic compound into quercetin *via* gut microbiota such as *Bacteroides uniformis* and *Bacteroides ovatus* which can convert rutin to quercetin (Selma, Espín, & Tomás-Barberán, 2009). The quercetin level ranged from 3.81–7.29 µg/mL in case of digested fraction as compared to 2.41–5.11 µg/mL of the control SBJ. The decline in quercetin in the later period of incubation could be due to biotransformation of quercetin into low molecular weight metabolites as indicated by several minor peaks in HPLC profile. Earlier, biotransformation of quercetin into 2-(3,4-dihydroxyphenyl) acetic acid, 3,4-dihydroxybenzoic acid, 2-(3-hydroxyphenyl) acetic acid, phloroglucinol, 3-(3-hydroxyphenyl) propionic acid, 3-(3,4-dihydroxyphenyl) propionic acid by gut microbiota has been reported (Bowey, Adlercreutz, & Rowland, 2003; Winter, Moore, Dowell, & Bokkenheuser, 1989). On the other hand, chlorogenic acid was observed to transfer into several other polyphenolics after 12 h of incubation. Chlorogenic acid has been reported to have very low bioavailability until it reaches the colon, where the gut microbiota such as *Escherichia coli*, *Bifidobacterium lactis*, and *Lactobacillus gasseri* hydrolyze it to release caffeic acid or other aromatic acid metabolites (Couteau, McCartney, Gibson, Williamson, & Faulds, 2001; Gonthier, Verny, Besson, Rémésy, & Scalbert, 2003). A minor quantity of ferulic acid was observed on the larger incubation period. The appearance of ferulic acid could be due to a metabolite from chlorogenic/caffeic acid (Gonthier et al., 2003).

3.4. Impact of digested SBJ on colonic microbiota

The human colon harbours a large and complex microbial flora and plays an important role in health and disease. The gut microbiota is influenced by a number of food components such as polyphenols, oligosaccharides *etc*. The fruits rich in polyphenols are reported to modulate colonic microbiota towards proliferation of beneficial bacteria

Table 3
Content of polyphenols of control and small intestine digested SBJ under colonic fermentation.

Incubation period (h)	Control SBJ (μg/mL)				Small intestine digested SBJ (μg/mL)				
	Rutin	Quercetin	Chlorogenic acid	Caffeic acid	Rutin	Quercetin	Chlorogenic acid	Caffeic acid	Ferulic acid
0	$10.23^{d} \pm 0.21$	$2.41^{a} \pm 0.09$	$2.18^{ab} \pm 0.19$	$0.94^{a} \pm 0.12$	$12.41^{g} \pm 0.28$	$3.81^{a} \pm 0.23$	$2.71^{d} \pm 0.13$	$1.37^{a} \pm 0.19$	–
12	$9.55^{c} \pm 0.13$	$2.92^{b} \pm 0.05$	$2.52^{c} \pm 0.11$	$1.62^{b} \pm 0.16$	$11.63^{f} \pm 0.22$	$4.75^{b} \pm 0.11$	$3.36^{e} \pm 0.22$	$2.65^{b} \pm 0.21$	–
24	$7.84^{b} \pm 0.15$	$3.82^{c} \pm 0.08$	$1.78^{b} \pm 0.21$	$3.72^{c} \pm 0.11$	$9.42^{e} \pm 0.25$	$5.86^{c} \pm 0.22$	$2.29^{d} \pm 0.15$	$4.92^{c} \pm 0.16$	–
36	$6.62^{a} \pm 0.11$	$4.63^{d} \pm 0.11$	$1.14^{d} \pm 0.09$	$4.41^{d} \pm 0.18$	$7.21^{d} \pm 0.19$	$6.87^{d} \pm 0.31$	$1.67^{c} \pm 0.23$	$5.16^{cd} \pm 0.26$	–
48	$6.42^{a} \pm 0.23$	$5.11^{d} \pm 0.14$	$1.03^{c} \pm 0.06$	$4.52^{d} \pm 0.17$	$5.18^{c} \pm 0.20$	$7.21^{d} \pm 0.42$	$0.96^{b} \pm 0.11$	$5.55^{d} \pm 0.21$	$0.67^{a} \pm 0.13$
60	$6.53^{a} \pm 0.16$	$5.05^{d} \pm 0.13$	$0.96^{b} \pm 0.15$	$4.67^{d} \pm 0.15$	$4.32^{b} \pm 0.12$	$7.29^{d} \pm 0.24$	$0.41^{a} \pm 0.08$	$5.61^{d} \pm 0.24$	$1.01^{b} \pm 0.16$
72	$6.33^{a} \pm 0.12$	$4.72^{d} \pm 0.10$	$0.85^{a} \pm 0.12$	$4.58^{d} \pm 0.23$	$3.01^{a} \pm 0.18$	$7.11^{d} \pm 0.31$	$0.19^{a} \pm 0.04$	$5.64^{d} \pm 0.19$	$1.66^{c} \pm 0.25$

a–g = Means in the rows with same superscript letter are not significantly different as measured by 2 sided Tukey's – post-hoc range test between replications.

(Mahajan, Goel et al., 2016). To investigate the effect of small intestine digested SBJ on gut microbiota, an *in vitro* single stage simulation experiment was conducted using healthy human faecal microbiota as inoculum. A blank was also prepared with the same conditions without the SBJ and control with undigested SBJ. To assess the impact, culture independent techniques such as DGGE was employed as the culture dependent techniques used for bacterial identification and analysis are time consuming and laborious and provides a limited view of the dynamics and diversity of the gut microbiota (González-Arenzana, Santamaría, Gutiérrez, López, & López-Alfaro, 2017). Recently, several studies have been done using PCR-DGGE based approach to study the effect of food component on gut microbiota (Dueñas et al., 2015; Li et al., 2016). For PCR-DGGE analysis, the samples were withdrawn at regular interval of 24 h for microbial community analysis, targeting population of LAB, Bacteroides/Prevotella and Bifidobacterial groups. The DGGE profiles (Fig. S2) were analyzed using Quantity one software (Bio-Rad, Hercules, USA) and evaluated for biodiversity indices such species richness (S) and Shannon diversity index (H).

The blank samples of the colonic fermentation possessed a Shannon diversity index of 0.60, 0.77 and 0.88 for Bacteroides/Prevotella, LAB and Bifidobacterial groups which decreased gradually by 10, 20 and 45% during 3 days of incubation. In general, the Shannon diversity index for each bacterial group increased in experimental small intestine digested SBJ as compared to the control group. The highest increase (71%) in diversity was observed for Bacteroides/Prevotella group, whereas diversity of LAB and Bifidobacterial group increased to 35% and 17%, respectively, whereas these values were 33%, 20% and 8.3% in the control group ($p < 0.05$) (Table 4). The higher diversity of for all the groups was observed after 24 h of incubation in experimental small intestinal digested SBJ ($p < 0.05$). Further detailed introspection of the results, using Sorenson's pairwise similarity coefficient (Cs) revealed that highest Cs value was observed with small intestine digested batch fermentation system throughout the 72 h study for all three groups; LAB, Bacteroides/Prevotella and Bifidobacteria (Table 5). This means that stability and establishment of gut microbiota were enhanced by SBJ. These results indicate that SBJ can have potential positive effects on gut microbiota. SBJ has been reported as a rich source of oligosaccharides and essential nutrients that enhance the growth of

Table 5
Sorenson's pairwise similarity coefficient (Cs) of different bacterial groups during colonic fermentation of Sea buckthorn berries juice.

Treatment	Lactic acid bacteria	Bacteroides/ Prevotella	Bifidobacteria
Blank	31.58^{a}	32.00^{b}	24.24^{a}
Control	41.38^{b}	22.00^{a}	25.81^{a}
Small intestine digested SBJ	43.24^{b}	41.38^{c}	52.17^{b}

a–c = Means in the column with same superscript letter are not significantly different as measured by 2 sided Tukey's – post-hoc range test between replications.

microbiota (Gunenc, Fang, & Hosseinian, 2015). Amino acids such as valine, histidine and glycine present in SBJ helps in growth of Bifidobacteria (Vasiljevic & Shah, 2008). Sea buckthorn berries also contain high amount of natural antioxidants such as flavonoids, carotenoids and ascorbic acid (Bal et al., 2011). Dietary polyphenols are generally biotransformed by the gut microbiota, initially by deglycosylation, followed by a breakdown of flavonoids into relatively simple and smaller aromatic carboxylic acids, commonly referred to as phenolic acids (Aura et al., 2008). In the colonic region, polyphenols are mainly metabolized through glucosidase, esterase, demethylation, decarboxylation and dehydroxylation activities by gut microbiota. These microbial activities may result into smaller metabolites such as short chain fatty acids and phenolic acids (Selma et al., 2009). Furthermore, these metabolites may modulate the growth of bacteria in the gut (Lee, Jenner, Low, & Lee, 2006).

Similar results have been observed in batch colonic fermentations with polyphenolic-rich extracts from different sources as reviewed by Mahajan, Goel et al. (2016). Molan, Lila, Mawson, and De (2009), found that the addition of blueberry extracts to a batch model significantly enhanced the growth of lactobacilli and bifidobacteria. Increase in number of *Bifidobacterium* sp. and *Lactobacillus-Enterococcus* sp. was observed on addition of commercial extract of pomegranate in a batch system (Bialonska, Kasimsetty, Khan, & Ferreira, 2009). Mandalari et al. (2010), reported that fermentations of almonds skin with faecal microbiota, significantly increased the populations of

Table 4
Diversity indices of Lactic acid bacteria (LAB), Bifidobacteria and Bacteroides/Prevotella during colonic fermentation.

Bacterial group	Diversity/richness	Control				SI digested SBJ			
		0 h	24 h	48 h	72 h	0 h	24 h	48 h	72 h
Lactic acid bacteria	Shannon diversity index (H)	0.82^{b}	0.83^{b}	0.99^{c}	0.76^{a}	0.78^{ab}	0.83^{b}	1.03^{cd}	1.06^{d}
	Richness (S)	7^{ab}	8^{abc}	10^{cde}	9^{bcd}	6^{a}	7^{ab}	11^{de}	12^{e}
Bifidobacteria	Shannon diversity index (H)	0.84^{b}	0.69^{a}	0.95^{cd}	0.91^{cd}	0.90^{c}	0.96^{d}	1.02^{e}	1.07^{e}
	Richness (S)	7^{ab}	5^{a}	9^{ab}	8^{ab}	8^{ab}	9^{ab}	10^{cd}	12^{d}
Bacteroides/Prevotella	Shannon diversity index (H)	0.61^{a}	0.60^{a}	0.78^{b}	0.83^{b}	0.60^{a}	1.01^{c}	1.01^{c}	1.03^{c}
	Richness (S)	4^{a}	4^{a}	6^{ab}	7^{a}	4^{a}	10^{c}	10^{c}	11^{c}

a–e = Means in the rows with same superscript letter are not significantly different as measured by 2 sided Tukey's – post-hoc range test between replications.

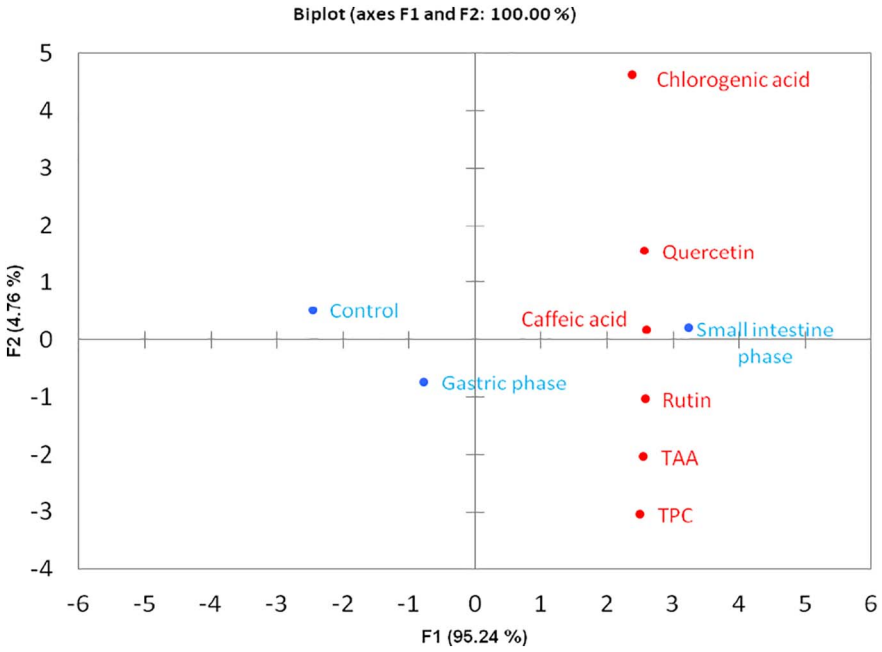

Fig. 3. Biplot for the PCA analysis of polyphenolic compounds released at different stages of digestion Sea buckthorn juice.

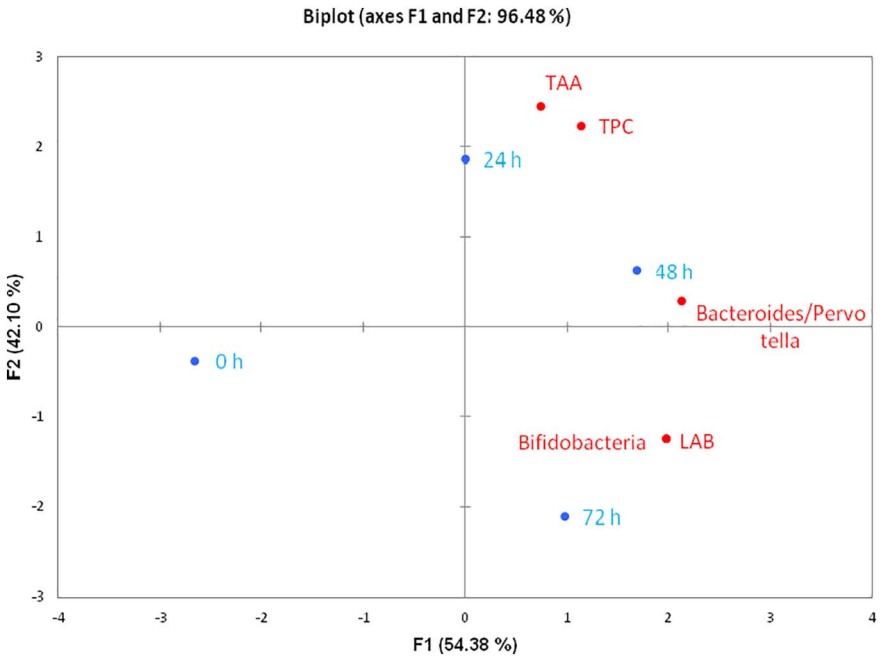

Fig. 4. Biplot for the PCA analysis of association of Total polyphenolic content (TPC), Total antioxidant activity (TAA) and microbial groups during colonic batch fermentation with digested Sea buckthorn juice.

bifidobacteria. Dolara et al. (2005), reported higher levels of Bacteroides, *Bifidobacterium* and *Lactobacillus* sp. in animal model when treated with red-wine polyphenols.

3.5. Principal Component Analysis (PCA)

PCA permits the visualization of the original arrangement of SBJ polyphenols during gastric & small intestine phase and effect of SBJ on colonic bacteria, in an n-dimensional space, by identifying the directions in which most of the information is retained. This method reveals the parameters that better explain the differentiation among phases of digestion by a small number of linear combinations of the different variables responsible for most of the variability in the data. In PCA, the biplot can show inter-unit distances among the units as well as display variances and correlations of the variables. The biplot graphic based on PCA for the most important factors (F1 and F2) is given in Figs. 3 and 4, respectively. The 100% of variability was represented by the first two factors in PCA biplot of digestion. The first factor (F1) explains 95.24% of the total variance with significant parameters of different phases of digestion, whereas F2 explains 4.76% of the total variance with significant parameters of polyphenolic compounds. From the PCA analysis, the rutin was found to be more associated with TPC and expressing higher TAA (Table 2) and all other polyphenolic compounds studied were closely related to small intestinal phase compared to other phases of digestion due to their release from the juice matrix as indicated in Scanning Electron Micrographs (Fig. 1).

PCA of colonic batch fermentation revealed that high percentage of variability represented by the first two factors (96.48%). The first factor (F1) explains 54.38% of the total variance with significant parameters of incubation period, whereas F2 explains 42.10% of the total variance with significant parameters of TPC and microbial biodiversity. From PCA biplot it was clear that increase in TPC contributed towards

proliferation of Bacteroides/Prevotella group as compared to LAB and Bifidobacteria (Fig. 3).

4. Conclusions

The present study, for the first time, revealed that the polyphenolic content and related antioxidative characteristics of Sea buckthorn berries increased after simulated digestion and fermentation by colonic microbiota. The digestion of the juice at different phases influences the gut microbial population towards proliferation of beneficial bacteria as indicated by increase in their diversity. Further studies are warranted for elucidation of absorption, structural modification and activity of biotransformed products towards reduced risk of developing chronic diseases.

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All authors have declared that they don't have any conflict of interest for publishing the research.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2017.11.032.

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