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Review

Review

The potential of berries to serve as selective inhibitors of pathogens and promoters of beneficial microorganisms

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Abstract

OBJECTIVES: Berries are distinct from other foods because of their unique compounds with bioprotective effects and antimicrobial/prebiotic properties. With new knowledge of how these unique phytochemicals differentially affect microbial communities, inhibit foodborne pathogens, and conserve beneficial species, the health claims associated with berries can be further substantiated. This review explores components of berries that have antimicrobial or prebiotic properties and incorporates new knowledge gained from both *in vitro* and *in vivo* experiments.
CONCLUSIONS: With the continued research efforts, antimicrobials and prebiotics derived from berries may provide an alternative to synthetic preservatives and antibiotics in addition to providing health benefits to consumers. Berries could be applied to food products or as dietary interventions through elucidating which compounds have antimicrobial properties and how pH and nutrient condition impact their efficacy. In addition, these compounds can be added to foods with beneficial microorganisms with minimal impact on their probiotic viability.

Key words: berries; probiotics; pathogens.

Introduction

Health-conscious consumers wish to know how dietary interventions can protect them from a wide range of maladies such as foodborne illness, metabolic syndrome, urinary tract infections (UTIs), and digestive disorders. However, this burgeoning market for natural remedies high in antioxidant requires reliable information on their safety and efficacy. Today, natural products from berries are being investigated as a new potential arsenal of antimicrobials and prebiotics because of their ability to selectively inhibit enteric pathogens while promoting beneficial microorganisms (Puupponen-Pimia *et al*., 2005a; Molan *et al*., 2009; Lacombe *et al*., 2012a).

Phytochemicals in berries have interactions and synergistic effects against pathogenic bacteria, however the exact mechanism of action remains unknown (Nohynek *et al*., 2006; Alakomi *et al*., 2007; Heinonen, 2007; Cesoniene *et al*., 2009). On the laboratory scale, berries have demonstrated inhibitory effects against enteric pathogens with regards to structural damage (Heinonen, 2007; Wu *et al*., 2009; Lacombe *et al*., 2010; Caillet *et al*., 2012; Lacombe, 2012b), gene expression (Wu *et al*., 2009), metabolism (Apostolidis *et al*., 2008), and cell membrane synthesis (Wu *et al*., 2008). In humans, berry extracts have exhibited various antimicrobial activities including the prevention of microbial adhesion to the urinary tract (Liu *et al*., 2008), the reduction of biofilm production in humans (Lee *et al*., 2009), and shift in colonic gut microbiota (Heinonen, 2007; Wu *et al*., 2009; Lacombe *et al*., 2010; Molan *et al*., 2010; Caillet *et al*., 2012; Lacombe, 2012a; Lacombe *et al*., 2012a; Vendrame *et al*., 2013), gene expression, metabolism, and cell membrane synthesis (Wu *et al*., 2008).

Research on functional foods that can promote gut health and beneficial microbiota has become a topic of interest in the field of preventive medicine. Berries are excellent sources of nutrients, fibre, and polyphenols, which may have a direct effect on microbial ecosystems (Wu and Prior, 2005; Del Bo *et al*., 2010a; Nile and Park, 2014). The chemistry of berry constituents can directly influence their bioavailability, metabolism, and biological effects *in vivo* (Prior *et al*., 2010; Nile and Park, 2014). The interaction of berries with microorganisms is important to study because the enzymatic transformations that occur with phenolics *in situ* can effect human physiology (Seeram *et al*., 2004). Dietary enrichment with berries demonstrated prebiotic capabilities with impacts on gut microbial population dynamics and gastrointestinal tract (GIT) health (Molan *et al*., 2010; Lacombe *et al*., 2013b; Vendrame *et al*., 2013). Impacts on intestinal microflora can have downstream effects in the body, including attenuation of indicators of metabolic syndrome and inflammation, although little is known about the mechanism of this process (Del Bo *et al*., 2010b; Vendrame *et al*., 2011).

The aims of this review are to discuss which components isolated from berries have antimicrobial and/or prebiotic properties, which microorganisms are affected, and examine their potential mode of action and application both *in vitro* and *in vivo*. The physiological state of a bacterium is an important consideration when studying its response to berry constituents. This article reviews the various definitions of injury and stress, sublethal injury of bacteria, and stress adaptation *in vitro* and how it applies *in vivo*.

Berries as Antimicrobials Against Pathogens

Extensive research has demonstrated berries from North and South America, Europe, and Asia have antimicrobial properties, with the consensus that inhibitory effects come from their antioxidant compounds (Puupponen-Pimia *et al*., 2001; Zheng and Wang, 2003). In nature, plants develop these compounds, called phytoalexins, to protect themselves against environmental threats and parasites (Prior *et al*., 1998). Many food processors and supplement manufacturers wish to utilize these qualities to create enhanced consumer products. To reduce health hazards and economic losses due to pathogenic microorganisms, it is necessary to define which compounds from the multitudes of berry species have antimicrobial properties, and which pathogens are susceptible (Nohynek *et al*., 2006; Heinonen, 2007; Badjakov *et al*., 2008; Cesoniene *et al*., 2009).

Foodborne pathogens

Important foodborne pathogens that were inhibited by berries include *Escherichia coli* O157:H7, *Listeria monocytogenes*, *Salmonella typhimurium*, *Bacillus cereus*, *Enterococcus faecalis*, *Helicobacter pylori*, *Pseudomonas aeruginosa*, *Campylobacter jejuni*, and *Staphylococcus aureus* (Apostolidis *et al*., 2008; Wu *et al*., 2008; Viskelis *et al*., 2009; Caillet *et al*., 2012; Lacombe, 2012b; Salaheen *et al*., 2014) (Tables 1 and 2). *In vitro* assays can provide important information about the potential antimicrobial potential of berries. Multiple assays are often used to determine which compounds have antimicrobial properties and the effective dosage. Agar diffusion assays are useful in the initial screening potential compound, but cannot provide a definitive minimum inhibitory concentration (MIC) or log reduction values. Liquid culture assays are advantageous in that they can provide both viable cells counts and absorbance data necessary to create growth curves for determining MICs. However, they present limitation if the target compound is not soluble the nutrient matrix and the colour of many berry compounds can interfere with absorbance readings. Berries belonging to the genus *Rubus* (cloudberry, blackberry, and raspberry) and *Fragaria* (strawberry) inhibited *S. typhimurium* (Puupponen-Pimia *et al*., 2001) at a concentration of 1 mg/ml on agar and in liquid culture over 24 h. Blackberry juice (10 per cent v/v) demonstrated a 2–4 log CFU/ml reduction in numbers of *E. coli* O157:H7, *S. typhimurium*, and *L. monocytogenes* in both skim and whole milk (Yang *et al*., 2014).

The genus *Vaccinium* contains berries that have a long history of therapeutic use in the USA, Europe, and Asia (Burdulis *et al*., 2009; Park *et al*., 2011; Lacombe *et al*., 2012a). Multiple cultivars of highbush blueberries (*Vaccinium corymbosum*), lowbush blueberries (*Vaccinium angustifolium*), and billberry (*Vaccinium myrtillus*) demonstrated

Table 1. Recent research pertaining to the antimicrobial properties of *Vaccinium* berries. BHI, brain heart infusion broth; TSB, tryptic soy broth.

Berry/fruit	Target bacteria	Method	Effective dose
Cranberry	*Pseudomonas aeruginosa*, *Listeria monocytogenes*, *Escherichia coli* O157:H7, *Salmonella typhimurium*	Juice concentrate in TSB inoculated with 6 $\log_{10}$ CFU/ml (Caillet *et al*., 2012)	66.5 µg/ml*
	L. monocytogenes, *E. coli* O157:H7, *S. typhimurium*	Juice concentrate on agar disc diffusion (Viskelis *et al*., 2009)	50 µl
	L. monocytogenes, *E. coli* O157:H7, *S. typhimurium*	Pressed powder on agar disc diffusion (Cesoniene *et al*., 2009)	224 mg/g
	L. monocytogenes, *E. coli* O157:H7, *S. typhimurium*, *Staphylococcus aureus*	Juice concentrate in BHI (Wu *et al*., 2008)	100 µl/ml**
Blueberry	*L. monocytogenes*, *S. enteritidis*	Blueberry phenolics extracts (100% v/v ethanol) added to 3 log CFU in TSB (Park *et al*., 2011)	24 ppm*
	S. typhimurium, *Campylobacter jejuni*, *L. monocytogenes*, *E. coli* O157:H7	Blueberry juice extract from whole berries inoculated with 8 log CFU/ml (Biswas *et al*., 2012)	100% v/v berry extract
	S. aureus, *L. innoca*, *Enterococcus faecalis*, *Bacillus cereus*, *P. aeruginosa*	Blueberry fruit and leaf extracts (Silva *et al*., 2013)	50 mg/ml
	L. monocytogenes, *S. enteritidis*	Blueberry extracts from four cultivars in TSB (Shen *et al*., 2014)	900 mg/ml

Effective dose is the concentration in which significant ($P < 0.05$) $\log_{10}$ reduction was achieved, usually reported as: *Minimum inhibitory concentration after 24 h at 37°C and **Significant growth reduction after 5 day at 21 h and 4°C.

Table 2. Recent research pertaining to the antimicrobial properties of *Rubus* and Fragaria berries. dimethyl sulphoxide DMSO.

Berry/fruit	Target bacteria	Method	Effective dose
Blackberry	*Helicobacter pylori*	Blackberry leaf extract in *Brucella* broth–bovine serum with 4% DMSO (Martini *et al*., 2009)	134–270 µg/ml
	Porphyromonas gingivalis, *Fusobacterium nucleatum*, *Streptococcus mutans*	Blackberry extract in liquid culture (González *et al*., 2012)	350–1400 µg/ml
	Escherichia coli O157:H7, *Salmonella typhimurium*, *Listeria monocytogenes*	Steam-pressed blackberry juice in liquid culture (Yang *et al*., 2014)	10% v/v juice
Raspberry	*S. typhimurium*	Solid-phase extract with sugars removed, in liquid culture (Puupponen-Pimia *et al*., 2005a)	1 mg/ml
	Bacillus subtilis, *Staphylococcus aureus*	Solid-phase extract with sugars removed, placed on agar disc (Nohynek *et al*., 2009)	500 µg
Strawberry	*S. typhimurium*	Solid-phase extract with sugars removed, in liquid culture (Puupponen-Pimia *et al*., 2005a)	1 mg/ml
	B. cereus, *Campylobacter jejuni*, *Clostridium perfringes*, *H. pylori*	Solid-phase extract with sugars removed, in liquid culture (Heinonen *et al*., 2009)	1 mg/ml

Effective dose is the concentration in which significant ($P < 0.05$) $\log_{10}$ reduction was achieved.

antimicrobial properties against foodborne pathogens and the resulting inhibition was independent of cultivar or whether the whole berry or just the skin was utilized (Burdulis *et al*., 2009; Park *et al*., 2011; Lacombe *et al*., 2012a). The American cranberry (*Vaccinium macrocarpon*) has demonstrated effectiveness against both Gram-negative and Gram-positive foodborne pathogens, in multiple nutrient conditions (Wu *et al*., 2008; Lacombe et al., 2012a, 2012b). Wu *et al*. (2008) observed a reduction of 8 log CFU/ml ($P < 0.05$) in *E. coli* O157:H7 compared to the control in nutrient-rich broth after 1 day of cranberry treatment (100 µl/ml) and bacteria were below detectable limits after 5 days of sampling. *Listeria monocytogenes* in nutrient-rich broth treated with cranberry extracts exhibited 3–5.3 log CFU/ml reduction in count compared to the control after 1 day (Wu *et al*., 2008).

The MICs of berry extracts varies based upon extracting method, microbial quantification method, and nutrient matrix. The quantification of polyphenols can differ based upon the assay and standardization of the assay, therefore making effective doses difficult to compare. In addition, some researchers used fresh berries as starting materials to derive their test compounds while others derived their compounds from powders (Prior *et al*., 1998). Each method has their advantage and disadvantages. Constituents derived from fresh berries may represent what is typically found in the wild; however, constituents are more subject to extrinsic factors, such as season and geographic location. Powdered berries require more processing, but can also better represent a composite of multiple harvests and are easy to standardize. To investigate the antimicrobial properties of berries, many researchers have extracted the phenolic constituents using various solvents such as water, ethanol, ethyl acetate, acetone, and methanol (Burdulis *et al*., 2009; Park *et al*., 2011; Biswas *et al*., 2012; Caillet *et al*., 2012; Lacombe *et al*., 2012a). The partial purification of these compounds can increase their antimicrobial activities and provide mechanistic insight into which components are most effective (Lacombe *et al*., 2010; Caillet *et al*., 2012). Anthocyanins plus proanthocyanidins demonstrated the lowest MICs followed by total extract, monomeric phenolic acid, anthocyanins, and proanthocyanidins. *Listeria monocytogenes* was the most susceptible to fraction treatment, followed by *E. coli* O157:H7, and *S. typhimurium* (Lacombe et al., 2012a, 2012b).

The degree of hydroxylation is reflective of the hydrophobicity of the compound and affects the antimicrobial activity of phytochemical compounds. The flavonol myricetin, as a pure compound, clearly inhibited the growth of human gastrointestinal pathogens; however, quercetin and kaempferol are more lipophilic in nature and demonstrated no inhibition (Puupponen-Pimia *et al*., 2001). Ellagitannins and anthocyanins could be components in cloudberries, raspberries, and strawberries causing the inhibition against *Salmonella* species because of its partial hydrophobicity (Puupponen-Pimia *et al*., 2001, 2005). Ellagic acid has been reported to exhibit a dose-dependent inhibitory effect on *H. pylori* isolated from peptic ulcer patients and *Vibrio cholerae*, *Shigella dysenteriae*, and *Campylobacter* spp. (Scalbert, 1991; Silva *et al*., 1997).

Berries are one of the few natural products that have demonstrated efficacy against foodborne viruses and parasites. Cranberry proanthocyanidins have antiviral properties against common causes of viral gastroenteritis (Su *et al*., 2010). Recent studies have demonstrated a 50 per cent reduction in total titre within the first 10 min of feline calicivirus, murine norovirus, bacteriophage MS2, and bacteriophage f-X174 with proanthocyanidins, with treatment (Su *et al*., 2010). However, these studies were done with viral surrogates due the difficulties in culturing human norovirus. Blueberry extracts demonstrated the inhibition of *Giardia dilodclialis* during its infectious life cycle by causing morphological distortion, and reducing the viability of the trophozoites (Anthony *et al*., 2007). Blueberry treatments increased spontaneous excystation of *Cryptosporidium parvum* oocysts and could be considered for use as oral supplements to reduce excretion of infectious oocysts. In addition, extracts could cause the modification of parasite morphology and truncation of the life cycle leading to the reduction/inhibition of attachment to the host enterocytes (Anthony *et al*., 2007).

Non-foodborne pathogens

UTIs are one of the more prevalent maladies that afflict women and most over-the-counter supplements for UTI either contain berry extract or enriched berry constituents in their formulations (Geerlings, 2011). The growing popularity in berry-derived supplements for UTIs stems from increased resistance rates for common antibiotic treatments (Geerlings, 2011). Recent studies have demonstrated that resistance to ciprofloxacin and norfloxacin, in urinary *E. coli* isolates, increased from 8 per cent at baseline to 23 per cent after 12 months of prophylaxis (Geerlings, 2011). Studies have demonstrated the anti-adhesional aspects of berries against uropathogenic *E. coli*. Much of the work surrounding the anti-adhesive activities of high-molecular-weight proanthocyanidins has focused on those with type-A linkages (Schmidt *et al*., 2004; Johnson *et al*., 2008; Lin *et al*., 2011). The degree of polymerization of the proanthocyanidins

is thought to be the main characteristic contributing to their anti-adhesional properties (Schmidt *et al.*, 2004). However, several other compounds, including organic acids, other polyphenolic compounds, and flavonol glycosides, have also been indicated as anti-adhesive compounds (Lin *et al.*, 2011). The hydrolyzable and condensed tannins from *Vaccinium* berries contain structures similar to those involved in the binding of bacteria to the surface of bladder and kidney cells (Lin *et al.*, 2011). Cranberries can inhibit the adherence of P-fimbriae of uropathogenic *E. coli* to the uroepithelial cell receptors (Liu *et al.*, 2008). Anthocyanins and proanthocyanidins from cranberries have the ability to raise the Gibbs free energy of association between uropathogenic *E. coli* and uroepithelial cells, therefore making adhesion thermodynamically unfavourable (Liu *et al.*, 2008). However, cranberries- and proanthocyanidins-enriched supplements are not sufficient for the treatment of severe urinary track infections and antibiotics are still highly recommended (Geerlings, 2011).

Cranberry proanthocyanidins have shown promise for treating oral infections, especially dental caries, and can inhibit the formation of biofilms by cariogenic bacteria (Eydelnant and Tufenkji 2008; Feghali *et al.*, 2012; González *et al.*, 2012). A preliminary human trial demonstrated that the daily use (6 weeks) of cranberry-containing mouthwash reduced *Streptococcus mutans* counts in saliva (Feghali *et al.*, 2012). The ability of cranberry proanthocyanidins to prevent sucrose-dependent biofilm formation by *S. mutans* has been attributed to their ability to inhibit the activity and production of fructosyltransferase and glucosyltransferase, which are involved in the production of exopolysaccharides (Feghali *et al.*, 2012). In addition, inhibition of non-sucrose-dependent biofilm formation has been attributed to the ability of cranberry proanthocyanidins to prevent bacterial co-aggregation, reduced bacterial hydrophobicity, and altered cell surface molecules (Feghali *et al.*, 2012).

Antimicrobial Mechanism of Functional Compounds From Berries

The current reviews and investigations pertaining to microbial inactivation by plant compounds do not provide one definitive mechanism of action, but suggest a concerted effort involving multiple pathways based upon the environmental conditions and the particular microorganisms involved (Friedman *et al.*, 2003; Puupponen-Pimia *et al.*, 2005b; Alakomi *et al.*, 2007; Kwon *et al.*, 2007; Apostolidis *et al.*, 2008). The mechanism of microbial inhibition by berry compounds is considered to be an accumulation of direct and indirect actions (Gyawali and Ibrahim, 2012). Direct actions are primarily considered to be the phytochemical reactions with the cell membrane causing inactivation of essential cellular enzymes. Indirect actions are considered to be phytochemical effects on nutrient availability or genomic expression resulting in impaired metabolism and function of the target microorganism. This information is necessary for formulation and optimization of berry products as antimicrobials or adjuvants in medicinal therapy.

The effect of low pH of inhibition

Acid stress can occur during the fermentation of foods or by the addition of preservatives such as organic acids (Wu *et al.*, 2008). The effect of low pH on *E. coli* O157:H7 was tested using a combination of citric, malic, and quinic acid, organic acids commonly found in cranberries (Wu *et al.*, 2008). The acid solutions were buffered at pH 4.7 and 3.5 and these solutions were then compared to cranberry concentrates at the same pH (25 and 100 µl/ml of cranberry extract). Treatment with cranberry extract exhibited a stronger effect than pH-buffered solutions, with an approximate increased reduction of 1 log CFU/ml (Wu *et al.*, 2008). Neutralization of the fractional components demonstrated that organic acids rely strictly on a low pH mechanism; however, the other components, namely, monomeric phenolics, anthocyanins, and proanthocyanidins, still retained their antimicrobial effects (Lacombe et al., 2010, 2012a, 2012b, 2013a, 2013b).

Inhibition under acidic conditions can be studied at the molecular level by looking at the level of stationary phase and stringent response regulatory gene transcripts. Much of a microorganism's ability to resist environmental stress is controlled by gene transcription (Allen *et al.*, 2004; Price *et al.*, 2004), which could be altered by berry components (Wu *et al.*, 2009). Looking at the environmental stress response network provides insights into the global effect that berry extracts have on microorganisms. E. coli treated with 5% v/v cranberry juice demonstrated the down regulation of cyclopropane fatty acyl phospholipid synthase (cfa), an enzyme related to cell membrane synthesis. In addition, acid-inducible genes such as hypothetical protein (*hdeA*) and outer membrane proteins *ompC*, *osmY*, and *slp* were down-regulated (Wu *et al.*, 2008), indicating a stress-induced response by the pathogen. The down-regulation of genes related to cell wall synthesis may be an adaptation to restrict the surface area available to cranberry inhibition. It has been proposed that the down-regulation of *ompC* and *ompF* is an important defence mechanism in *E. coli* O157:H7, by preventing the acidification of the cytoplasm (Allen *et al.*, 2004).

Berry effect on energy transduction in bacterial cell membranes

Pathogens can recover from sublethal injury under favourable conditions; therefore, it is important to investigate the effect of berry constituents on microbial physiology. Most injured cells have damaged permeability barriers (surface structures and the cytoplasmic membrane) that render them susceptible to many selective agents or antimicrobials. Membrane damage was visualized under transmission electron microscopy for treatments of 5 per cent v/v of cranberry and lowbush blueberry fraction against *E. coli* O157:H7. All berry treatments demonstrated aggregation of the cytoplasm, while the control cells remained intact. Membrane damage ranged from localized membrane damage with organic acid, monomeric phenolic acid, and proanthocyanidins to a lack of a distinguishable morphology in the presence of anthocyanins plus proanthocyanidins and anthocyanins, for both blueberries and cranberries (Lacombe *et al.*, 2012b). Under the fluorescence staining of SYTO9 and propidium iodine, proanthocyanidins from cranberries and blueberries (5 per cent v/v) resulted in the highest membrane permeability in *E. coli* O157:H7, followed by anthocyanins plus proanthocyanidins, anthocyanins, total phenols, and monomeric phenolic acids, respectively (Lacombe *et al.*, 2012b). However, proanthocyanidins demonstrated the highest recovery on MacConkey Sorbitol agar, followed by total phenolics, anthocyanins, monomeric phenolic acids, and anthocyanins plus proanthocyanidins. Therefore, combinatorial treatments of berry constituents may be necessary to ensure the successful inhibition of pathogens.

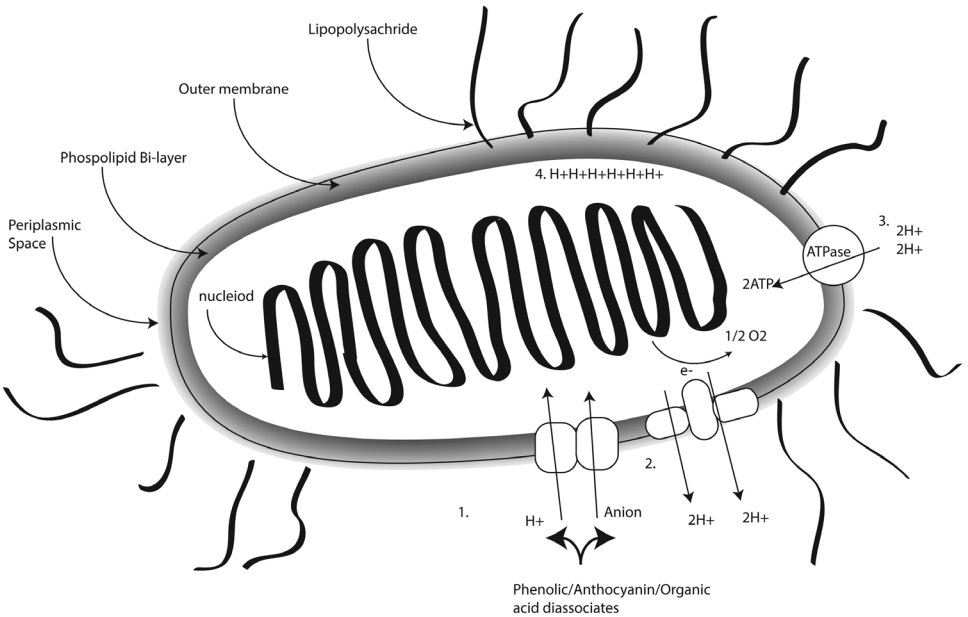
The physiology and structure of bacterial outer membranes is considered to be the major components in their ability to resist chemical stress (Nohynek *et al.*, 2006). The outer membrane surrounds the cell to create a hydrophilic surface, which protects against low pH, bile salts, digestive enzymes, and other antimicrobial obstacles found in the GIT (Nohynek *et al.*, 2006). Increases

in membrane permeability resulted in the disruption of outer membrane structures necessary for metabolism and the inhibition of ATP production and causes the leakage of protons and potassium ions (Gill and Holley, 2006). Berry polyphenols, such as ellagitannins, anthocyanins, and proanthocyanidins, demonstrated a synergistic effect on membrane damage by increasing the permeability of *S. typhimurium* (Nohynek *et al.*, 2006). The destabilization effect of these components decreased with the addition of Mg^{2+}. A similar effect was observed with ethylenediaminetetraacetic acid, a chelating agent that disintegrates membranes by removing divalent cations (Ca^{2+} and Mg^{2+}) that stabilize the membrane (Nohynek *et al.*, 2006).

Polyphenols are capable of hydrophobic interactions and hydrogen bonding with membrane proteins (Apostolidis *et al.*, 2008). They can sequester ions required for protein stability (Guo *et al.*, 2007) and donate or accept electrons along the membrane interface (Kwon *et al.*, 2007). A large number of phenolic compounds act specifically by interfering with one of the basic cellular functions, namely, energy transduction. In energy-transducing membranes, phenolics may inhibit electron flow by binding directly to specific components of the electron transfer chain and, even more importantly, they can dissipate the electrochemical proton gradient, short-circuiting the chemiosmotic proton cycle and preventing ATP synthesis (Escher *et al.*, 1999). The phytochemicals isolated from berries may have antimicrobial properties because they have the unique ability to donate protons, which cause hyper acidification at the plasma membrane and also sequester electrons from the respiration process (Lacombe *et al.*, 2012b). In the case of antioxidants such as phenolic compounds and other weak organic acids, the chemiosmotic theory can be broken down into three steps (Figure 1). First uncoupling is viewed as a shuttle mechanism, in which charged species migrate across the membrane driven by the electrical potential. Protons are then taken up from the aqueous phase, and neutral phenols diffuse back across the membrane driven by the concentration gradient of phenols that has been built up by the migration processes (Escher *et al.*, 1999). This intrinsic uncoupling activity has been correlated with the antimicrobial capability of the phenolics (Escher *et al.*, 1999), and this effect is contingent on the environment; at acidic pH, the equilibrium may change due to proton gradients, and, at neutral pH, REDOX uncoupling can interfere with electrical gradients (Kwon *et al.*, 2007). A buffered solution of HCl at pH 3 provided the same membrane hyperpolarization in *E. coli* O157:H7 as organic acids (pH 4.8) and proanthocyanidins (pH 6) (Lacombe *et al.*, 2012b). In addition, the neutralization of berry constituents demonstrated membrane depolarization of *E. coli* O157:H7. This mechanism explains how neutral and acidic polyphenols can destabilize membranes because they can both interact at the membrane as charged species and uncouplers of oxidative phosphorylation. This was confirmed by testing the membrane potential of *E. coli* O157:H7 with Bis(1,3-dibutylbarbituric acid) trimethine oxonol (DiBAC4), a negatively charged, lipophilic distributional probe for measuring membrane potential (Lacombe *et al.*, 2012b). After treatment at native and neutral pH, berry extracts demonstrated membrane hyperpolarization at their native pH, while monomeric phenolic, anthocyanins plus proanthocyanidins, and proanthocyanidins demonstrated membrane depolarization at neutral pH (Lacombe *et al.*, 2012b).

Berry Role in Promoting Beneficial Species

When considering antimicrobials as preventative measures against foodborne diseases and other ailments, it is important to consider possible impacts upon beneficial microorganisms. Recent studies demonstrated that extracts from berries have antimicrobial effects against foodborne pathogens while conserving probiotic species. Phenolic extracts of eight berries commonly consumed in Finland inhibited the growth of selected Gram-negative bacteria and were not active against Gram-positive probiotic lactic acid bacteria (LAB—Puupponen-Pimia *et al.*, 2005b; Lacombe *et al.*, 2012a). Organic acids, monomeric phenolics, anthocyanins, and proanthocyanidins,



1. Anion Model of Toxicity: Phenolic oraganic acid dissassociates and anions/H+ accumulate in the cell (Van Immersal et al., 2006)
2. Chemi-Osmotic Model: Electron chain attempt to create H+ gradient and is disrupted (Kwon et al., 2005; Escher 1998)
3. To balance the energy deficit H+ is pumped in to generate ATP (Kwon et al., 2005
4. Hyperacidification of the cytosol (Kwon et al., 2005)

Figure 1. Schematic diagram of chemiosmotic uncoupling caused by berry constituents.

derived from cranberries and blueberries, impacted the growth and viability of *S. typhimurium*, *E. coli* O157:H7, and *C. jejuni* strains without affecting the growth of probiotic species such as *Bifidobacteria bifidum* and *Lactobacillus bulgaris* (Biswas *et al.*, 2012). Pathogens such as *L. monocytogenes*, *S. typhimurium*, and *E. coli* O157:H7 are twice to four times as susceptible to treatments with cranberry and lowbush blueberry phytochemical constituents when compared to probiotic *Lactobacillus rhamnosus*. In addition, Puupponen-Pimia *et al.* (2001) demonstrated that *S. typhimurium* and *E. coli* CM 871 were strongly inhibited while the growth of *Lactobacillus acidophilus* and *Bifidobacterium lactis* strains was unaffected by the presence of billberry. Blackberry juice inhibited *L. monocytogenes*, *S. typhimurium*, *C. jejuni*, and *E. coli* O157:H7 (Biswas *et al.*, 2012) but had no inhibitory effect on *Lactobacillus casei*, *L. rhamnosus*, and *Lactobacillus plantarum*.

While the reason why most LAB demonstrate more tolerance to berry constituents, there may be some evidence in the genome that explain what components of membrane physiology are advantageous. Most LAB grow in the presence of antimicrobials such as polyphenols, acids, and ethanol (Behr *et al.*, 2006; Torres *et al.*, 2007). To counteract stress, LAB have evolved several defence strategies, namely, reduced generation of oxidizing molecules during metabolism, enzymatic or non-enzymatic detoxification oxidizers, and repair of damaged cell components (Behr *et al.*, 2010). The bile-salt hydrolase gene has recently been proposed to be an intestinal niche-specific molecular marker for lactobacilli and has been credited for the species' success in colonizing the GIT (Pfeiler *et al.*, 2009). Approximately 17 per cent of all proteins encoded by the LAB genome are involved in biosynthesis and function of the cytoplasmic membrane (Behr *et al.*, 2006). Wine LABs increase their membrane fluidity to counteract damage caused by ethanol and native phenolics (Torres *et al.*, 2007). In addition, LABs rely heavily on energy-transducing systems to survive in constantly changing and often-hostile environments (Torres *et al.*, 2007). Most of these metabolic energy-generating systems contribute to the prevention of a lethal decrease of the internal cytoplasmic pH. Non-enzymatic detoxification mechanism of oxidative molecules in LAB is connected to their extraordinary high levels of intracellular manganese and can cycle between the oxidation states 2 and 3 altering the redox potential. Mn2 can act as a scavenger of toxic oxygen species (Behr *et al.*, 2010), whereas Mn3 and its complexes are able to oxidize different substrates and contribute to the overall oxidative potential (Behr *et al.*, 2010).

Evidence of the prebiotic capabilities of berries

One of the major advances in the field of GIT microbiology is the realization of the effect diet has on the composition of the community and how it affects the health of the host (Claus *et al.*, 2011; Scott *et al.*, 2011). *In vitro* human fecal batch cultures have demonstrated the enrichment of *Lactobacillus* and *Bifidobacteria* with the addition of 1 g/l gallic acid and 200 mg/l of anthocyanins (Hidalgo *et al.*, 2012). Antioxidant effects of polyphenols and other nutrients observed *in vitro* are not completely transferable to health effects observed *in vivo*, therefore it is important to use *in vivo* models to understand how they are metabolized (Branning *et al.*, 2009; Hakansson *et al.*, 2009; Jacobs *et al.*, 2009; van Duynhoven *et al.*, 2009; Del Bo *et al.*, 2010a; Kemperman *et al.*, 2010; Molan *et al.*, 2010; van Dorsten *et al.*, 2010; Vendrame *et al.*, 2011). In humans, the intestinal absorption of dietary polyphenols is often slow and largely incomplete; however, up to 85 per cent of anthocyanins enter the colon intact and can be used as substrates for microbial metabolism (Kahle *et al.*, 2006). The microbial role in the metabolism of ingested phenolic compounds in not well understood, but recent evidence suggests that bacterial metabolism increases the availability of essential nutrients and promotes detoxification (Kahle *et al.*, 2006). New metabolites like gallic, syringic, and homogentisic acids appeared due to bacterial enzymatic action on anthocyanins; the former have been described as being more bioavailable or bioactive than the original molecule (Hidalgo *et al.*, 2012).

In vivo research in humans and rats demonstrated that berry-enriched diets increased *Bifidobacteria* species after dietary treatment and lowered concentrations of pro-inflammatory markers (Molan et al., 2009, 2010; Vendrame *et al.*, 2011). Recent studies using the Sprague-Dawley (SD) rat model fed polyphenols extracted from blueberry and blackberry by gavage and demonstrated an increase in *Lactobacillus* and *Bifidobacteria* in the gut (Molan et al., 2009, 2010). A metagenomic study demonstrated that the genetic signature in the colon of SD rats fed a diet enriched in lowbush wild blueberries shifted towards greater biodiversity (Lacombe *et al.*, 2013b). Hierarchical analysis showed significant reduction in the relative abundance of the genes *Lactobacillus* and *Enterococcus* after 6 weeks of wild blueberry intake. Genetic signatures associated with phylum Actinobacteria, the order Actinomycetales, and several novel genera under the family Bifidobacteriaceae and Coriobacteriaceae were also in higher abundance in SD rat fed blueberries. The same study documented a 20 per cent increase in xenobiotic degradation and a two-fold increase in benzoate degradation in the proximal colon of rats fed an 8 per cent blueberry diet (Lacombe *et al.*, 2013b). The genome sequence of *Bifidobacterium longum* has a large number of predicted proteins (more than 8 per cent) related to the catabolism of non-digestible plant polymers, including enzymes involved in the degradation of complex polysaccharides and xenobiotics (Lacombe *et al.*, 2013b). Cleavage of the glycosidic bond in the anthocyanin structure is proposed as the first step in bacterial anthocyanin bioconversion, involving β-glucosidase activity (Kahle *et al.*, 2006; Kemperman *et al.*, 2010). The intestinal microbiota is considered an appropriate target for therapeutic interventions in the form of dietary supplements and/or food ingredients, with the specific aim of influencing community composition and restoring functional capacity (Juskiewicz *et al.*, 2011). In termite hind guts, members of the phylum Actinobacteria have demonstrated their involvement in xenobiotic metabolism and these microorganisms could possibly contribute to the degradation of benzoate compounds derived from berries (Le Roes-Hill *et al.*, 2011). The authors indicated that although the microbiome of rats differs from humans, the applied model was a powerful tool to study population dynamics and related metabolic functions (Lacombe *et al.*, 2013a). In humans, dietary treatment with blueberries increased the population of *Bifidobacteria* more than twofold, demonstrating the prebiotic activity of berry polyphenols (Vendrame *et al.*, 2011).

Berry's role in the prevention of dysbiosis

The use of berries as prebiotics has been tested in clinical trials with the objective to improve the well-being of patients. The capability for anaerobic digestion of berries in the GIT is reflected with increased *Bifidobacteria* populations and other microbes that catabolized the diverse berry compounds, especially polyphenols (Turnbaugh *et al.*, 2006; Vieira-Silva and Rocha, 2010; Vendrame *et al.*, 2011) (Figure 2). For clinicians, these findings are very important due to the growing interest in prebiotics as functional foods and the perceived benefit of increasing the numbers of beneficial bacteria in the GIT to realize their health benefits.

The microbial enzymatic metabolism has demonstrated its importance for the conversion of many classes of compounds including flavonoids, isoflavonoids, lignans, phenolic acids, fibre, and tannins (Molan *et al.*, 2009; Kemperman *et al.*, 2010; Laparra *et al.*,

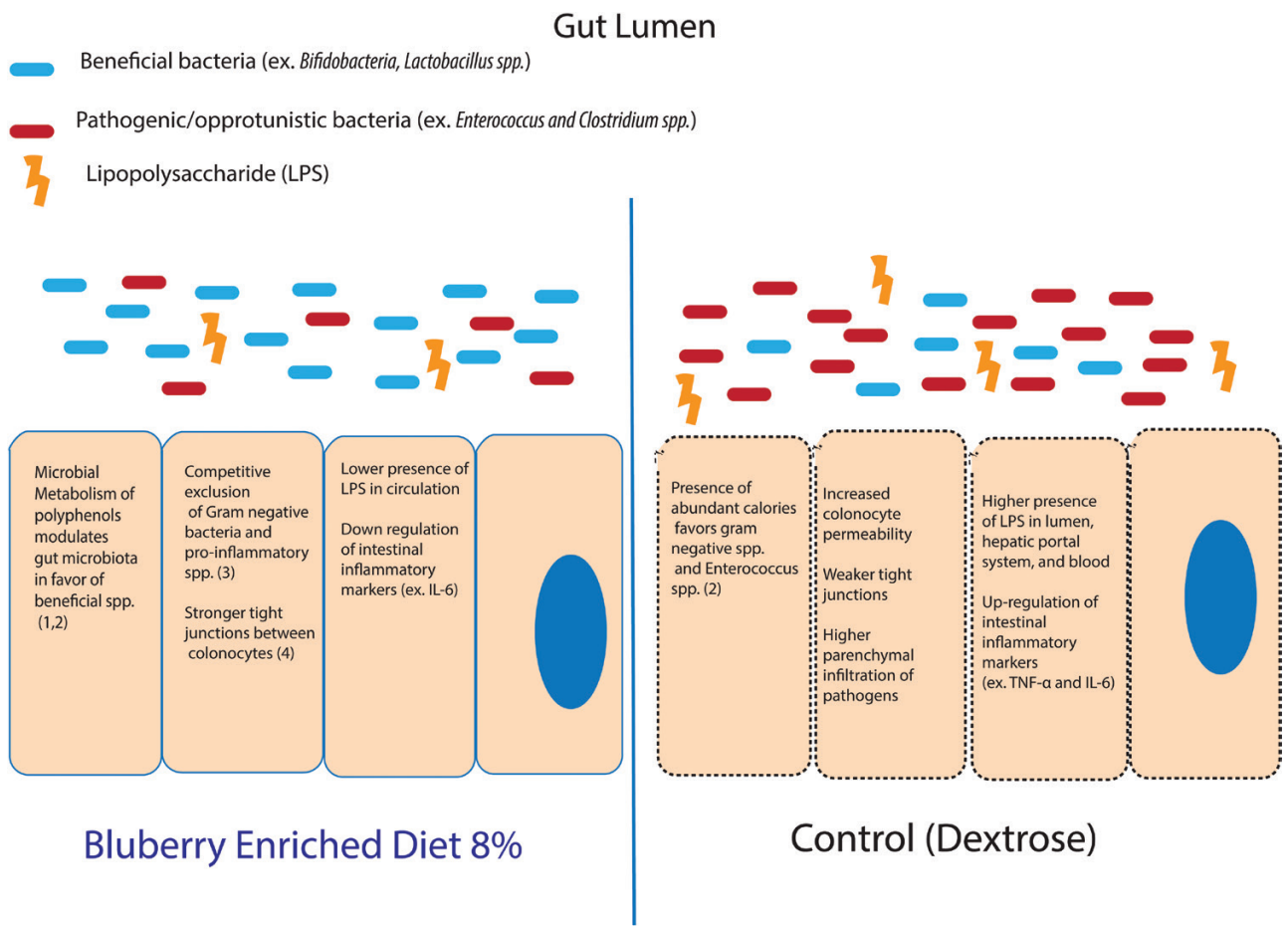


Figure 2 Possible effects of the berry-enriched diet on the gut lumen (Branning *et al.*, 2009; Hakansson *et al.*, 2009; Vendrame *et al.*, 2011; Lacombe *et al.*, 2013b).

2010; Molan *et al.*, 2010; Vendrame *et al.*, 2011). While berries have been valued in traditional medicine, they have only recently been used to affect clinical biomarkers. The high-molecular-weight polyphenols have been recognized as important in preventive medicine (Jacobs *et al.*, 2008; Kemperman *et al.*, 2010; Matsuo *et al.*, 2010). In some cases, proanthocyanidin constituents or anthocyanin pigments have been identified as the active agents, but in many other cases, interactions between co-occurring phytochemical constituents potentiate the bioactivity of berry extracts. The phytochemical constituents that are found in many edible berry fruits have increasingly been linked to modulation of biomarkers associated with conditions of diabetes, overweight/obesity, and cardiovascular disease, all components of metabolic syndrome (Branning *et al.*, 2009; Hakansson *et al.*, 2009; Jacobs *et al.*, 2009; van Duynhoven *et al.*, 2009; Del Bo *et al.*, 2010a; Kemperman *et al.*, 2010; Molan *et al.*, 2010; van Dorsten *et al.*, 2010; Vendrame *et al.*, 2011). Flavonoids and their derivatives are the largest and most important group of plant phenolics and have shown various biological effects including inhibition of low-density lipoprotein oxidation, as well as antimicrobial and anticarcinogenic capacities (Ruel *et al.*, 2008; Hamer and Mishra, 2009; Basu *et al.*, 2011). The downstream effects of a berry-enriched diet are demonstrated by an increase in plasma antioxidant capacity and provides protection to the lymphocytes against oxidative DNA damage and lower vascular reactivity and sensitivity to an α-adrenergic agonist in the aorta of SD rats (Del Bo et al., 2010a, 2010b).

Health-promoting properties attributed to beneficial bacteria include modulation of colonic microbiota by inhibiting a wide range of pathogens, improvement of lactose digestion, reduction of serum cholesterol, stimulation of the immune system through cytokine modulation, reinforcement of intestinal epithelial cell tight junctions, and increased mucus secretion (Laparra and Sanz, 2010) (Figure 2). Shifts in the gut microbiota are considered as one of the many factors involved in the pathogenesis of both inflammatory bowel disease and irritable bowel syndrome (Laparra and Sanz, 2010). In active ulcerative colitis (UC) patients, the numbers of fecal lactobacilli decrease, indicating that a reduction in intestinal *Lactobacillus* species may be a sign of mucosal inflammation (Hakansson *et al.*, 2009). Significant increases in Enterobacteriaceae and *Clostridium* spp. in feces have been one of the most frequently isolated anaerobes from the inflamed mucosa of UC patients. In addition, the biodiversity of the microbiota was shown to be lower for UC patients than for healthy controls and *E. coli* (or related Enterobacteriaceae) were significantly associated with UC (Hakansson *et al.*, 2009). Recent research has demonstrated a significant reduction in *Enterococcus* spp. in mice fed diets supplemented with blueberries (Barnett *et al.*, 2012). The permeability of the colonic epithelium is an important aspect of gastrointestinal health, and increases in permeability can allow for gut-derived bacteria and toxins to infiltrate the liver via the portal circulation (Laparra and Sanz, 2010). Berries reduced the degree of parenchymal infiltration and *Enterococcus* and *Clostridium* spp. translocations to the liver in SD rats (Branning *et al.*, 2009; Hakansson *et al.*, 2009).

Developing therapeutic regimens to combat colorectal cancer without significant side effects is of great interest to clinicians and researchers. Recent studies investigated the protective effect of

blueberry husks to delay or prevent colon carcinogenesis, and pathological abnormalities of the liver. The pathogenesis of colorectal carcinogenesis associated with colonic inflammation is believed to involve progression from inflamed and hyperplastic cryptal cells, through dysplasia, to adenoma and carcinoma. UC associated dysplasia is a likely precursor lesion or marker of carcinomas, and it is likely that colorectal carcinomas evolve through stages of increasingly severe epithelial dysplasia before becoming invasive lesions (Hakansson *et al.*, 2009). Lipopolysaccharides (LPS) associated with the cell wall of Gram-negative bacteria are highly inflammatory compounds. LPS are associated with disturbed mucosal integrity and bacterial translocation from the GIT. Translocated LPS can cause extensive damage to a variety of organs, including the liver (Hakansson *et al.*, 2009). The action of LPS from Gram-negative bacteria is mediated via Toll-like receptor 4, which initiates up-regulation of inflammatory cytokine expression in colitis-associated cancer lesions from patients with UC. Dietary enrichment with blueberries increased the viable count of fecal lactobacilli and subsequently lowered levels of LPS in the liver and blood plasma together with a reduction of inflammatory cytokines (Hakansson *et al.*, 2009).

Conclusion

Berries represent an important source of phenolic compounds in the American diet, and the market for berries has increased over the year due to increased knowledge of their health benefits. The ability of berries to conserve probiotic species while adversely affecting pathogens provides a major advantage to processors of fermented products looking for antimicrobials. The demonstration of the influence of berries on microbial ecosystems with respect to health benefits represents a tremendous opportunity for science as well as industry. Several factors impact the suitability of berries as a prebiotic and outcome for overall health differ depending on the target site of probiotics and berries as well as the mode of application (Rauch and Lynch, 2012). This obstacle of implementation is magnified by the lack of standardization of the target population, study design, and definition of end points, making it difficult to validate and compare outcomes between studies (Rauch and Lynch, 2012). Precise genotypic and phenotypic characterization of the gut microbiome or target pathogens is needed along with more investigation into cultivar viability and processing, food matrix or probiotic carriers, and mechanism of action of berries on beneficial microorganisms (Rauch and Lynch, 2012).

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