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A systematic literature review of the effect of anthocyanins on gut microbiota populations

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Abstract

Background: Evidence has shown that anthocyanins, a subclass of polyphenol, are metabolised in the gut, modulate bacterial species and exert bioactive effects through this interaction. Methods: A systematic literature review was undertaken to determine the level of current evidence for the association between anthocyanin intake and changes in gut microbiota populations. The studies included were also assessed for the different techniques used in microbiota determination. Following the PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) guidelines, scientific databases, including Scopus, PubMed, ScienceDirect, Web of Science and MEDLINE, were searched up to June 2017. Details on population/sample, study design, intervention/control, dosage and method of microbiota determination were extracted. Results: Six studies (three in vitro, two animal and one human trials) were included in the review, which showed that anthocyanins induced a significant proliferative effect on Bifidobacterium spp., known for their wide use in probiotics and for the treatment of irritable bowel syndrome. There was also an observed inhibition of Clostridium histolyticum, which was shown to be pathogenic in humans. The depth of analysis is an important consideration for the choice of microbiota determination technique with respect to a comprehensive, high-resolution microbiota analysis or analysis of the main microbiota taxa. Conclusions: Very limited research has been carried out in the area of anthocyanins and gut microbiota; beneficial effects have generally been observed, and further clinical trials in humans are needed to confirm changes to gut microbes in relation to dietary anthocyanin intake and potential health benefits. Journal of Human Nutrition and Dietetics

Systematic Review

A systematic literature review on the effect of anthocyanins on gut microbiota populations

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Abstract

Context: Evidence has shown that anthocyanins, a sub-class of polyphenol, are metabolised in the gut, modulate bacterial species and exert bioactive effects through this interaction.

Objective: A systematic literature review was undertaken to determine the level of current evidence for the association between anthocyanin intake and changes in gut microbiota populations. Included studies will also be assessed for the different techniques used in microbiota determination.

Data sources: Following the PRISMA guidelines, scientific databases including Scopus, PubMed, ScienceDirect, Web of Science, and MEDLINE were searched up to June 2017.

Data extraction: Details on population/sample, study design, intervention/control, dosage and method of microbiota determination were extracted.

Result: Six studies (3 *in vitro,* 2 animal and 1 human trials) were included in this review which showed that anthocyanins induced a significant proliferative effect on *Bifidobacterium spp.,* known for their wide use in probiotics and for the treatment of Irritable Bowel Syndrome (IBS). There was also an observed inhibition of *Clostridium histolyticum,* which have been shown to be pathogenic in humans. Depth of analysis is an important consideration on the choice of microbiota determination technique, in regard to a comprehensive, high-resolution microbiota analysis or analysis of the main microbiota taxa.

Conclusion: Very limited research has been carried out in the area of anthocyanins and gut microbiota; beneficial effects have generally been observed and further clinical trials in humans are needed to confirm changes to gut microbes in relation to dietary anthocyanin intake and the potential health benefits.

Systematic Review Registration: PROSPERO registration number CRD42017073750

Keywords: Anthocyanins, Gut Microbiota, Metabolism, Health benefits, Systematic review

Introduction

Anthocyanins are a group of water-soluble plant pigments responsible for the deep rich purple/red/blue colours observed in plant-based foods. They are one of the major subclasses of flavonoids, a class of polyphenols. Their role in human health has been established in epidemiological studies including improved vision (1), cognition and blood pressure (2, 3), and protective effects against cardiovascular disease risk factors (4).

The metabolism of anthocyanins has been well reviewed (5). Briefly, anthocyanins consumed through the diet have shown to be poorly absorbed by the body, with only a small proportion of intact anthocyanins able to pass through the gastrointestinal wall using active transporters like the sodium-dependent glucose transporter 1 (SGLT1) and facilitative glucose transporter 2 (GLUT 2) (6). Anthocyanins that are absorbed in the GI tract are mainly found as methylated, sulphated or glucuronidated forms but also as intact glycosides in very low concentrations in biological fluids such as blood (plasma) and urine (10 to 2000 nM) (5). Even though bioavailability of intact anthocyanins in the body is reportedly low, a considerable amount of (food) matrix-bound anthocyanins can reach the large intestine and the colon. Here they undergo further intensive metabolism and degradation and are absorbed into the blood stream, with some of the subsequent metabolites showing potential to be more biologically active than the intact anthocyanins (5, 7). The recent interest in the gut microbiota and its role in human health has implications for furthering the understanding of the effect of anthocyanins on gut microbiota, and subsequent health benefits (8).

The gut microbiota, which is the microbe population living in the intestine, contain tens of trillions of microorganisms, and is made up of at least 1000 different species of identified bacteria (9). The majority of the bacteria in the gut are categorised under seven phyla, namely Firmicutes, Bacteroidetes, Proteobacteria, Fusobacteria, Verrucomicrobia, Cyanobacteria and Actinobacteria with the Firmicutes and Bacteroidetes phyla making up over 90% of the human gut microbiota (10, 11). Some of the essential functions of the gut microbiota include vitamin production, regulation of lipid metabolism, short chain fatty acid production as fuel for epithelial cells and regulation of gene expression (12) as well as an important functional role in the gut-brain axis relationship (13). A heathy gut, which comprises of effective digestion and absorption of food, absence of GI illness, normal and stable intestinal microbiota, and effective immune status, is required in order to sustain a host homeostasis (14). Irregularities and imbalances in the microbiota at different ages have been linked to

different diseases and metabolic conditions across the lifespan, ranging from allergies in young infants to Inflammatory Bowel Disease in young adults (15). Research on the gut microbiota continues to shed light on the health effects of changes to the gut microbiota modulated by diet.

Different techniques have been described for the determination of gut microbiota. Advancement in the taxonomical composition of the gut microbiota was limited until the 21st century due to bacteriological culture being the only method available to determine its composition. Even till date, only about 30% of the gut microbiota has been cultured (10). Further understanding of the human gut has been improved by advanced techniques independent of bacteriological culture. Prior to these advancements, culture and biochemical typing were the gold standards for identification of bacteria species but have now been overtaken by methods that are able to produce a more representative information of the overall microbiota (16). Examples of some of these techniques are sequencing of the 16S rRNA gene or its amplicons, fluorescence *in situ* hybridization (FISH), quantitative polymerase chain reaction (qPCR), denaturing gradient gel electrophoresis (DGGE) and terminal restriction fragment length polymorphism (T-RFLP). With these methods researchers are able to get qualitative and quantitative information on the diversity of the gut microbiota as well as changes in the gut microbiota in relation to an intervention or disease (17). Improved scientific methods of gut microbiota determination have also presented the issue of suitability of the different techniques used and how the results compare to one another.

Alterations of the gut microbiota have been shown to induce and promote or reduce the risk of chronic diseases. This hypothetical mechanism has been applied to the field of obesity and obesity-related non-alcoholic fatty liver disease in relation to prebiotics / probiotics (18, 19) and anthocyanins (20) as gut modifiers. With this question in mind, given this rapidly advancing area of research in gut health, a systematic literature review has been undertaken to summarise the current evidence on the effect of anthocyanins on the gut microbiota. The included studies will also be assessed for the different techniques used in microbiota determination. It is hypothesised that supplementation of the diet with anthocyanin rich foods promotes the proliferation of healthy anaerobic bacterial populations, while inhibiting the pathogenic species evidenced in different gut microbiota determination techniques.

Methods

A systematic literature review was conducted according to recommendations of the PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) statement and checklist (21) (figure 1 and table S1). Scientific databases including Scopus, PubMed, ScienceDirect, Web of Science, and MEDLINE were searched up to June 2017. A combination of search terms with truncations included the following keywords: anthocyanins and gut microbiota or colon microbiota (Table 1). The review was registered on PROSPERO (International prospective register of systematic reviews) as CRD42017073750 (22).

Eligibility criteria for included studies (table 2):

1. carried out either *in vitro*, on animal or conducted in human subjects;
2. that quantified the effect of anthocyanin on gut microbial population and described the method used;
3. that utilised anthocyanin extracts or anthocyanin-rich foods with anthocyanin (individual compounds or total) content measured and stated;
4. that assessed changes in gut microbiota associated to anthocyanin metabolism in the gut;
5. reported in the English language for reasons of time efficiency and cost of translation not being feasible for this review.
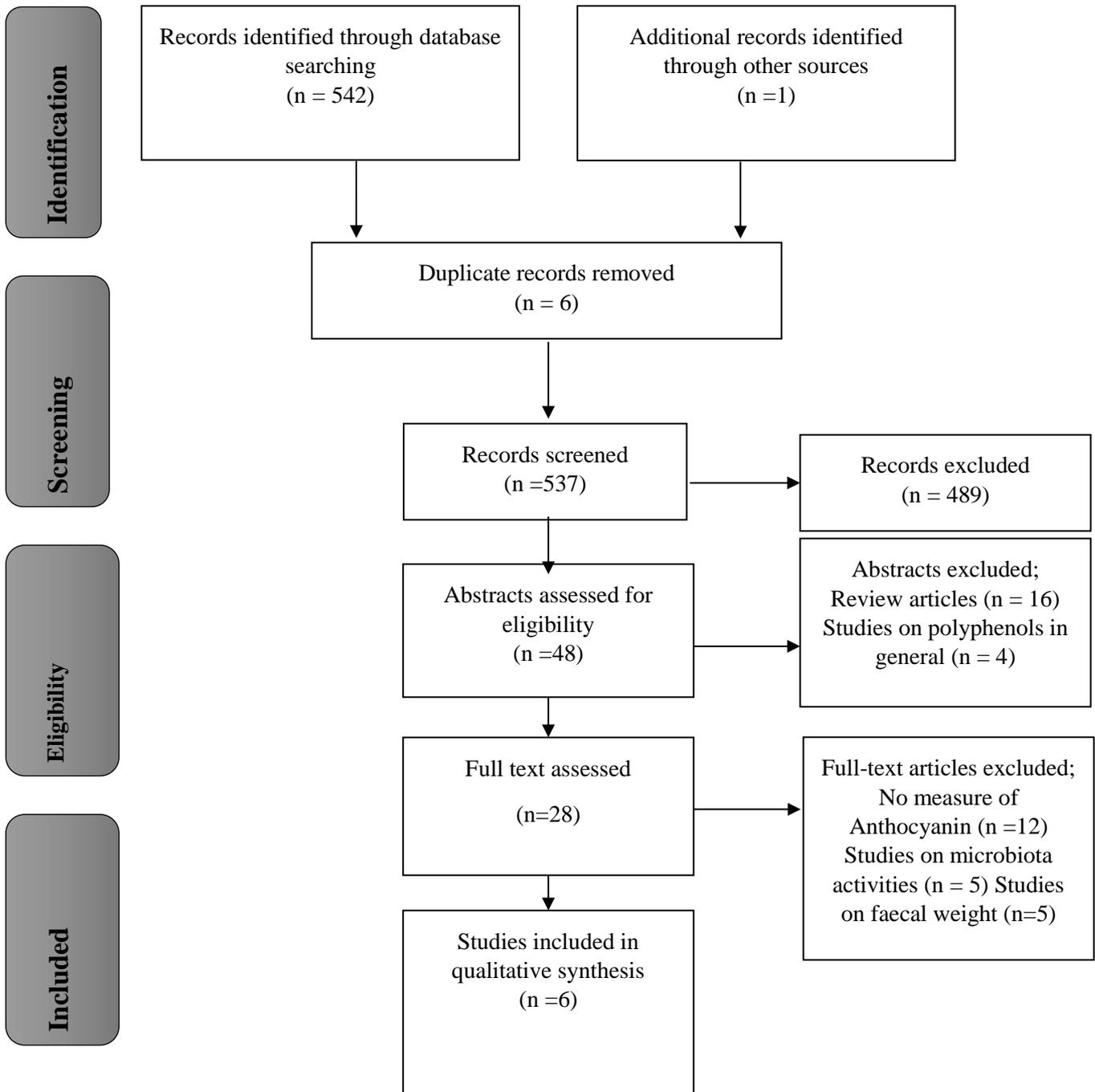
Exclusion criteria

Studies were excluded if:

1. they measured the stability of anthocyanins in the gut without quantifying effects on gut microbial population;
2. there was no measure of anthocyanin content in food/beverage source. One study (23) met this criteria but was included because anthocyanin content was measured indirectly through the use of anthocyanin metabolites in the urine and was the only included clinical trial;
3. they measured total polyphenols or flavonoids in general and not specifically the subclass of anthocyanins.

Review articles were excluded from the search but were considered for hand searching. Hand searching of reference lists was undertaken for all included studies and review articles. All selected abstracts and citations were exported from the scientific databases to the reference management software ENDNOTE X7 (Thompson Reuters, New York, NY, USA). Following extraction and selection of publications according to the above eligibility criteria a tabular

Figure 1 PRISMA flowchart for study selection

Identification

Records identified through database searching
(n = 542)

Additional records identified through other sources
(n =1)

Duplicate records removed
(n = 6)

Screening

Records screened
(n =537)

Records excluded
(n = 489)

Abstracts assessed for eligibility
(n =48)

Abstracts excluded;
Review articles (n = 16)
Studies on polyphenols in general (n = 4)

Eligibility

Full text assessed
(n=28)

Full-text articles excluded;
No measure of Anthocyanin (n =12)
Studies on microbiota activities (n = 5) Studies on faecal weight (n=5)

Included

Studies included in qualitative synthesis
(n =6)

summary was developed for this review which included the population, design, intervention, anthocyanin dosage, health outcomes and results.

Table 1: Scopus search strategy

TITLE-ABS-KEY (anthocyan* AND "gut microbiota") AND (LIMIT-TO (DOCTYPE , "ar") OR LIMIT-TO (DOCTYPE , "cp") OR LIMIT-TO (DOCTYPE , "ip")) AND LIMIT-TO (EXACTKEYWORD , "Human") OR LIMIT-TO (EXACTKEYWORD , "Intestine Flora") OR LIMIT-TO (EXACTKEYWORD , "Humans") OR LIMIT-TO (EXACTKEYWORD , "Polyphenols") OR LIMIT-TO (EXACTKEYWORD , "Metabolism") OR LIMIT-TO (EXACTKEYWORD , "Polyphenol") OR LIMIT-TO (EXACTKEYWORD , "Article") OR LIMIT-TO (EXACTKEYWORD , "Nonhuman") OR LIMIT-TO (EXACTKEYWORD , "Anthocyanins")) AND (LIMIT-TO (LANGUAGE , "English"))

Results

The database searches returned 542 articles and one additional study was identified through hand searching of reference lists. Following removal of duplicates and screening of records, 28 full text articles were evaluated and only six studies were eligible and therefore included in the review **(fig 1).** The included studies are summarised in **Table 3** and briefly described below.

In vitro studies

Three *in vitro* studies were included in this review. Anthocyanins were tested individually or in a mixture, in the case of enocianin, a commercial food colouring from *Vitis Vinifera* grape peel which contains a mixture of anthocyanins including malvidin-3-glucoside (200 mg/L and 20 mg/L respectively), delphinidin-3-glucoside (60 mg/L and 6mg/L respectively), petunidin-3-glucoside (85 mg/L and 8.5 mg/L respectively), peonidin-3-glucoside (traces), and cyanidin-3-glucoside (traces). With high and low concentrations reflecting different levels of intake, anthocyanins showed proliferative effects on beneficial bacterial population and inhibitory effects on pathogenic species. Incubation of malvidin-3-glucoside with human faecal slurry at 0 (control), 5, 10 and 24 h, showed a significant, ($p<0.05$), increase in total

bacteria at 24h. Significant proliferative effects were also observed in the bacteria species with beneficial effects such as *Bifidobacterium spp.* and *Lactobacillus spp.* known for their wide use in probiotics and for the treatment of ulcerative colitis, Irritable Bowel Syndrome (IBS) and constipation (24). A reduction, although not statistically significant, was observed in the potentially harmful *Clostridium histolyticum* following 24 h incubation. For enocianin, a statistically significant increase ($p<0.05$) was observed in *Lactobacillus spp.* and *Bifidobacterium spp.* following all incubation durations. Bacterial levels were significantly higher than those observed with maldivin-3-glucoside, however, no significant change was observed in *C.coccoides- Eubacterium rectale* group which are known for the critical roles they play in immune homeostasis (25) or the pathogenic *C. histolycum* group (26).

Similar effects have also been observed with purple sweet potato anthocyanin extracts (cyanidins and pelargonidin based anthocyanins) using faecal samples. Following fermentation at 0, 6, 12, and 24 h, a significant increase ($p<0.05$) was observed for the total bacterial count. In comparison to the control medium, a steady increase was observed in the numbers of the *Bifidobacterium spp.* following incubation with purple sweet potato anthocyanins while a significant increase was observed in the bacterial population of *Lactobacillus/Enterococcus spp.* On the contrary, the control media had a steady increase in the *Bacteroides-Prevotella* and *Clostridium histolycum* during fermentation, whereas in the media containing anthocyanins, levels were significantly reduced ($p<0.05$).(27)

On the release, metabolism, and effect on gut microbial growth of cyclodextrin encapsulated anthocyanins (cyanidin-3-glucoside, delphinidin-3-glucoside and malvidin-3-glucoside) on faecal slurry, between 0 and 24h, malvidin-3-glucoside showed no significant effect on the population of *Bifidobacterium spp., Clostridium coccoides/Eubacterium rectale* group, *Lactobacillus/Enterococcus spp., C. histolycum* group, *bacteroides spp.,* or *Clostridium* cluster IX. However, a significant increase was observed for the members of the domain *Bacteria (*EUBmix probe). Cyanidin-3-glucoside and delphinidin-3-glucoside on the other hand showed a significant ($p<0.05$) inhibition of the *C. histolycum* group (28).

All three *in vitro* studies utilised the FISH technique for enumeration of bacterial populations.

Animal studies

Using six different berries (blackberry, blackcurrant, black raspberry, blueberry, Concord grape, and maqui berry) with structurally diverse anthocyanin profiles, Overall et. al. (2017)

(29) supplemented the diets in a mouse model of polygenic obesity. Anthocyanins from these individual berries was normalised to 400µg/g total anthocyanins and supplemented with animal chow for each berry category. Six-week old mice were fed LFD (Low-fat diet) or HFD (High-fat diet) for six weeks to initiate the development of obesity in the HFD animals. Thereafter, the HFD animal group were randomised to different berry supplemented diets for a further 12 weeks. Faecal samples were collected from the cages, weighed, and pooled at different time points (weeks 4, 8, 9 and 12) and analysed at the end of the study. Using quantitative real-time PCR, analysis of the bacterial phyla relative abundance in the faecal samples showed that berry supplementation with blackberry and black raspberry did not change the gut microbial population shift that was observed with either LFD or HFD. Supplementation with Concord grape showed a significant increase of the Actinobacterial populations from 2% to 8% in comparison to HFD controls which were similar in kcal/g but without anthocyanin supplementation. Blueberry and blackcurrant supplementation also showed a significant increase in the populations of obligate anaerobes Bacteroidetes from 7% to 10%-12% and Actinobacteria from 2% to 9%-15% which are some of the bacteria species beneficial to humans. Translating the anthocyanin consumption in these animal studies to the human context , the authors suggested it was equivalent to consuming 2.4 mg/kg/day or 145 mg/day of total anthocyanins for an average adult (30) which could be achieved by daily consumption of 1–2 servings of fresh anthocyanin-rich berries (31).

Similarly, Lacombe et. al. (2013) (32) examined the effect of dietary supplementation of lowbush wild blueberries (LWB) on the colonic microbial population of Sprague Dawley rats. Following control diets or LWB-supplemented diets for six weeks, analysis of the colon contents of the rats using shotgun sequencing showed a significant reduction in the relative abundance of the genera *Lactobacillus* and *Enterococcus* associated with the berry intervention. There was also a significant (p<0.05) two-fold increase in the relative abundance of Bifidobacteriaceae and Coriobacteriacaea and the phylum to which they belong, Actinobacteria, in the LWB-supplemented group.

Human clinical trials

A randomised crossover clinical trial tested the association between changes in faecal microbiota following wine interventions. Nine male participants were randomized to receive daily 275 mL of alcoholised red wine or dealcoholized red wine or 100mL of gin for 20 days. Participants' usual baseline dietary habits and pattern and lifestyle were maintained, and

additional alcoholic beverages were avoided for the duration of the study. At baseline, and following each intervention period, faecal samples were collected from participants. No significant differences in daily energy and dietary intake were observed between baseline and after each intervention. Quantification of the microbial content of faecal samples was done using quantitative real-time PCR. Results showed that in comparison to gin, both red wine and dealcoholized red wine significantly (p = 0.001) increased the faecal concentration of *Bifidobacterium, Enterococcus and Eggerthella lenta.* Although the anthocyanin content of the red wine intervention used in this study was not measured prior, results showed that the lowest to the highest changes in Bifidobacteria tertiles was associated with a higher excretion of four phenolic metabolites related to anthocyanin metabolism in participants(23).

Table 2: PICOS criteria for inclusion and exclusion of studies

Parameter	Inclusion	Exclusion
Participants	Humans, animals and cell cultures (*In vitro)*	None
Intervention	Quantified anthocyanins	Other polyphenols or flavonoids
Comparator	Negative controls or different foods/diets/nutrients	None
Outcomes	Changes in gut microbiota	Stability of anthocyanins in the gut
Study design	Randomised and non-randomised experiments	None

Table 3: Summary of studies included in the systematic review

Reference	Population/ Sample	*In vitro* / *in vivo*	Design (Intervention/control)	Anthocyanin Dosage mg/100g (reported dosage)	Method of microbiota determination	Result
Hidalgo, et al (2012) [26]	pH-controlled, stirred, batch-culture fermentation system reflective or mimicking of the distal human large intestine conditions	*In vitro*	Enocianin-commercial food colouring from *Vitis Vinifera* grape peel anthocyanin-rich extract / malvidin-3-glucoside (individual anthocyanin)	34.5 mg (345mg/L)	Fluorescent in situ hybridization (FISH)	Anthocyanins significantly enhanced the growth of *Bifidobacterium spp.* and *Lactobacillus-Enterococcus spp.* This increased proliferation was higher after enocianin treatment. p<0.005
Zhang, et al. (2016) [27]	Fresh faecal samples from 8 healthy volunteers (25-30y)	*In vitro*	Purple sweet potato anthocyanins / Fructooligosaccharide (FOS) (prebiotic)	706mg (7.06mg/g) treatment: 1% (w/v)	Fluorescent in situ hybridization (FISH)	Purple sweet potato anthocyanins induced proliferation of *Bifidobacterium* and *Lactobacillus/Enterococcus spp.* and inhibited the growth of *Bacteroides-Prevotella* and *Clostridium histolyticum.* They did not affect the total bacteria count.
Flores, G., et al (2015) [28]	Fresh faecal samples from 3 healthy volunteers	*In vitro*	Individual anthocyanins / a negative control (w/out anthocyanins)	2mg (20mg/L)	Fluorescent in situ hybridization (FISH)	Significant growth of the domain *Bacteria* and slight inhibition of the *Clostridium histolyticum* group
Overall, et al. (2017) [29]	Seventy-six 6-wk old male mice/ faecal sample (cage collected)	*In vivo* (Animal study)	Berry supplementation / High fat diet (without berry supplementation)	0.04mg (1.14mg/mouse/day)	Quantitative real-time PCR	Significant increase in obligate anaerobic bacterial and Actinobacteria population in the gut (relative abundance).

Reference	Population/ Sample	*In vitro* / *in vivo*	Design (Intervention/control)	Anthocyanin Dosage mg/100g (reported dosage)	Method of microbiota determination	Result
Lacombe, et al (2013) [32]	Nine males, three-week old Sprague-Dawley/ colon content samples (faeces)	*In vivo* (Animal study).	Blueberry-enriched diet, (AIN93+8% w/w Lowbush Wild Blueberry powder substituting for dextrose) / Control diet (AIN93) without blueberry supplementation for 6 weeks	(24.06 ± 5.2mg/day)	Microbiome shotgun sequencing	Significant increase in the relative abundance of *Actinomycetales*, and several novel genera under the family Bifidobacteriaceae and Coriobacteriaceae and significant reduction in the relative abundance of *Lactobacillus* and *Enterococcus*.
Boto-Ordóñez, et al (2014) [23]	9 adult men (45-50y)/ fresh faecal samples	*In vivo* (Randomised crossover-controlled trial (3 consecutive periods of 20 days each))	Randomised crossover-controlled trial (3 consecutive periods of 20 days each); Red wine (RW)/ dealcoholized red wine (d-RW) / gin	9.72mg in RW and d-RW/ nd in gin (272mL (26.44mg anthocyanin))	Quantitative real-time PCR	Significant change in bacteria species (*Bifidobacterium, enterococcus, and eggerthella lenta*) concentration following RW and d-RW intervention (p<0.001)

Discussion

Findings from this systematic review support the hypothesis that consumption of foods high in anthocyanins promotes the proliferation of healthy anaerobic bacterial populations, while inhibiting the pathogenic species. Specifically, the presence of anthocyanins significantly increased the microbial population of *Bifidobacterium spp.* and *Lactobacillus-Enterococcus spp.* This observation was consistent in *in vitro,* animal and human studies (23, 26, 27, 32). These gram-positive bacteria species have been shown to exert beneficial effects in the treatment of diarrhoea and other specific diseases including inflammatory bowel disease (IBD), necrotizing enterocolitis and colorectal cancer (33).

A number of factors including diet, age and antibiotics are important determinants of the gut microbiota profile, and the influence of these factors continue to change throughout the lifetime (34). The most significant of these factors is diet. In relation to anthocyanin consumption, a diet rich in fruit and vegetables has been shown to significantly alter the gut microbiota. Even though studies have observed the absence of significant increase in total microbial population when samples were incubated with anthocyanins, Zhang, et al. (2016) (27) suggested that the absence of this effect could be due to purple sweet potato anthocyanins including anthocyanin monomers not being able to affect total bacterial population that these purple sweet potato anthocyanins could enhance inhibition and proliferation of different species at similar rates. In addition Flores et al (2015) (28) attributed this to the amount of anthocyanin used in their intervention study (20mg/L) which could have been too low to exert any significant changes. Contrary to these observations, with higher concentration (200mg/L) of anthocyanins, Hidalgo et. al. (2012) (26) observed a significant (p<0.05) proliferative and inhibitive effect in microbiota population which was more evident in samples that were supplemented with a mixture of anthocyanins showing the synergistic effect of anthocyanin subclasses. Another possibility is that the observed simultaneous proliferative effect on the beneficial bacteria and inhibitory effect on harmful bacteria by anthocyanins could explain the absence of any significant change in total bacterial population as there exists a natural balance between beneficial and harmful bacteria in the gut (35).

Even though observations in *in vitro* models may not be extrapolated to *in vivo* systems, comparison of composition and concentration of anthocyanins across studies which had a significant effect on gut microbiota showed that lower concentrations with a variety of anthocyanins (Malvidin-, delphinidin-, petunidin-, peonidin- and cyanidin-glycosides) (26)

had as much effect as higher concentration with only one class of anthocyanins (cyanidins glycosides) (27). Although anthocyanins were not the highest concentration of flavonoids or polyphenols in wine (23), it is important to note that anthocyanins, not other polyphenols, was associated with the increased level of *Bifidobacteria* in faecal samples. This was due to the observed increase in microbial metabolites in urine presumably derived from anthocyanins. The anthocyanin content of all interventions used in the included studies was determined using High Performance Liquid chromatography (HPLC) coupled to different detectors. HPLC has been identified as the most reliable method of measuring total anthocyanin content as well as individual anthocyanins in foods and biological matrices in comparison to the colorimetric method that measures only total anthocyanins (36).

With these observed beneficial effects, the exact mechanism of action of anthocyanins in the gut remains unclear, Overall et. al., (2017) (29) observed an increased oxygen tension in all gut compartments associated with high-fat diets which was attenuated by supplementing the diet with berries and berry anthocyanins. As a result, they suggested anthocyanins may reduce oxygen tension in the gut lumen and therefore promote the proliferation of oxygen-sensitive bacterial population. However, in the small intestine, there exists high level of oxygen which limits bacterial growth, such that only fast growing, facultative anaerobes with the ability to bind to epithelial/mucus are believed to survive (37). This highlights the difference in microbiota composition along the lower GI tract and an important consideration in sampling.

On the issue of sampling, all the studies, but one, included in this review utilised faecal samples for the quantification of anthocyanin effects on gut microbiota. In place of faecal samples, rectal mucosal biopsy has been proposed as a better alternative in gut microbiota research in terms of assessment but not participant burden (38). Durbán et al. (2011) (39) did a comparative study on the bacterial community composition between faecal samples and rectal mucosal biopsies. Samples were collected from an un-prepped healthy population. Comparison of the two samples showed a significant difference in the bacterial diversity between faecal and rectal mucosal samples from the same participant. Another study compared healthy subjects to IBS subjects, where they observed that there was a reduction in bacterial abundance and diversity in mucosal samples in comparison to stool samples from the same participants (40). There is a possibility that significant differences exist in the microbial community across the six major subdivisions of the human colon (cecum, ascending colon, transverse colon, descending colon, sigmoid colon, and rectum). However,

the degree to which composition and functions differ remain unclear. Studying the microbial diversity of the human colon, Eckburg et. al. (2005) (10) observed significant inter-individual variability and differences between faecal and mucosa community composition. This discrepancy highlights the importance of sampling sites and raises the suggestion that rectal mucosal biopsy samples are more appropriate than faecal samples and should be used instead or together with faecal samples (17). Regardless, faecal samples are still the preferred over rectal mucosa biopsy due to ease of collection and being less invasive.

Another important consideration in gut microbiota research is the methods of determination and quantification. Although bacterial culture, which was the gold standard in the past, is inexpensive, it produces a limited representation of the gut microbiota diversity and results in undervaluation of real changes. Culture independent techniques have become popular and are now the most commonly used techniques (41). Most of the different culture independent techniques are based on the analysis of the 16rRNA gene. The 16rRNA genes are highly protected across bacterial species. They also have distinctive characteristics that allows for identification of different species. (17). Studies included in this review employed different techniques including FISH, qPCR and shotgun sequencing in the determination and quantification of the gut microbiota. These techniques have different pros and cons. The qPCR for example, although fast, is unable to identify unknown species and has an amplification bias in that the primer set used in multi-template PCR is required to have a shared sequence across the targets. As often is the case, this shared sequence is absent in the targets. When a primer has one incongruity with some targets, the amplification efficiency is significantly reduced and as a result, a large bias in the amplification will occur (42). Even though the FISH technique has no PCR bias, it is dependent on probe sequences which mean that it is also unable to identify unknown species. The shotgun sequencing on the other hand, although more adequate than the aforementioned techniques, is expensive and analysis of data involves a complex software (17). The major limitation of the PCR and FISH methods in comparison to the shotgun sequencing is their inability to identify unknown species. This limitation does not seem to have affected study results as changes in bacteria species were measured with already known species hence addressing this limitation. The availability of various methods requires deciding the suitability of a given methodology in gut microbiota research. This decision will be dependent on the depth of analysis. So far, the shotgun sequencing techniques produces the most potent data including projections of microbiota function (17). Although a comparative study of 16S amplicon and shotgun sequencing on

water samples found that less than 50% of phyla identified via 16S amplicon sequencing were recovered from shotgun sequencing while also identifying ~27% more families (43).

A notable strength of this systematic review is the side by side comparison of different methods of microbiota determination as well as effects of anthocyanins. At the time of writing this article, to our knowledge, this systematic review appears to be the first to determine the effect of anthocyanins on gut microbiota as well as comparing different methods of microbiota determination. Another possible strength is the specific focus on anthocyanins and their effect on gut microbiota. The synergistic effect of compounds in food (nutrients) presents some difficulty in clearly defining causal association in epidemiological research. Although nutrients are not consumed in isolation, determining the specific effects of particular nutrients is an important aspect of nutrition research.

As an emerging area of research in the last decade, there are very few *in vitro* and animal studies and even fewer clinical trials that have been carried out. Consequently, there are limitations in the comparison of results and generalisation of conclusions due in part to the diverse sources of anthocyanins, methods of gut microbiota determination and quantification as well as the possibility of synergistic effects of anthocyanins and/with other phytochemicals and nutrients present in the food sources resulting in the observed benefits. Another notable limitation of this review is the different control diets/samples used which restricts the generalisation of results. Although a limitation, it is important to note that these controlled samples/diets all had in common the absence of anthocyanins demonstrating the modulatory effects of these natural plant pigments.

It is imperative to advance the understanding of the direct or indirect beneficial effects of anthocyanins on bacterial growth through research. It would also be noteworthy to measure concurrently these observed effects and identify further anthocyanin metabolites as well as other health effects, for example vascular function and cognition to further understand complex *in vivo* processes such as the gut-brain axis relationship.

Conclusion

In conclusion, research on the gut (microbiota) as a metabolic organ is still emerging and characterisation of bacteria species present in the gut is ongoing. Results from this review observed beneficial effects such as significant proliferative effect on *Bifidobacterium spp.*,

known for their wide use in probiotics and for the treatment of Irritable Bowel Syndrome and inhibition of *Clostridium histolyticum*, which have been shown to be pathogenic in humans Research on the possible effect of anthocyanins on gut microbiota population is still in its early stages and conclusions and generalisations cannot be made due to the limited evidence base and varied techniques employed in studies as well as differences in anthocyanin sources, composition, digestive stability, metabolism, biotransformation of anthocyanins in either a food matrix or supplement isolated. This also makes it difficult to understand or elucidate the exact mechanism of anthocyanins may exert these effects. Therefore, the complete effect and exact mode action of anthocyanins on gut microbiota population needs more research clarification, in part by conducting more well-designed human clinical trials, reaching a consensus on anthocyanin dose and form as well as using a uniform approach to control diets. Determination of key techniques to measure (44) and confirm these findings is also vital in this field.

Transparency declaration

The lead author on behalf of the authorship team affirms that this manuscript is an honest, accurate and transparent account of the study being reported and that there has been no omission of any important aspects. The reporting of this study was done according to the recommendations of the PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) statement and checklist (21).

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