Phenolic Composition and Evaluation of the Antimicrobial Activity of Free and Bound Phenolic Fractions from a Peruvian Purple Corn (*Zea mays* L.) Accession

Lena Gálvez Ranilla, Ashish Christopher, Dipayan Sarkar, Kalidas Shetty, Rosana Chirinos, and David Campos

Abstract: Beneficial effects on overall gut health by phenolic bioactives-rich foods are potentially due to their modulation of probiotic gut bacteria and antimicrobial activity against pathogenic bacteria. Based on this rationale, the effect of the free and bound phenolic fractions from a Peruvian purple corn accession AREQ-084 on probiotic lactic acid bacteria such as *Lactobacillus helveticus* and *Bifidobacterium longum* and the gastric cancer-related pathogen *Helicobacter pylori* was evaluated. The free and bound phenolic composition was also determined by ultra-performance liquid chromatography. Anthocyanins were the major phenolic compounds (310.04 mg cyanidin-3-glucoside equivalents/100 g dry weight, DW) in the free phenolic fraction along with hydroxycinnamic acids such as *p*-coumaric acid derivatives, followed by caffeic and ferulic acid derivatives. The bound phenolic form had only hydroxycinnamic acids such as ferulic acid, *p*-coumaric acid, and a ferulic acid derivative with ferulic acid being the major phenolic compound (156.30 mg/100 g DW). These phenolic compounds were compatible with beneficial probiotic lactic acid bacteria such as *L. helveticus* and *B. longum* as these bacteria were not inhibited by the free and bound phenolic fractions at 10 to 50 mg/mL and 10 mg/mL of sample doses, respectively. However, the pathogenic *H. pylori* was also not inhibited by both purple corn phenolic forms at same above sample doses. This study provides the preliminary base for the characterization of phenolic compounds of Peruvian purple corn biodiversity and its potential health benefits relevant to improving human gut health.

Keywords: *B. longum*, *H. pylori*, *L. helveticus*, phenolic compounds, purple corn

Practical Application: This study provides insights that Peruvian purple corn accession AREQ-084 can be targeted as a potential source of health-relevant phenolic compounds such as anthocyanins along with hydroxycinnamic acids linked to its dietary fiber fraction. Additionally, these phenolic fractions did not affect the gut health associated beneficial bacteria nor the pathogenic *H. pylori*. Purple corn can be targeted for design of probiotic functional foods integrated with their anthocyanin linked-coloring properties.

Introduction

Purple corn (*Zea mays* L.), which belongs to the *Kculli* race, has been cultivated in the Peruvian Andean region since approximately 900 B.C. and is domestically used for infusing color into traditional beverages and desserts especially unfermented *chichas* and *mazamorras,* which are maize and tapioca flour jellies (Grobman and others 1961). Because of its high anthocyanin content, which has been reported to be even higher than those found in blueberry, a natural colorant agent rich in anthocyanins is commercially obtained from purple corn and is widely used in Asia, South America, and Europe (Jing and Giusti 2007; Lao and others 2017). Anthocyanins, an important group of phenolic compounds and water-soluble pigments, are well known for their potential health benefits according to various *in vivo* studies and some clinical trials (Matsumoto and others 2002; Lala and others 2006; Cassidy and others 2013; Zhu and others 2013). Among anthocyanins, cyanidin-3-glucoside, pelargonidin-3-glucoside, and peonidin-3-glucoside with their malonyl derivatives have been found in Peruvian purple corn cobs or seeds along with other non-anthocyanin phenolic compounds such as phenolic acids and flavonol derivatives (Pascual-Teresa and others 2002; Aoki and others 2002; Pedreschi and Cisneros-Cevallos 2007; Lao and Giusti 2016; Paucar-Menacho and others 2017).

Human health benefits associated with purple corn have been mainly attributed to their high anthocyanin contents. Several *in vitro* and *in vivo* studies with purple corn have reported anthocyanin-linked functional properties such as antioxidant, anti-inflammatory, anti-carcinogenic, and anti-hyperglycemic activity (Hagiwara and others 2001; Tsuda and others 2003; Ranilla and others 2009; Li and others 2012; Ramos-Escudero and others 2012; Long and others 2013). Furthermore, a previous study also reported a reduced risk of hypertension among Peruvian adults due to the daily consumption of anthocyanin-rich purple corn extract for a 3-wk period (Finkel and others 2013).

The antimicrobial potential of purple corn has not been studied extensively. Zhao and others (2009) pointed out that a certain purple corn hybrid from China showed a high *in vitro* antimicrobial

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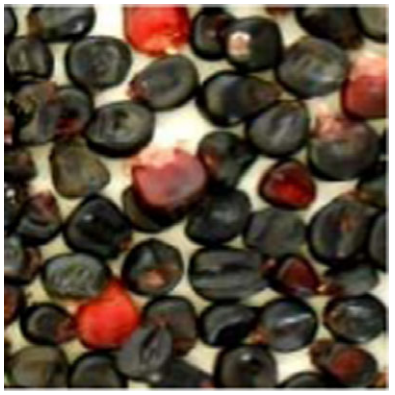


Figure 1–Evaluated purple corn accession AREQ-084.

activity against pathogenic bacteria such as *Salmonella* Enteritidis and *Staphylococcus aureus* due to its high anthocyanin content and composition. Other plant-based foods rich in anthocyanins such as berries have also shown antimicrobial properties against some pathogenic Gram-negative bacteria (Puupponen-Pimiä and others 2001). These studies were mainly focused on the anthocyanin-linked free phenolic fraction; however, the potential antimicrobial activity of the cell wall-bound phenolic fraction from purple corn has not been studied yet.

In general, the predominant phenolic acids in cereal grains such as corn are mostly hydroxycinnamic acid derivatives, which are found in insoluble bound forms linked to cell wall polysaccharides (Dewanto and others 2002; Guo and Beta 2013). This bound phenolic fraction is not absorbed in the small intestine and it reaches the colon where it is partially released by colon microbiota leading to a myriad of health benefits (Shahidi and Yeo 2016). In our previous study, the Peruvian purple corn accession AREQ-084 had the highest total phenolic contents in its free and bound forms along with high total anthocyanin contents among different purple corn accessions (Huaman-Alvino and others 2016). Therefore, the aim of this study was to investigate the effect of the free and bound phenolic fractions from the Peruvian purple corn accession AREQ-084 on the growth of two species of beneficial lactic acid bacteria with probiotic potential such as *Lactobacillus helveticus* and *Bifidobacterium longum* as well as the antimicrobial activity against a gastric and gastric cancer-relevant pathogen *Helicobacter pylori*. The total phenolic content, total anthocyanins and the phenolic acid profile by UPLC in the free and bound phenolic fractions from purple corn accession AREQ-084 were also evaluated. Results from this study provide preliminary insights about the health-relevant of purple corn relevant for human gut health.

Materials and Methods

Materials

The purple corn accession AREQ-084 (Figure 1) was provided by the germplasm bank of the Maize Research Program from the Agrarian University of La Molina (UNALM), located in Lima city, Peru. This accession was originally collected from the Peruvian region of Arequipa, located at 1600 m above sea level within the period of 1970 to 1978. Corn grains were then successively regenerated by the Maize Research Program in experimental fields with similar environmental conditions as those from the region of origin. Corn sample used in the current study was harvested in 2010. Ears were partially dried in the field (within sacks) to 15% of moisture. A composite grain sample derived from 100 selected plants was obtained. Then the grains were dried in a forced air drier at 40 °C for 3 d to 8% of humidity and finally stored at 5 °C and 70% to 90% of relative humidity in storage rooms. A representative sample (200 to 300 g of mature dried seeds) derived from the above composite sample was used in current study. Seeds were milled to a fine powder (500 μm) and the flour was stored at −20 °C until analysis. All chemicals and solvents used were of high-performance liquid chromatography (HPLC) or analytical grade.

Strains used

The probiotic lactic acid bacteria strains used were *L. helveticus* (ATCC 15009) and *B. longum* (ATCC 15708). *H. pylori* (ATCC 43579, originated from human gastric samples) was obtained from the American Type Culture Collection (Manassas, Va., U.S.A.).

Extract preparation

The extraction of the free and bound phenolic fractions was performed based on the optimized methodology reported by Fuentealba and others (2017) with some modifications as follows. For the free phenolic fraction extraction, a sample of 5 g of powdered purple corn was mixed with 20 mL of methanol/acetone/water (45:45:10, v/v/v) acidified with 0.1% HCl in a flask and the headspace was flushed with nitrogen gas. The mixture was agitated in an orbital shaker at 200 rpm for 63 min at ambient temperature and in the absence of light. The homogenate was then centrifuged at 2665 × *g* for 15 min, the supernatant was recovered and a second extraction was applied on the residue under the same solvent conditions for 30 min. After centrifugation, both supernatants were combined and then vacuum-evaporated to dryness at 45 °C and reconstituted in 10 mL with Milli-Q water. The final extract was corrected to a pH of 6.5 to 7 and kept at −20 °C until analysis.

The extraction of the bound phenolic fraction was performed by mixing 0.5 g of powdered purple corn with 2 mL of methanol/acetone/water (45:45:10, v/v/v) acidified with 0.1 % HCl. The free phenolic fraction was extracted under same experimental conditions as stated above. Supernatants were discarded and the residue was suspended in 20 mL of 3 N NaOH, flushed with nitrogen gas and hydrolyzed under agitation at room temperature for 88 min. The mixture was acidified to a pH of approximately 2.5 with concentrated HCl and the bound phenolic fraction was extracted six times with 10 mL of ethyl acetate. The supernatant was recovered after centrifugation at 2665 × *g* for 5 min. The ethyl acetate fractions were mixed, vacuum-evaporated to dryness at 45 °C, and reconstituted in 5 mL of Milli-Q water. The final extract was corrected to a pH of 6.5 to 7 and stored at −20 °C until analysis.

Total phenolic contents

The total phenolic contents were analyzed according to the Folin–Ciocalteu method (Singleton and Rossi 1965). An aliquot of 0.5 mL of the free or bound phenolic extract was transferred into a test tube and mixed with 0.25 mL of Folin-Ciocalteu reagent (1 N) and 1.25 mL of 1.2 N Na_2CO_3. Tubes were allowed to stand for 30 min in a dark place. The absorbance was read at 755 nm. A standard curve with gallic acid in water at various concentrations was used and results were expressed as mg of gallic acid equivalents (GAE) per 100 g of sample in dry weight (DW).

Total anthocyanin contents

Total anthocyanins were determined based on the method of Abdel-Aal and Hucl (1999) with some modifications. Powdered purple corn sample (1 g) was weighed in a 50 mL centrifuge tube and 20 mL of acidified ethanol (ethanol and 1 N HCl, 85:15, v/v) were added. The solution was mixed and adjusted to pH 1 with 4 N HCl, the tube was flushed with nitrogen gas and agitated for 30 min, and then centrifuged at 2665 × *g* for 15 min. A second extraction on the residue was performed under same conditions as stated above for additional 15 min. Both supernatants were collected and poured into a 50-mL volumetric flask and made up to volume with acidified ethanol. Absorbance readings at 535 nm were taken and corrected for background absorbance at 700 nm (due to turbidity). Using the molar extinction coefficient of 25965 $cm^{-1}M^{-1}$ and a molecular weight of 449.2 g/mol, total anthocyanin contents were calculated and expressed as mg of cyanidin-3-glucoside equivalents per 100 g of sample in DW.

Phenolic acid profiles by ultra-performance liquid chromatography

Free and bound phenolic fractions from purple corn were filtered (pore size 0.2 μm) and then injected in a Acquity ultra-performance liquid chromatography (UPLC) H-class system (Waters, Milford, Mass., U.S.A.) equipped with a diode array detector, a quaternary pump, an auto-sampler, and controlled by the Empower software (Waters). Phenolic compounds were separated using an Acquity UPLC BEH C18 analytical column (100 × 2.1 mm i.d., 1.7 μm particle size; Waters) and protected with an Acquity UPLC BEH C18 VanGuard Pre-column (5 × 2.1 mm i.d., 1.7 μm particle size; Waters); the column temperature was thermostat stabilized at 30 °C and samples were kept at 5 °C in the vial tray. The injection volume was 5 μL, the flow rate was 0.2 mL/min and eluates were monitored from 200 to 700 nm. The solvents used for gradient elution were (A) formic acid: water (1:999, v/v, pH 2.5) and (B) 100 % acetonitrile. Initial conditions were: 98% (A) and 2% (B) and a gradient of solvent (B) was used: from 2% to 15% for 3 min, then from 15% to 45% for 6 min, from 45% to 98% for 1 min and maintained in these conditions for the next 3 min, then returned to initial conditions in 1 min and followed for a re-equilibration time of 3 min (total run time 17 min). Detected phenolic acid compounds were identified by comparison of their retention times and spectral characteristics with those of pure standards. Quantification of phenolic acids were performed using the corresponding calibration curves ($r = 0.9990$) built with pure standards (ferulic acid, *p*-coumaric, and caffeic acid) diluted in methanol and results were expressed as mg per 100 g sample DW.

Lactic acid bacteria proliferation assay

This assay was performed according to Ranilla and others (2012). Initially, collected LAB strains from ATCC were preserved in MRS broth (with 20% glycerol) and kept at freezer (−80 °C). During experiment, frozen stocks (100 μL) from the lactic acid bacterial strains were inoculated into 10 mL MRS broth (Difco) and incubated for 24 h at 37 °C under reduced oxygen condition. Then, 100 μL of the 24 h grown strain were re-inoculated into 10 mL MRS broth for 24 h at 37 °C. Extracts containing the free and bound phenolic fractions from purple corn were filter sterilized using sterile filters Millex GP 0.22 μm (Millipore Corp., Bedford, Mass., U.S.A.). Filter-sterilized sample extracts (1 mL) and 100 μL of the 48 h grown strain (diluted 100 times with sterile distilled water) were added into 9 mL of MRS broth tubes and incubated at 37 °C for 48 h. A control with 1 mL of sterile distilled water instead of sample extract was also included. From the 10 mL reaction volume, 100 μL of the serially diluted samples were plated on MRS agar (Difco) and incubated in anaerobic BBL GasPak jars (Becton, Dickinson and Co., Sparks, Md., U.S.A.) with BD GasPak EZ anaerobe container system sachets (Becton, Dickinson and Co; producing an anaerobic atmosphere with less than 1% of oxygen) at 37 °C for 48 h to determine the CFU/mL. The plate counting was done at time 0 and after 6, 12, 24, and 48 h of bacteria growth. The effect of the free and bound phenolic fractions from purple corn on lactic acid bacteria proliferation was compared at same sample concentration (10 mg/mL in final medium), additionally, the free phenolic fraction was evaluated at different sample concentrations (10, 25, and 50 mg/mL in final medium).

Preparation of starter culture of *H. pylori*

H. pylori was cultured according to Stevenson and others (2000). Standard plating medium (*H. pylori* agar plates) were prepared by using 10 g of special peptone (Oxoid Ltd., Basingstoke, England) per liter, 15 g of granulated agar (Difco Laboratories, Becton, Dickinson and Co.) per liter, 5 g of sodium chloride (Fisher Scientific, Waltham, Mass., U.S.A.) per liter, 5 g of yeast extract (Difco) per liter, 5 g of beef extract (Difco) per liter of water. The composition of the broth medium was the same as the standard plating medium without the use of granulated agar.

H. pylori stock was prepared using media (10 g of special peptone, 5 g of sodium chloride, 5 g of yeast extract, and 5 g of beef extract per liter of water) and stored in −80 °C freezer. Then a volume of 100 μL of stock of *H. pylori* was added into test tubes containing 10 mL of sterile broth media. Tubes were incubated at 37 °C for 24 h and then 100 μL of the 24 h culture was subcultured into 10 mL of sterile broth media for another 24 h under reduced oxygen environment. After incubation, 100 μL of the active culture was spread using spread plate technique on to *H. pylori* agar plates to make bacterial lawn for the agar-diffusion assay.

Agar-diffusion assay

The antimicrobial activity of the free and bound phenolic fractions from purple corn was evaluated by the agar-diffusion method and based on the method reported by Ranilla and others (2012). The assay was done aseptically using sterile 12.7 mm diameter paper disks (Schleicher & Schuell, Inc., Keene, N.H., U.S.A.) to which 100 μL of filter-sterilized sample extracts were added. Saturated disks were placed onto the surface of seeded agar plates. Controls consisted of disks with sterilized-distilled water only. Treated plates were incubated at 37 °C for 48 h in BBL GasPak jars (Becton, Dickinson and Co.) with BD GasPak Campy container system sachets (Becton, Dickinson and Co.; producing a microaerophilic environment). The diameter of clear zone (no growth) surrounding each disk was measured and the zone of inhibition was determined and expressed in mm. The antimicrobial activity of the free and bound phenolic fractions from purple corn was evaluated at same sample dose (10 mg/disk, reflecting the sample concentration in final medium for the lactic acid bacteria proliferation assay), whereas the free phenolic fraction was additionally evaluated at different sample doses (10, 25, and 50 mg/disk, reflecting the sample concentration in final medium for the lactic acid bacteria proliferation assay).

Statistical analysis

Extractions (free and bound phenolic fractions) were performed in triplicate and results were expressed as means ± standard deviation. Only the lactic acid bacteria proliferation assay was

Table 1–Total phenolic contents, total anthocyanins, and phenolic acid profiles by UPLC-PDA in the free and bound phenolic fractions from purple corn accession AREQ-084.

Compound	Phenolic fraction		Total
	Free	Bound	
Total phenolic contents (mg GAE/100 g DW)	247.86 ± 33.26	206.60 ± 39.42	454.45 ± 62.26
Total anthocyanin contents (mg cyanidin-3-glucoside equivalents/100 g DW)	310.04 ± 34.16	ND	310.04 ± 34.16
Phenolic acids (mg/100 g DW):			
p-Coumaric acid	ND	33.49 ± 1.44	33.49 ± 1.44
p-Coumaric acid derivatives[a]	3.86 ± 0.80	ND	3.86 ± 0.80
Ferulic acid	ND	156.30 ± 14.41	156.30 ± 14.41
Ferulic acid derivatives[b]	1.44 ± 0.35	18.78 ± 7.16	20.21 ± 7.12
Caffeic acid derivatives[c]	1.78 ± 0.20	ND	1.78 ± 0.20
Total phenolic acids (mg/100 g DW)	7.08 ± 1.34	208.57 ± 17.86	215.65 ± 18.91

[a]Quantified as *p*-coumaric acid.
[b]Quantified as ferulic acid.
[c]Quantified a caffeic acid.
ND, not detected.

performed by duplicate and results from these experiments were subjected to the Kruskall–Wallis test ($P < 0.05$) for evaluating statistical differences among treatments. The Statgraphics Centurion XV software package (StatPoint Inc., Rockville, Md., U.S.A.) was used.

Results and Discussion

Total phenolic contents, anthocyanins contents, and phenolic acid profiles by UPLC

The total phenolic and anthocyanin contents and the phenolic acid profiles measured by UPLC in the Peruvian purple corn accession AREQ-084 are shown in Table 1. The major phenolic group in purple corn kernels has been mainly associated to anthocyanins in comparison to other non-pigmented corn varieties where phenolic acids such as hydroxycinnamic and hydroxybenzoic acid derivatives are more common (Gonzalez-Muñoz and others 2013; Das and Singh 2015). In this study, both the free and bound phenolic fractions had almost similar total phenolic contents corresponding to 54.54% and 45.46% with respect to the final total phenolic content (free and bound), respectively. Anthocyanins were the major phenolic compounds found in the free phenolic fraction, while hydroxycinnamic acid derivatives (*p*-coumaric, ferulic, and caffeic acid derivatives) were major phenolic acids detected in lower concentrations by UPLC (7.08 ± 1.34 mg/100 g DW). The bound phenolic fraction was only composed of hydroxycinnamic acid derivatives, mainly ferulic acid followed by *p*-coumaric acid and a ferulic acid derivative. This phenolic acid fraction represented around the 96.7% of the total phenolic acid content (free and bound).

Only few previous studies have reported phenolic characterization of kernels from Peruvian purple corn because most studies were mainly focused on ground-dried cobs or commercial anthocyanin-rich extracts made from Peruvian purple cobs (Pascual-Teresa and others 2002; Pedreschi and Cisneros-Zevallos 2007; Jing and Giusti 2007; Quispe and others 2011). This is because overall high anthocyanin contents have been found in cobs from purple corn compared to kernels (738 and 4065 mg cyanidin-3-glucoside/100 g DW for kernels and cobs from Peruvian purple corn, respectively; Cevallos-Casals and Cisneros-Zevallos 2003). However, the same study reported that whole kernels also significantly contribute to the total anthocyanin contents considering the total corn ear weight. Therefore, information of this study would provide the scientific foundation for the diversification of the use of purple corn kernels especially for human health relevant applications.

Comparable values of total anthocyanin contents were found by Ramos-Escudero and others (2012) and Paucar-Menacho and others (2017; 201 and 363.6 mg cyanidin-3-glucoside/100 g DW, respectively) when seeds were evaluated from different commercial Peruvian purple corn varieties (INIA-601 from Cajamarca-Peru and PMV-581 from Lima-Peru, respectively). Moreover, anthocyanin values found in this study were higher than those reported by Montilla and others (2011; 1.97–71.68 mg cyanidin-3-glucoside/100 g DW) and Zilic and others (2012; 59.7 mg cyanidin-3-glucoside/100 g DW) who studied different purple corn kernels from Bolivia and a dark blue sample from the United States, respectively.

Similar to this study, hydroxycinnamic acid derivatives such as ferulic and caffeic acid derivatives were detected in the free phenolic fraction from Peruvian purple corn kernels (Paucar-Menacho and others 2017). In addition, hydroxybenzoic acid derivatives, flavonols derivatives such as kaempferol, quercetin, and isorhammetin derivatives and a flavanone (naringenin) have been reported in samples from Peru (Ramos-Escudero and others 2012; Paucar-Menacho and others 2017).

The composition of the bound phenolic fraction from the evaluated Peruvian purple corn accession was in agreement with some studies that investigated purple corn kernels from different origins. Ferulic and *p*-coumaric acids were also found in the bound fraction of purple or dark blue-pigmented corn seeds from Mexico, Bolivia, United States, and Chile (Lopez-Martínez and others 2009; Montilla and others 2011; Zilic and others 2012; Gonzalez-Muñoz and others 2013, respectively). Higher ferulic acid and total phenolic acid contents were found in this study (156.3 and 208.57 mg/100 g DW, respectively) in comparison to amounts reported for 2 Chilean purple corn accessions (127 to 128.9 and 147.08 to 149.81 mg/100 g DW for ferulic acid and total phenolic acid contents, respectively; Gonzalez-Muñoz and others 2013). In addition, several black and purple corn samples from Mexico have shown lower ferulic acid contents (151 to 154 mg/100 g DW) than those found in this study (Lopez-Martínez and others 2009).

Effect of the free and bound phenolic fractions from the Peruvian purple corn accession on proliferation of probiotic lactic acid bacteria and the inhibition of *H. pylori*

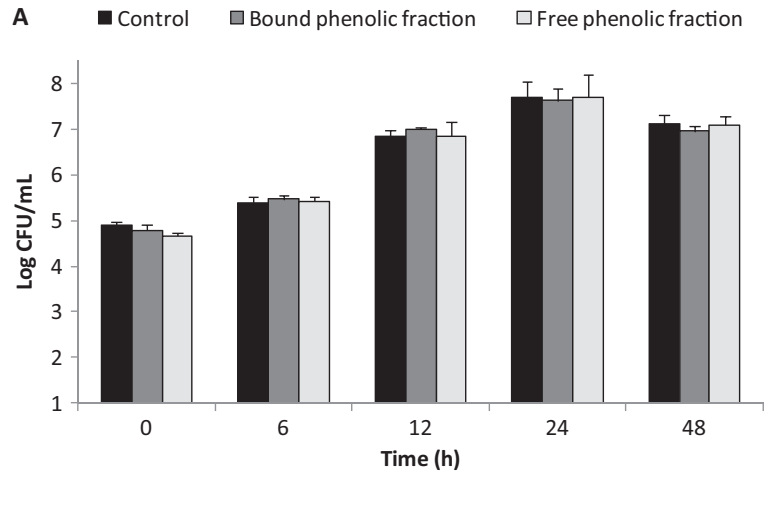
The effect of the free and bound phenolic fraction from the purple corn accession at one sample dose (10 mg/mL) on the

growth of two probiotic lactic acid bacteria strains such as *L. helveticus* and *B. longum* is shown in Figure 2. In addition, the effect of the high anthocyanin-free phenolic fraction on probiotic bacteria proliferation was also evaluated at three higher sample doses (10, 25, and 50 mg/mL; Figure 3) as this phenolic group significantly contributed to the total phenolic contents in purple corn.

The growth of *L. helveticus* and *B. longum* was not affected by either of the two phenolic fraction extracts (free and bound) at 10 mg/mL of sample dose during the incubation time (0–48 h; $P > 0.05$). Further, higher free phenolic sample doses at 25 and 50 mg/mL did not affect the proliferation of lactic acid bacteria when compared to the control treatment ($P > 0.05$; Figure 2).

Puupponen-Pimiä and others (2001) found that anthocyanin rich-berry extracts at 1 mg/mL of sample concentration (equivalent to a content of 0.0009 and 0.26 mg cyanidin-3-glucoside equivalents/mL of total anthocyanins in final medium for cloudberry and blueberry, respectively) did not inhibit the growth of *Lactobacillus rhamnnosus*, *Lactobacillus paracasei, Lactobacillus plantarum*, and *Bifidobacterium lactis* using the liquid culture method. Furthermore, pure anthocyanin compounds such as cyanidin-3-glucoside and delphinidin chloride were also evaluated in same study by the agar diffusion assay at ranges of 0.003 to 0.03 and 0.05 to 0.5 mg, respectively against several *Lactobacillus* strains and no inhibition was observed. Cyanidin-3-glucoside is the major anthocyanin compound in Peruvian purple corn kernels followed by pelargonidin and peonidin derivatives (Aoki and others 2002; Paucar-Menacho and others 2017). In this study, evaluated sample doses (10, 25, and 50 mg/mL) of the free phenolic fraction corresponded to an average of 0.03 to 0.15 mg cyanidin-3-glucoside equivalents/mL of total anthocyanin contents in final medium. This range was almost similar to those studied by Puupponen-Pimiä and others (2001), indicating that the number of beneficial probiotic lactic acid bacteria may not be affected at such a level of anthocyanin content and therefore can compatible for synergistic and health targeted uses with such bacteria enriched foods and beverages.

Other studies have reported that the antimicrobial action by phenolic compounds not only depends on the applied dose but also on their chemical structure and if the phenolic compound is evaluated in isolated form or in a mixture. For instance, the *in vitro* incubation of isolated malvidin-3-glucoside (dose of 0.2 mg/mL) with human fecal slurry for 24 h allowed an increase of 0.3 log cells/mL of *Bifidobacterium* spp. whereas no effect was observed on *Lactobacillus* spp. growth in relation to the control treatment. In contrast, there was a decrease in the pathogenic *Clostridium histolyticum* group (Hidalgo and others 2012). In the same study, the incubation of a mixture of anthocyanins (malvidin-3-glucoside, dephinidin-3-glucoside, petunidin-3-glucoside, and cyanidin-3-glucoside) at a dose of 0.3 mg/mL with human fecal slurry resulted in a slight increase (+0.2 log cells/mL more than the control) of *Bifidobacterium* spp. but a significant increase in



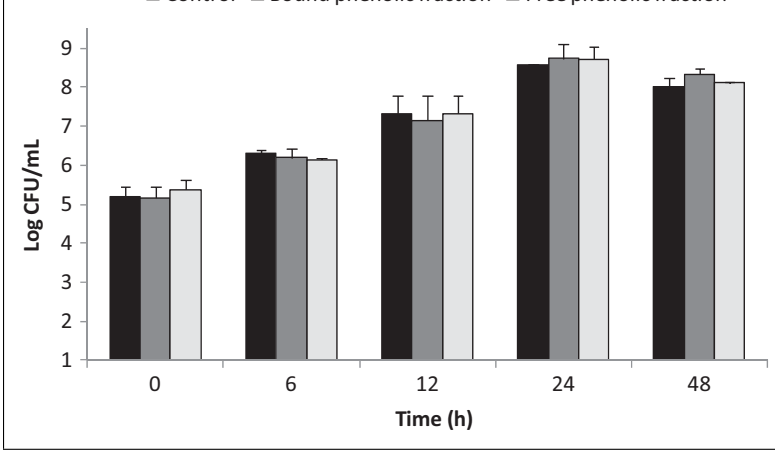


Figure 2–Effect of the free and bound phenolic fraction from a purple corn accession (Areq-084) at 1 sample dose (10 mg/mL) on probiotic bacteria growth: *L. helveticus* and *B. longum.* Results are expressed as means of independent duplicate experiments.

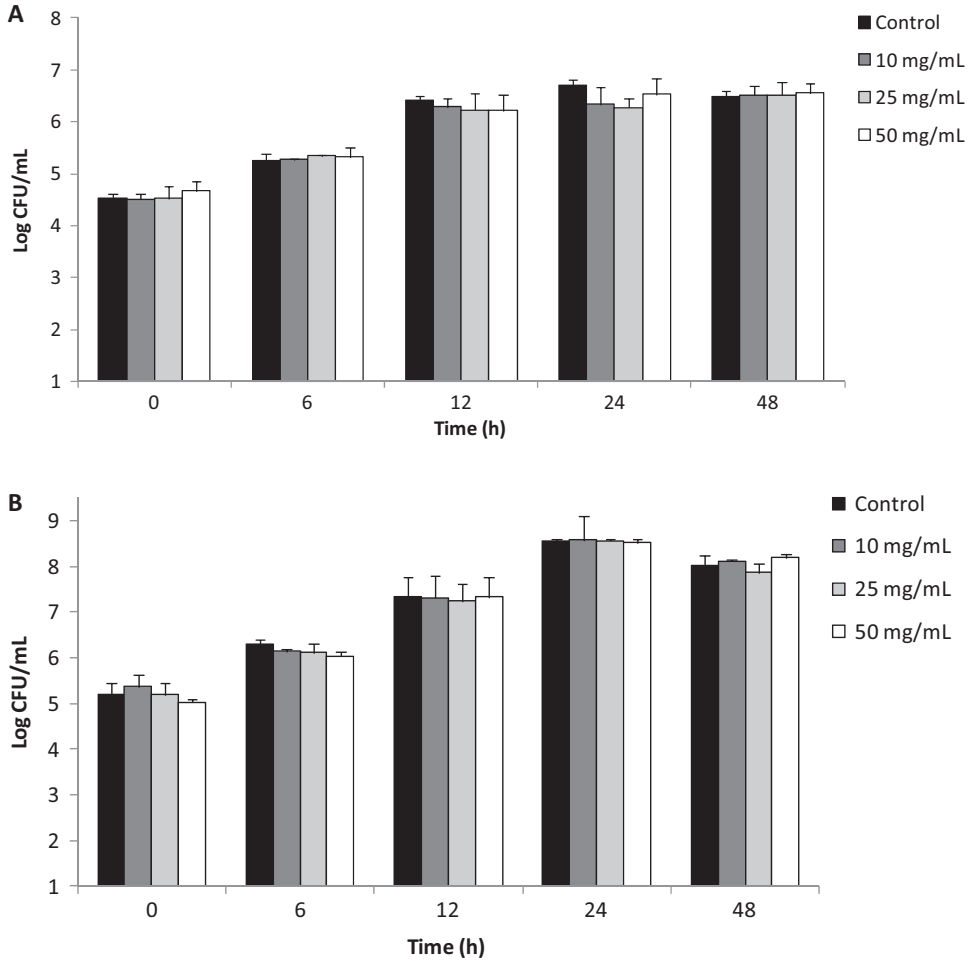


Figure 3–Effect of the free phenolic fraction from a purple corn accession (Areq-084) at 3 sample doses (10, 25, and 50 mg/mL) on probiotic bacteria growth: *L. helveticus* and *B. longum.* Results are expressed as means of independent duplicate experiments.

Lactobacillus spp. growth (+0.8 log cells/mL more than the control) whereas no effect was observed on *C. hystoliticum* growth after 24 h of incubation. Hidalgo and others (2012) evaluated anthocyanin ranges (0.02 to 0.3 mg/mL as total anthocyanins) that were estimated to reflect lower and upper levels of anthocyanin daily intake in humans and were similar to those used in this study (0.03 to 0.15 mg cyanidin-3-glucoside equivalents/mL of total anthocyanin contents). In another study, isolated phenolic compounds such as hesperidin and quercetin (at a dose of 0.1 mg/mL) inhibited the growth of *Bifidobacterium* species after 48 h of incubation up to 10% and 20%, respectively in relation to the control whereas no effect was observed in probiotic bacteria when ferulic acid was used at the same dose and time of incubation (Gwiazdwoska and others 2015). The free phenolic extract from purple corn in this study was composed by a mixture of phenolic compounds such as anthocyanins (likely cyanidin-3-glucoside, pelargonidin-3-glucoside, peonidin-3-glucoside, and their malonyl derivatives as was found in other purple corn samples; Lao and others 2017) and phenolic acids. This phenolic composition is different from phenolic profiles from other sources; therefore, it is expected that the antimicrobial effect of these new phenolic combination would be different compared to results from above investigations.

According to *in vivo* studies, anthocyanins are rapidly absorbed through the stomach and small intestine but their overall bioavailability is very low since less than 1% of the ingested anthocyanins are absorbed and excreted in the urine; whereas large amounts of the ingested anthocyanins enter the colon and are exposed to the action of microbial population (McGhie and Walton 2007). Anthocyanins are then degraded to sugar and phenolic components by microbiota action, with the phenolic components further degraded by disruption of the C-ring to yield phenolic acids and aldehydes (McGhie and Walton 2007). The metabolism of cyanidin-3-glucoside in human microbiota-associated rats has been described in other *in vivo* study and determined protocatechuic acid, 2,4,6-trihydroxybenzaldehyde, and 2,4,6-trihydroxybenzoic acid (gallic acid) as the main colonic metabolites (Hanske and others 2013). Cyanidin-3-glucoside derivatives from purple corn may follow similar gut-linked metabolic biotransformations, but further *in vivo* studies are needed to elucidate the effect of the specific anthocyanin profile from purple corn on human gut microbiota.

The bound phenolic fraction, rich in hydroxycinnamic acid derivatives, did not affect the growth of probiotic lactic acid bacteria at 10 mg/mL of sample dose which corresponds to an average of 0.02 and 0.016 mg/mL of total phenolic acids and ferulic acid contents, respectively in final medium. Probiotic lactobacilli such as *L. plantarum*, *L. fermentum*, and *L. brevis* were not inhibited by doses less than 0.125 mg/mL of certain hydroxycinnamic acids

derived from the metabolism of flavanol monomers and dimers (Cueva and others 2010). However, a significant inhibition of *Lactobacillus* species was observed when higher doses (1 mg/mL) were used (Cueva and others 2010). Moreover, Puupponen-Pimiä and others (2001), using the agar diffusion technique, reported that pure phenolic acids such as trans-cinnamic acid, *p*-coumaric acid, caffeic acid, chlorogenic acid, and ferulic acid had antimicrobial activity against certain Gram-negative bacteria (*Escherichia coli* and *Salmonella enterica*) only at the highest evaluated dose (0.5 mg/well), whereas Gram-positive bacteria such as *L. rhamnosus*, *L. reuteri*, *L. paracasei*, *L. johnsonii*. *L. crispatus*, and *L. plantarum* were not affected at dose ranges of 0.05 to 0.5 mg/well. Same study also reported that differences in cell surface structure between Gram-negative and Gram-positive bacteria would determine different sensitivities towards phenolic compounds. Phenolic doses used in above investigation were higher than those used in the current study which can explain obtained results; however, the phenolic composition may also have a significant influence as discussed above.

Bound phenolic compounds are not absorbed in the small intestine because they are ester-linked to cell wall polysaccharides; they then enter the colon where they undergo fermentation by the colon microbiota giving rise to potentially absorbable metabolites (Shahidi and Yeo 2016). An *in vivo* animal study has shown that rat colonic microflora exhibit esterase activity that releases certain ferulic acid derivatives from dietary cereal bran which is later partially absorbed and then enters the circulatory system (Andreansen and others 2001). Grape dietary fiber (GDF) rich in polyphenols stimulated the proliferation of large intestine-linked *Lactobacillus* species by 1-log whereas the growth of *Bifidobacterium* was barely affected in rats fed daily with GDF during a period of 4 weeks (Pozuelo and others 2012). It is most likely that the growth stimulation of *Lactobacillus* observed in the above study may be related among other factors to the continuous exposure of gut bacteria to bound phenolic compounds of GDF. These conditions were different compared to those applied in the current *in vitro* study; nevertheless, this is the first approach regarding the effect of bound phenolic compounds from purple corn on probiotic lactic acid bacteria.

H. pylori, is a Gram-negative, microaerophilic bacterium which has been considered as the cause of one of the most important infections affecting the human stomach because this bacterium inhabits the inner mucosa, causing not only gastritis but also gastroduodenal ulcers and, in the more severe stages, tumors (De Monte and others 2015). Because of the increase in antibiotic resistance and host-linked side effects associated to common *H. pylori* therapies, the investigation of naturally occurring antimicrobial compounds from food diversity could potentially be an alternative or used in synergy with drugs for preventing and treating *H. pylori* infection and also countering antibiotic resistance. The free phenolic extract derived from a Peruvian purple corn accession was screened for potential antimicrobial activity against *H. pylori* at a range of 10 to 50 mg/disk of sample dose corresponding to a final concentration of 0.03 to 0.15 mg cyanidin-3-glucoside equivalents/disk (total anthocyanin content). In addition, the bound phenolic fraction was assayed at 10 mg/mL of sample dose corresponding to a final concentration of 0.02 mg/disk of total phenolic acid contents. No inhibitory activity of free and bound phenolic fraction of purple corn accession against *H. pylori* was observed in this study. Previous reports have shown that similar or lower anthocyanin concentrations than those used in current study had inhibitory activity against other Gram-negative bacteria. Lacombe and others (2010) have shown that an anthocyanin concentration of 0.029 mg cyanidin-3-glucoside equivalents/mL was inhibitory against *E. coli* O157:H7 when the antimicrobial activity of a high anthocyanin-fraction from cranberry was evaluated. Zhao and others (2009) reported that a anthocyanin-rich extract obtained from a Chinese purple corn sample strongly inhibited the growth of *Salmonella* Enteritidis and *Staphylococcus aureus* in a dose dependent manner whereas no inhibition was observed on *E. coli* strain using the cylinder-plate assay and at lower anthocyanin doses (0.003 to 0.03 mg cyanidin-3-glucoside equivalents/well) than those evaluated in our current study. Only few studies have reported the antimicrobial action of anthocyanins against *H. pylori*. Kim and others (2012) determined that a dose of 0.045 mg cyanidin-3-glucoside/mL (similar or lower than ranges used in the current study: 0.03 to 0.15 mg cyanidin-3-glucose equivalents/disk) did not show any effect on *H. pylori* bacterial number; however, *H. pylori* virulence factors such as cytotoxin-associated protein A (CagA) and vacuolating cytotoxin A (VacA) decreased. The pretreatment of human gastric epithelial cell lines with anthocyanins isolated from soybean coat in a range of 0.0125 to 0.05 mg/mL (similar or lower than ranges used in this study) decreased the *H. pylori*-induced inflammation markers such as reactive oxygen species (ROS) and interleukin-8, and inhibited the *H. pylori*-induced inducible nitric oxide synthases and cyclooxygenase-2 mRNA expression (Kim and others 2013). It is most likely that the antimicrobial action of anthocyanins from purple corn would be more related with the decrease of *H. pylori* virulence factors and induced inflammatory markers than with the direct effect on *H. pylori* number. Additionally, purple corn sample doses investigated in this study might have inhibitory activity against other Gram-negative bacteria strains. However, this premise should be further confirmed in future studies.

Different phenolic compounds and high-phenolic food ingredients have been shown to exert anti-*H. pylori* activity under multiple mechanisms and synergies, and chemical structure, concentrations and composition of phenolic compounds seems to be important factors that influence the antimicrobial activity against pathogenic bacteria (Nohynek and others 2006; Lacombe and others 2010; Takeuchi and others 2014).

Conclusion

When consumed as a part of diet, anthocyanin and other phenolic acids of plant-based foods significantly contribute to diverse health benefits such as improvement of gut health by modulating the composition and population of human gut microbiome. Based on this scientific rationale, characterization of anthocyanin and other phenolic acids of free and bound phenolic fractions of Peruvian purple corn accession from the Andean region and their impact on growth of beneficial probiotic lactic acid bacteria and antimicrobial activity against *H. pylori* were evaluated for the first time. Major phenolic compounds in the free phenolic fraction from the Peruvian purple corn accession AREQ-084 were anthocyanins whereas the bound phenolic fraction was composed by hydroxycinnamic acids, mainly ferulic acid, followed by *p*-coumaric acid and ferulic acid derivatives. The free and bound phenolic fractions did not inhibit the proliferation of beneficial probiotic lactic acid bacteria such as *L. helveticus* and *B. longum*. Further, both phenolic fractions were also not effective against the pathogenic *H. pylori*. Although no inhibitory activity of free and bound phenolic fractions of purple corn was observed against *H. pylori,* however the lack of inhibitory activity against beneficial lactic acid bacteria has important human health relevance.

Results of this study indicates that phenolic compounds of this Peruvian purple corn can be potentially used as functional food ingredients when targeted for other health applications and will be potentially compatible without having any negative side-effect on gut health associated beneficial bacteria. This study provides initial scientific insights on potential role of free and bound phenolic fractions of purple corn on potential gut microbiome and can be explored further to determine antimicrobial properties of these phenolic compounds against other pathogenic bacteria and microorganisms, including antimicrobial synergies with other single antimicrobial agents that could overcome antimicrobial resistance. Finally, future *in vivo* studies for better understanding the potential interaction between gut microbiota and phenolic compounds from purple corn are recommended.

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Authors' Contributions

Lena Gálvez Ranilla designed the study, performed the chemical analysis and part of the *in vitro* antimicrobial assays, interpreted the results, and drafted the manuscript.

Ashish Christopher and Dipayan Sarkar coordinated and performed the experimental antimicrobial assays and reviewed the manuscript.

Kalidas Shetty contributed to the design of the study, interpreted the results, and approved the final version of the manuscript.

Rosana Chirinos and David Campos were responsible for the experimental work related to the analysis of purple corn phenolic extracts by UPLC-PAD and reviewed the manuscript.

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