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Berry Phenolics: Antimicrobial Properties and Mechanisms of Action Against Severe Human Pathogens

Liisa J. Nohynek, Hanna-Leena Alakomi, Marja P. Kähkönen, Marina Heinonen, Ilkka M. Helander, Kirsi-Marja Oksman-Caldentey, and Riitta H. Puupponen-Pimiä

Abstract: *Antimicrobial activity and mechanisms of phenolic extracts of 12 Nordic berries were studied against selected human pathogenic microbes. The most sensitive bacteria on berry phenolics were* Helicobacter pylori *and* Bacillus cereus. Campylobacter jejuni *and* Candida albicans *were inhibited only with phenolic extracts of cloudberry, raspberry, and strawberry, which all were rich in ellagitannins. Cloudberry extract gave strong microbicidic effects on the basis of plate count with all studied strains. However, fluorescence staining of liquid cultures of virulent* Salmonella *showed viable cells not detectable by plate count adhering to cloudberry extract, whereas* Staphylococcus aureus *cells adhered to berry extracts were dead on the basis of their fluorescence and plate count. Phenolic extracts of cloudberry and raspberry disintegrated the outer membrane of examined* Salmonella *strains as indicated by 1-*N*-phenylnaphthylamine (NPN) uptake increase and analysis of liberation of [^{14}C]galactose-lipopolysaccharide. Gallic acid effectively permeabilized the tested* Salmonella *strains, and significant increase in the NPN uptake was recorded. The stability of berry phenolics and their antimicrobial activity in berries stored frozen for a year were examined using* Escherichia coli *and nonvirulent* Salmonella enterica *sv. Typhimurium. The amount of phenolic compounds decreased in all berries, but their antimicrobial activity was not influenced accordingly. Cloudberry, in particular, showed constantly strong antimicrobial activity during the storage.*

Introduction

Fruits and berries contain a variety of phenolic compounds located in plant tissues, often in the surface layer of the plant, fruit or berry, which is in connection to their main natural function, to protect the plant against environmental stress and pathogens. Berries are rich in phenolic compounds, which are classified into four main groups: flavonoids, phenolic acids, lignans, and polymeric tannins (1). Flavonoid anthocyanins are common in bilberry, black and red currant, chokeberry, strawberry, and raspberry, in which they appear as colored substances (2–4). Ellagitannins are complex phenolic polymers, which are the main phenolic compound in red raspberry and cloudberry. Strawberries also contain ellagitannins, although the amounts are lower (5,6). The most common phenolic acids present in berries are derivatives of either hydroxycinnamic acids or hydroxybenzoic acids. Lingonberry, strawberry, and cranberry are examples of berries containing lignans (7).

The phenolic content of vegetables is known to decrease during long-term storage in the freezer, and the variety of phenolics changes (8). There are, however, only a few reports on the effect of frozen storage on berry phenolics and their composition (9–11), although this knowledge is important because most Nordic berries are frozen due to the short harvesting season.

Antimicrobial activity of plant phenolics has been intensively studied, and, in addition to controlling invasion and growth of plant pathogens, their activity against human pathogens has been investigated to characterize and develop new healthy food ingredients, medical compounds, and pharmaceuticals (1,12–14). Berries are an important part of the Nordic diet, but they have also been used as natural antimicrobial pharmaceuticals. Bilberry has been used, for example, for gastrointestinal (GI) disorders, and cranberry has been a well-known treatment in urinary infections. Research on the effects of berries on human health, especially on the well-being of the GI tract and anticancer activity, has been active during recent years (5,12,14–16). For example, cranberry has been reported to control the growth of *Listeria monocytogenes* (16–19) and to possess compounds suppressing adhesion and growth of *Helicobacter pylori* (20–22) and bacteria causing urinary tract infections (23–25). High molecular weight galactans in black currant seed extracts were reported to inhibit adhesion of *H. pylori* to human gastric mucosa (26).

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The control of human GI tract pathogens by diet or by natural medicinal components is actively examined (22,27–29). Minimal use of antibiotics is recommended due to the threat of the spread of antibiotic resistance among normal human GI tract microbial flora, and therefore alternative antimicrobial compounds are sought (28,30). *Helicobacter pylori* is a causative agent of gastritis, and untreated infection may lead to chronic gastritis, peptic ulceration, and gastric cancer. The organisms respond poorly to treatment because of inactivity of medicine in the acidic stomach environment and the ability of the bacteria to embed into mucus of the stomach. Promising results have been obtained with tea catechins (31) and cranberry phenolics to eradicate *H. pylori* (21). *Campylobacter jejuni*, *Escherichia coli*, and *Salmonella enterica* sv. Typhimurium have caused foodborne and waterborne outbreaks of GI tract infections in humans, and *Bacillus cereus*, *Clostridium perfringens*, and *Staphylococcus aureus* are causative agents of food poisoning by producing toxin in food, followed by toxic symptoms in humans. *Staphylococcus aureus* and *S. epidermidis* belong to normal flora of humans but are able to cause diseases from skin infections to sepsis, usually among people with impaired host defenses (32,33). Opportunistic human pathogen *Candida albicans* is a common organism in normal human flora but also the most common pathogenic yeast-causing infection both on the skin and in the human body (33).

Many gram-negative bacteria are difficult food contaminants and pathogens. Therefore, their control in food processing systems and food is vital. The cells of gram-negative bacteria are surrounded by an outer membrane (OM), which provides the bacterium with a hydrophilic surface (34). The gram-negative OM functions as a barrier for many external agents, protecting the cells from the detergent action of bile salts and degradation by digestive enzymes (34,35). The effect is mainly due to the presence and features of lipopolysaccharide (LPS) molecules in the outer leaflet of the membrane, resulting in many gram-negative bacteria in an inherent resistance to hydrophobic antibiotics (for example, macrolides, novobiocins, rifamycins, and actinomycin D), detergents [for example, bile salts, sodium dodecyl sulfate (SDS), and Triton X-100], and hydrophobic dyes (for example, eosin, methylene blue, brilliant green, and acridine dyes) (36). The gram-negative bacteria regulate OM permeability characteristics by the presence of hydrophilic channels known as porins, which allow nutrients with relatively small molecular weight (<600 Da) to enter the inner parts of the cell. These water-filled pores generally exclude the entry of hydrophobic substances (34,35). The influx of lipophilic compounds into gram-negative bacterial cells is limited not only by the OM permeability barrier but also by their active efflux by ubiquitous, "multidrug" efflux pumps, which are usually energized by the proton motive force (34,37). Although the OM of gram-negative bacteria is an efficient barrier against many external agents, it is possible to specifically weaken the OM by various agents (for example, permeabilizers) that disintegrate the LPS layer (35). A classical example of these is ethylenediaminetetraacetic acid (EDTA), which can disorganize and weaken the interactions between LPS molecules by chelating the OM-stabilizing divalent cations (Ca^{2+} and Mg^{2+}) (35). Certain small terpenoid and phenolic compounds found in herbal plants (for example, essential oil components) have been reported to disintegrate the OM, releasing LPS and increasing the permeability of the cytoplasmic adenosine triphosphate (ATP) (38,39). Relatively few data, however, are available concerning the antimicrobial mechanisms from berry-derived purified compounds, although growth-inhibitory and antimicrobial activity of berry samples has been reported (1, 14,16,40).

The aim of this work was to evaluate the antimicrobial activity of berry phenolics on several human pathogenic bacteria and to reveal some of the mechanisms involved in the antimicrobial activity of crude phenolic extracts and purified phenolic fractions derived from berries. This study complements our previous research on antimicrobial activities of berries and berry phenolics (1,16,40). In addition, the stability of phenolic compounds in berries during long-term storage in the freezer was followed, and, to our knowledge, this is the first report of the effect of storage on antimicrobial activity of berries. This knowledge is important for the food industry because it almost entirely uses frozen berries.

Materials and Methods

Bacterial Strains and Growth Conditions

The bacterial strains used with their complete code and origin are listed in Table 1. All the strains were stored at –70°C and revived on agar plate. *Clostridium perfringens* E-861^T was grown in reinforced clostridial medium (RCM) broth or on RCM agar (Difco, Sparks, MD), and de Man Rogosa Sharpe medium (Oxoid, Hampshire, UK) was used for growth of *Lactobacillus rhamnosus* strains E-800 and E-666. The strains were incubated in anaerobic jars ($H_2/CO_2/N_2$, 10:5:85; Anoxomat WS8000, Mart∃ Microbiology, Lichtenvoorde, Holland) at 37°C for 2–3 days. Microaerophilic strain *Campylobacter jejuni* E-1008^T was grown in brain heart infusion (BHI) (Difco) with K_1 vitamin (1 mg l^{-1}) and hemin (5 mg l^{-1}) for 2–3 days, and *H. pylori* NCTC 11637 was grown in BHI or on sheep blood agar (Oxoid) for 4–6 days. Both strains were incubated in anaerobic jars (Anoxomat WR8000) in microaerophilic atmosphere (6% O_2) at 37°C. Aerobic bacterial strains *B. cereus* E-727; *E. coli* E-564^T; *Salmonella* sv. Typhimurium strains SH-5014 and E-1151; *Staphylococcus aureus* strains E-045, E-530, and E-531^T; and *S. epidermidis* E-768 ^{T} were grown in or on nutrient medium (Difco) for 1 day. *Bacillus cereus* was incubated at 30°C, and the other aerobic strains were incubated at 37°C. *Candida albicans* NCPF 3179 was grown in YM (Difco) for 1–2 days at 37°C. Liquid aerobic bacterial cultures were grown with constant agitation of 150 rpm, and *C. albicans* cultures were shaken with 100 rpm.

Table 1. Microbial Strains Used in the Study[a]

Strain	Catalog Code[b]	Origin
Bacillus cereus	VTT **E-96727**	ATCC, strain 9139
Campylobacter jejuni	VTT **E-981008**^{T}	LMG, strain 8841
Candida albicans	NCPF 3179	The Institute of Dentistry, University of Helsinki (Finland)
Clostridium perfringens	VTT **E-98861**^{T}	ATCC, strain 13124
Escherichia coli	VTT **E-94564**^{T}	ATCC, strain 11775
Helicobacter pylori	NCTC 11637	H. Rautelin, University of Helsinki
Lactobacillus rhamnosus	VTT **E-97800**	Own isolate, VTT Biotechnology (Espoo, Finland)
L. rhamnosus GG	VTT **E-96666**	ATCC, strain 53103
Salmonella enterica sv. Infantis	VTT **E-97738**	Own isolate, VTT Biotechnology
S. enterica sv. Typhimurium	SH-5014	I. Helander, VTT Biotechnology
S. enterica sv. Typhimurium	VTT **E-981151**	National Public Health Institute (Helsinski, Finland)
Staphylococcus aureus	VTT **E-70045**	ATCC, strain 6538
S. aureus	VTT **E-94530**	ATCC, strain 25923
S. aureus	VTT **E-94531**^{T}	ATCC, strain 12600
S. epidermidis	VTT **E-97768**^{T}	DSM, strain 20044

a: Abbreviations are as follows: ATCC, American Type Culture Collection (Manassas, VA); LMG, Laboratorium voor Microbiologie, BCCM/LMG Bacteria Collection, Universiteit Gent (Gent, Belgium); NCPF, National Collection of Pathogenic Fungi, Public Health Laboratory (Bristol, UK); NCTC, National Collection of Type Cultures, PHLS Central Public Health Labortory (London, UK); DSM, DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Braunschweig, Germany).

Berry Material

Fresh berry samples of bilberry (*Vaccinium myrtillus*), lingonberry (*V. vitis-idaea*), cranberry (*V. oxycoccus*), red raspberry (*Rubus idaeus*, var. Ottawa), cloudberry (*R. chamaemorus*), strawberry (*Fragaria ananassa* Senga Sengana), black currant (*Ribes nigrum* var. Öjeby), sea buckthorn berry (*Hippophae rhamnoides*), chokeberry (*Aronia mitschurinii*), highbush bilberry (*V. myrtillus*), rowanberry (*Sorbus aucuparia*), and crowberry (*Empetrum nigrum*) were purchased from the market during the growing season of July–August 2001, 2002, and 2003. For analysis of antimicrobial activity, the whole berries were stored frozen in the freezer at –18°C until used for preparation of phenol extracts.

For analysis of storage stability of berry phenolics, fresh berry samples (5 kg) of bilberry, black currant, cloudberry, and red raspberry were purchased from a market during the growing season of July–August 2000. Samples were vacuum-packed immediately and stored in a freezer at –20°C up to 12 mo until used for preparation of phenolic extracts.

Preparation and Chemical Analysis of Berry Extracts

Extraction and analyses of berry phenolics were carried out according to Kähkönen et al. (41) at 0, 3, 9, and 12 mo of frozen storage. Extraction of phenolic compounds was performed by homogenizing (Ultra-Turrax T25 mixer, Janke & Kunkel, Germany) for 1 min 2 g of lyophilized berries with 20 ml of solvent (acetone–water 70:30 vol/vol) in a centrifuge tube. Tubes were centrifuged (4,000 rpm, 15 min), and the clear supernatant was collected. The procedure was repeated twice with another 20 ml of solvent. Supernatants were combined and taken to dryness, and the solid residues were dissolved in Milli Q purified water. Interfering sugars were removed by solid-phase extraction (C18) prior to analyses of total phenolics by Folin-Ciocalteau assay (42) and phenolic profile analysis by high-performance liquid chromatography (HPLC).

Phenolic berry isolates were further fractionated using Amberlite XAD-7 column chromatography (43). Sample was introduced into the column (diameter 40 mm, length 300 mm), and free sugars and organic and phenolic acids were washed out with 6% CH_3CN (CH_3CN:TFA:H_2O 6:0.5:93.5 vol/vol/vol). Elution was continued with CH_3CN (CH_3 CN:TFA 99.5:0.5) to yield a fraction containing flavonols, anthocyanins, and ellagitannins. To separate ellagitannins from the other phenolics, this fraction was introduced into a column of similar size packed with Sephadex LH-20. Sample was introduced into the column, and flavonols and anthocyanins were eluted with 50% MeOH. Ellagitannins were eluted with aqueous acetone (70:30 vol/vol). For analyses of antimicrobial activity, the berry extracts and ellagitannin and anthocyanin fractions were used as freeze-dried powder.

Phenolic profiles were determined using an analytical HPLC method described by Kähkönen et al. (41). The separation of phenolic compounds was carried out on a Nova-Pak C18 column (150 × 3.9 mm, 4 mm; Waters, Milford, MA) with a WISP 712 autosampler, three 501 pumps, a PDA996 diode array detector, and a Millennium 2020C/S software data module (Waters). On the basis of spectral identification, phenolics were quantified into six subclasses: flavanols and proanthocyanidins [expressed as (+)catechin equivalents, detection wavelength 280 nm], hydroxybenzoic acids (as gallic acid equivalents, 280 nm), ellagitannins (as ellagic acid equivalents, 280 nm), hydroxycinnamic acids (as chlorogenic acid equivalents, 320 nm), flavonols (as rutin equivalents, 365 nm), and anthocyanins (as cyanidin 3-glucoside equivalents, 520 nm). The standard compounds used were obtained from Extrasynthese (Genay, France) and Sigma Chemical Co. (St. Louis, MO). All organic solvents used were of HPLC

grade. The results were expressed as averages and standard deviations of triplicate measurements (milligrams per gram of freeze-dried extract).

Antimicrobial Activity

Liquid culture analysis: Comparison of the growth curves in liquid cultures was used for analysis of antimicrobial activities of berry phenolics. Microbial cultures for antimicrobial assays were revived from –70°C storage cultures on agar plate, and fresh inoculants for testing antimicrobial activities were grown in liquid cultures to late logarithmic or to stationary phase. Lyophilized berry extracts were suspended in 10 ml of growth media to a final concentration of 1 mg ml^{-1}, and 1% of liquid microbial inoculum was added. Cultures with no berry material were used as controls. Liquid cultures were incubated as described previously for each strain, and microbial growth was followed by plate count of samples taken four to six times during the growth period of 1–6 days, depending on the growth rate of the studied microbe. The number of culturable cells in the samples was determined by diluting the sample with peptone saline (Maximal Recovery Diluent, Lab M, Amersham, UK), plating in duplicate on the media, and incubating as indicated for each strain. The effect of the tested berry material on the growth of microbial strains in liquid cultures was evaluated by measuring the difference in the plate counts of test and control cultures during the growth period. Each experiment was performed two or three times.

Adherence of bacterial cells to berry material: The adhesive properties of berry extracts and the effect of this phenomenon on the determination of antimicrobial activity were studied by sonication (Amdent 830 Piezo, Amlab, Sweden) of samples obtained from liquid cultures. *Salmonella* sv. Typhimurium strain E-1151 and *L. rhamnosus* E-666 were used as test organisms. Liquid cultures with and without berry extract were sonicated (two times for 10 s, with an interval of 20 s) before sampling. The samples were used for plate count analysis to evaluate the number of cultivable cells.

Fluorescence staining: The viability of bacterial cells in liquid cultures with berry extracts was evaluated using nucleic acid probes SYTO9 and propidium iodide (PI) in the LIVE/DEAD BacLight bacterial viability kit (L7012, Molecular Probes, Leiden, The Netherlands) according to the manufacturer's instructions. SYTO9 stains all bacterial cells with green fluorescence, and PI stains with red fluorescence, penetrating only bacterial cells with damaged membranes. Stained samples were observed by epifluorescence microscopy (Olympus BX60, Olympus Optical Co., Tokyo, Japan) provided with a digital image analyzer (analySIS version 3.0, Soft Imaging System, Münster, Germany).

Storage Stability of Berry Phenolics

The effect of storage of berries in the freezer for 0, 3, 9, and 12 mo on their phenolic contents and antimicrobial activities was evaluated. Fresh and frozen berries were lyophilized and ground for preparation of phenolic extracts, which were analyzed for total phenols and main phenolic fractions as described previously. Antimicrobial activities of these extracts were studied in liquid cultures using bacterial strains *Escherichia coli* E-564^T, *S.* Typhimurium SH-5014, *L. reuteri* E-849, and *L. rhamnosus* strains E-666 and E-800.

Mechanism of Antimicrobial Activity

Permeability assays: Permeability properties of the OM of Salmonella strains were determined by 1-*N*- phenylnaphthylamine (NPN, Sigma-Aldrich, Steinheim, Germany) uptake. Bacterial strains used were *S. enterica* sv. Typhimurium E-1151, *S. enterica* sv. Typhimurium SH-5014 (an rfaJ mutant producing rough LPS of chemotype Rb2) (44), and *S. enterica* sv. Infantis E-738. A stock solution of NPN (0.5 M) was prepared in acetone and diluted to 40 μM into 5 mM *n*-heptadecanoic acid methyl ester (HEPES, pH 7.2) for the fluorometric assays. The uptake of the hydrophobic fluorescent probe NPN by bacterial cells in buffer suspensions was measured using black fluorotiter plates (cat. no. 9502867, LabSystems, Helsinki, Finland) and the automated fluorometer Fluoroskan Ascent FL (LabSystems) as described earlier (45,46). Briefly, cells grown into $OD_{630} = 0.5 \pm 0.02$ were harvested by centrifugation and suspended into half volume of 5 mM HEPES (Sigma-Aldrich, Steinheim, Germany) buffer. Aliquots (100 μl) of this cell suspension were pipetted into fluoroplate wells, which contained NPN (10 μM) and as test substances either EDTA (1.0 and 0.1 mM, Riedel-de-Haen, Seelze, Germany), HEPES buffer (control pH 7.2), phenolic berry extracts, ellagitannin or anthocyanin fractions (1 mg ml^{-1}), or HCl (pH 5.0) to make up a total volume of 200 μl. Fluorescence was monitored within 3 min from four parallel wells per sample (excitation, 355 nm; half bandwidth, 38 ± 3 nm; emission, 402 nm; half bandwidth, 50 ± 5 nm). Each assay was performed at least three times. The background fluorescence of the phenolic berry extracts was subtracted from the fluorescence values.

LPS release: The effect of EDTA and selected phenolic berry extracts as well as ellagitannin and anthocyanin fractions on LPS release was studied using radiolabeling of LPS, and release of [^{14}C]galactose-LPS ([^{14}C]Gal-LPS) was monitored according to Alakomi et al. (47). D-[$^{1-14}$C]Gal was obtained from Amersham Pharmacia Biotech (Buckinghamshire, UK) with a specific activity of 49.4 μCi $mmol^{-1}$.

Smooth *S. enterica* sv. Typhimurium E-1151 and *S. enterica* sv. Infantis E-738 cells were grown in Luria-Bertani broth (38) supplemented with 2 mM $CaCl_2$ at 37°C with shaking (200 rpm) to $OD_{630} = 0.5 \pm 0.02$ and supplemented with [^{14}C]Gal (0.1 μCi ml^{-1}) to label their LPS. Briefly, labeling with [^{14}C]Gal was performed for 5 min at 37°C with

shaking (200 rpm). A 1-ml aliquot was then removed to check the level of incorporation of radiolabel (Wallac 1410 liquid scintillation counter, Pharmacia, Espoo, Finland). The remaining cells were collected by centrifugation (1,000 *g*, 10 min, 25°C) and washed with 10 mM Tris/HCl buffer (pH 7.2) at room temperature. After centrifugation, the cells were resuspended in the same buffer to $OD_{630} = 0.5 \pm 0.02$, divided into portions, and supplemented with EDTA (0.1 or 1.0 mM), phenolic berry extracts, ellagitannin or anthocyanin fractions, or HCl to obtain pH 3.5 or 3.8; buffer was used as control. After adding the test substance, the suspensions were incubated at 37°C for 10 min with shaking (100 rpm). Samples were taken for radioactivity measurements, and the remaining cells were centrifuged twice (1,500 *g*) at room temperature. After centrifugation, samples from the cell-free supernatants were taken for radioactivity measurements. The amount of radioactivity in the cell-free supernatant was taken as the measure of liberated LPS, and the percentage value for LPS release was calculated by comparison with radioactivity of similar volumes of untreated and uncentrifuged bacterial suspensions.

Statistical analysis: Results from the permeability assays were analyzed statistically using two-tailed unpaired Student's *t*-test to determine differences; levels of significance are denoted as $*P < 0.02$, $**P < 0.01$, and $***P < 0.001$.

Results

Antimicrobial Activity of Berry Phenolics on Human Pathogens

The antimicrobial activity of Nordic berry extracts was screened against selected human pathogens (Table 2). Microbial strains had different sensitivities against berry extracts, and antimicrobial effects of studied berries were variable. The phenolic extract of cloudberry possessed the strongest antimicrobial activity, followed by raspberry and strawberry with even antimicrobial effects. The weakest antimicrobial effects were measured with chokeberry, rowan berry, crowberry, and buckthorn berry.

Liquid cultures of *Helicobacter pylori* NCTC 11637 and *B. cereus* E-727 were the most sensitive bacterial strains against the berry extracts. No cultivable cells of *H. pylori* were detected with phenolic berry extracts during the incubation period, with a detection limit of 100 cells per milliliter. The number of cultivable cells of *B. cereus* decreased by 1 to 4 logarithms compared with the inoculum of about 10^6 cells per milliliter during the incubation period of 24 h with the berry extracts. Cloudberry, raspberry, and strawberry were clear inhibitors of microbial growth of all cultures, including *Campylobacter jejuni* E-1008^T and *Candida albicans* NCPF 3179, which were not inhibited by the other berry extracts. The cell count in *Campylobacter jejuni* E-1008^T and *Candida albicans* NCPF 3179 cultures with cloudberry, raspberry, and strawberry decreased during the first 5 h of incubation 0.5 to 1.5 logarithms, but, for the duration of the incubation period of 24 h, the cell count increased. Cranberry extract was effective against *B. cereus* E-727 and *Clostridium perfringens* E-861^T. The sensitivity to cloudberry and bilberry phenolics of *Staphylococcus aureus* strains E-075, E-530, and E-531^T was similar, showing a bacteriocidic effect with cloudberry and a bacteriostatic effect with bilberry. *Staphylococcus epidermidis* strain E-768^T tolerated slightly better the tested berry extracts in liquid cultures than *S. aureus* E-075 (Table 2).

Stability of Berry Phenolics and their Antimicrobial Activity

The stability of phenolic compounds and their antimicrobial activity were studied in berries stored frozen up to 1 yr. During the frozen storage the content of ellagitannins, hydroxycinnamic acids, anthocyanins, and flavonols in the berries decreased to 50–75%, 55–78%, 57–68% (with the exception of cloudberry anthocyanins), and 0–45%, respectively, of the original amount (Table 3). Bilberry anthocyanins (97% remaining) and cloudberry ellagitannins (95%) were the most stable compared with the other berry phenolics after 3 mo of storage. The ellagitannins in cloudberries were also better preserved than in raspberries during 12 mo of frozen storage. The amount of both flavonols and hydroxycinnamic acids was low. The total content of phenolic compounds measured spectrophotometrically indicated not only a decrease in phenolic content but also an increase in the cloudberry phenolics.

The effect of frozen storage on phenolic content in berries was not directly comparable with their antimicrobial activities. In general, antimicrobial activity of berry extracts against gram-negative bacteria *E. coli* E-564^T and *Salmonella* Typhimurium SH-5014 was constant or increased in berries stored frozen for several months to a year (Table 4). A clear increase in antimicrobial effect was observed with black currant against *S.* Typhimurium SH-5014 after 9-mo storage. The effect of bilberry and raspberry against both strains varied during the year and also cloudberry slightly against *E. coli* E-564^T. Cloudberry extracts were strongly bacteriocidic against *S.* Typhimurium SH-5014 during the research period of 1 yr. Probiotic bacterial strains *L. rhamnosus* GG E-666 (Table 4) and E-800 (data not shown) were not affected by berry extracts at any time during the experiment.

Immobilization Properties of Berry Extracts

In addition to the plate count method, fluorescent probes SYTO9 and PI were used to evaluate the viability of bacterial cells in selected liquid cultures. On the basis of plate count, cloudberry extract showed the strongest antimicrobial effect on all the strains. The number of cultivable *S.* Typhimurium E-1151 cells decreased by 3 to 4 logarithmic units during first few hours of incubation detected by plate count, followed by an increase during the incubation period of 24 h.

Table 2. Antimicrobial Activities of Phenolic Berry Extracts (1 mg ml^{-1}) on Human Pathogenic Microbial Strains[a]

Berry Extract (1 mg ml^{-1})	*Bacillus cereus* E-727	*Campylobacter jejuni* E-1008^{T}	*Clostridium perfringens* E-861^{T}	*Helicobacter pylori* NCTC 11637	*Staphylococcus aureus* E-045	Staphylococcus epidermidis E-768^{T}	*Candida albicans* NCPF 3179
Cloudberry	++++	+++	+++++	+++++	+++++	++++	+++
Chokeberry	+++	–	+	ND	++	+	–
Bilberry	++++	–	+++	+++++	+++	ND	–
Black currant	++++	–	+	+++++	+++	ND	–
Lingonberry	++++	–	–	ND	ND	++++	–
Raspberry	++++	+++	+++	+++++	++++	++++	++
Cranberry	++++	–	+++	ND	ND	+++	–
Strawberry	++++	++	++	+++++	++++	+++	+++
Highbush bilberry	++++	–	++	ND	++	++	–
Rowan berry	++++	+	–	ND	+	ND	–
Crowberry	++++	–	–	ND	ND	ND	–
Buckthorn berry	++++	–	+	ND	ND	ND	–

a: Abbreviation is as follows: ND, not determined. –, no inhibitory effects; +, weak inhibition; ++, clear inhibition; +++, strong inhibition; ++++, very strong inhibition; +++++, death of the culture.

Table 3. Effect of Frozen Storage on the Phenolic Content (mg g^{-1} dry wt) of Berries[a]

Berry	Storage (mo)	Total Phenolics	Ellagitannins	Hydroxycinnamic Acids	Anthocyanins	Flavonols
Black currant	0	470	ND	14	210	0.5
	3	340	ND	12	180	0.3
	9	410	ND	9	170	0.3
Bilberry	0	600	ND	22	340	2
	3	450	ND	19	330	0.9
	9	390	ND	12	290	0.8
	12	490	ND	12	230	0.9
Cloudberry	0	460	200	9	3	0.5
	3	410	190	6	3	<0.1
	9	380	150	5	0.7	<0.1
	12	560	150	7	4	<0.1
Raspberry	0	530	200	8	62	0.2
	3	560	180	8	51	0.2
	9	440	130	4	39	<0.1
	12	470	100	5	35	0.2

a: Abbreviation is as follows: ND, not detected.

Table 4. Stability of Antimicrobial Activity of Phenolics Extracted From Fresh Berries and From Berries Stored Frozen for 3, 9, and 12 mo[a]

Berry Extract (1 mg ml^{-1})	Storage (mo)	*Escherichia coli* E-564^T	*Salmonella* sv. SH-5014	*Lactobacillus rhamnosus* GG E-666
Bilberry	0	++/++	++++/++++	–/–
	3	+/++	+/+	–/–
	9	+/+	++/+++	–/–
	12	+/++	++++/++++	–/–
Black currant	0	+/+	+/+i	–/–
	3	+/+	+/+	–/–
	9	+/++	++/+++	–/–
	12	ND	ND	ND
Raspberry	0	+++/++++	++++/++++	–/–
	3	++/++	++/+++	–/–
	9	+++/+++	+++++/+++++	–/–
	12	++/+++	+++++/+++++	–/–
Cloudberry	0	++++/++++	+++++/+++++	–/–
	3	+++/+++	+++++/+++++	–/–
	9	++++/++++	+++++/+++++	–/–
	12	++/+++	+++++/+++++	–/–

a: Abbreviation is as follows: ND, not determined. Antimicrobial activity was screened twice with *E. coli* E-564^T, *S. enterica* sv. Typhimurium strain SH-5014, and *L. rhamnosus* GG E-666. –, no inhibitory effects; +, weak inhibition; ++, clear inhibition; +++, strong inhibition; ++++, very strong inhibition; +++++, death of the culture.

Fluorescence staining showed how the majority of viable bacterial cells with green fluorescence was attached to cloudberry material. A few free green cells were seen in the liquid phase as well as cells with red fluorescence indicating dead bacteria. An effect similar to cloudberry was observed with strawberry extract, whereas immobilization of *S.* Typhimurium E-1151 cells by bilberry extract was very weak. Sonication of the liquid culture released *S.* Typhimurium E-1151 cells from the cloudberry extract followed by 100-times higher numbers of culturable cells in the plate count (Fig. 1).

Fluorescence staining of liquid culture of *Staphylococcus aureus* E-075 with cloudberry and strawberry extracts visualized cells with red fluorescence irreversibly attached to berry extract. Cell death was also detected by constantly decreasing numbers of cultivable cells by 1 to 3 logarithmic units with strawberry and by 4 to 5 logarithmic units with cloudberry during the incubation period of 24 h. Attachment and slight bacteriocidic effect of bilberry extract on *S. aureus* cells was visible after 3 h of incubation, whereas, after 8–24 h of incubation, most of the cells were in the liquid and viable, as determined by fluorescence staining and plate count.

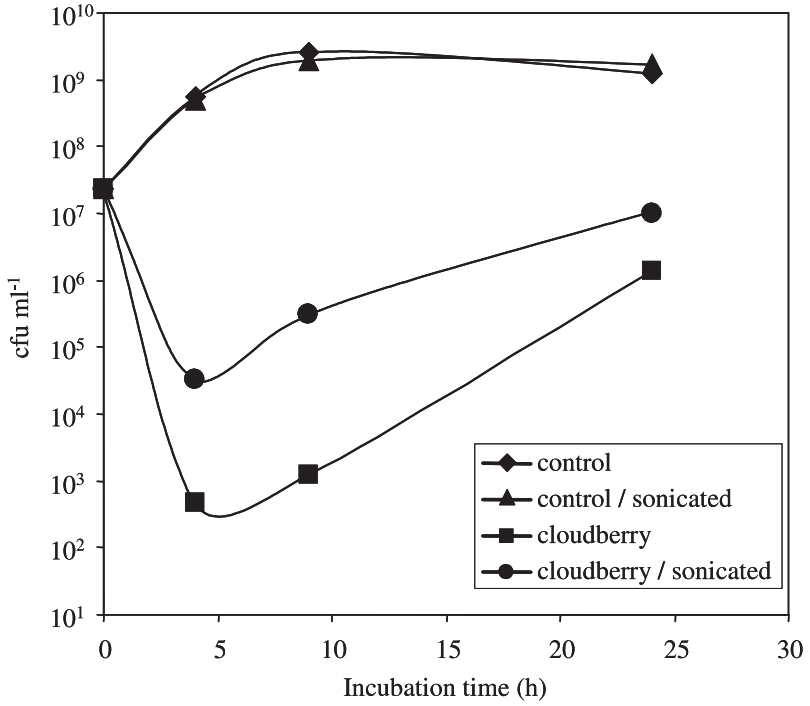


Figure 1. Effect of sonication on plate count of *Salmonella enterica* sv. Typhimurium VTT E-981151 in liquid culture with and without phenolic cloudberry extract.

Effect of Tested Samples on the Uptake of NPN

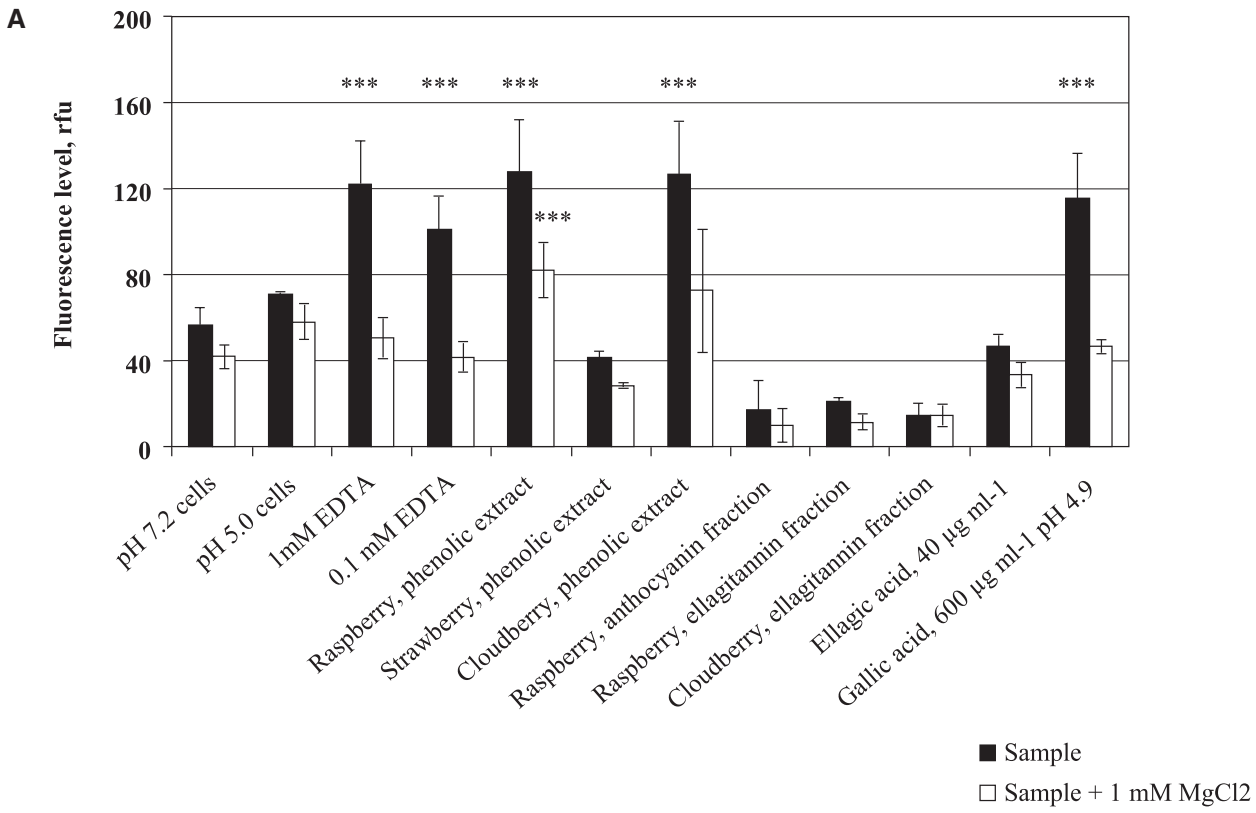
To reveal changes in OM permeability, two smooth and one rough (LPS chemotype Rb2) *Salmonella enterica* strains were selected for NPN uptake studies. Detailed results of the NPN uptake experiments with EDTA, HCl (pH 5.0), gallic acid (pH 4.9), ellagic acid (pH 7.0), phenolic berry extracts (pH 4.8–5.1), and ellagitannin and anthocyanin fractions (pH 6.8–7.0), including the effect of the addition of $MgCl_2$ in the assay buffer, are shown in Fig. 2A–C. For the smooth *S.* Typhimurium E-1151 and *S.* Infantis E-738 strains, EDTA, gallic acid, and phenolic berry extracts of cloudberry and raspberry brought about a significantly higher ($P < 0.001$) NPN uptake than control treatments (pH 7.2 and 5.0; Fig. 2A and B). Addition of 1 mM $MgCl_2$ into the buffer abolished the permeabilizing activities of EDTA and gallic acid, and the NPN uptake of target cells was in the same level as in the corresponding control cells (Fig. 2A and B). In a similar manner, 1 mM $MgCl_2$ addition almost totally abolished the activity of phenolic extracts of cloudberry and raspberry. In the ellagic acid and strawberry phenolic extract–treated cells, the NPN uptake was at the same level as in the control cells. Whereas in cells treated with anthocyanin and ellagitannin fractions derived from raspberry or ellagitannin fractions derived from cloudberry, the NPN uptake was at a lower level than in the control cells (Fig. 2A and B).

EDTA and ellagic acid did not increase the NPN uptake of rough *S.* Typhimurium SH-5014 cells (Fig. 2C). However, raspberry and cloudberry phenolic extracts as well as gallic acid increased the NPN uptake of SH-5014 ($P < 0.001$). As shown in Fig. 2C, addition of 1 mM $MgCl_2$ into the buffer used in the NPN assay abolished the OM-disintegrating activity of gallic acid almost totally, whereas only part of the activity of phenolic extracts of raspberry and cloudberry was removed by $MgCl_2$ addition ($P < 0.001$).

Phenolic extracts of black currant, bilberry, lingonberry, and cranberry (1 mg ml^{-1}) had no NPN uptake-enhancing activity on tested *Salmonella* strains (data not shown).

Release of LPS

Table 5 summarizes the results of specific labeling of [^{14}C]Gal-LPS and LPS release. The amounts of 1 mM EDTA-induced [^{14}C]Gal-LPS in *S.* Typhimurium E-1151 and in *S.* Infantis E-738 were 26% and 24%, respectively. Treatment with 0.1 mM EDTA did not induce release of [^{14}C]Gal-LPS from *S.* Typhimurium E-1151. However, 0.1 mM EDTA-induced [^{14}C]Gal-LPS from *S.* Infantis E-738 was 20%. Phenolic extracts of cloudberry, black currant, cranberry, and bilberry (1 mg ml^{-1}) liberated LPS, as indicated by the amount of releasable [^{14}C]Gal-LPS. Phenolic berry extracts resulted in a final pH of 3.5–3.8 in the LPS assay buffer. The pH control treatments (pH 3.5 and 3.8) also efficiently liberated LPS, and the difference between the amount of phenolic berry extract–releasable [^{14}C]Gal-LPS was not significant compared with the pH 3.5– or pH 3.8–releasable [^{14}C]Gal-LPS. The amount of raspberry ellagitannin and anthocyanin fractions liberating [^{14}C]Gal-LPS from *S.* Typhimurium E-1151 cells was similar to that in pH 7.2 control cells. Likewise, cloudberry ellagitannin fraction did not liberate LPS from *S.* Typhimurium E-1151 cells because the amount of liberated



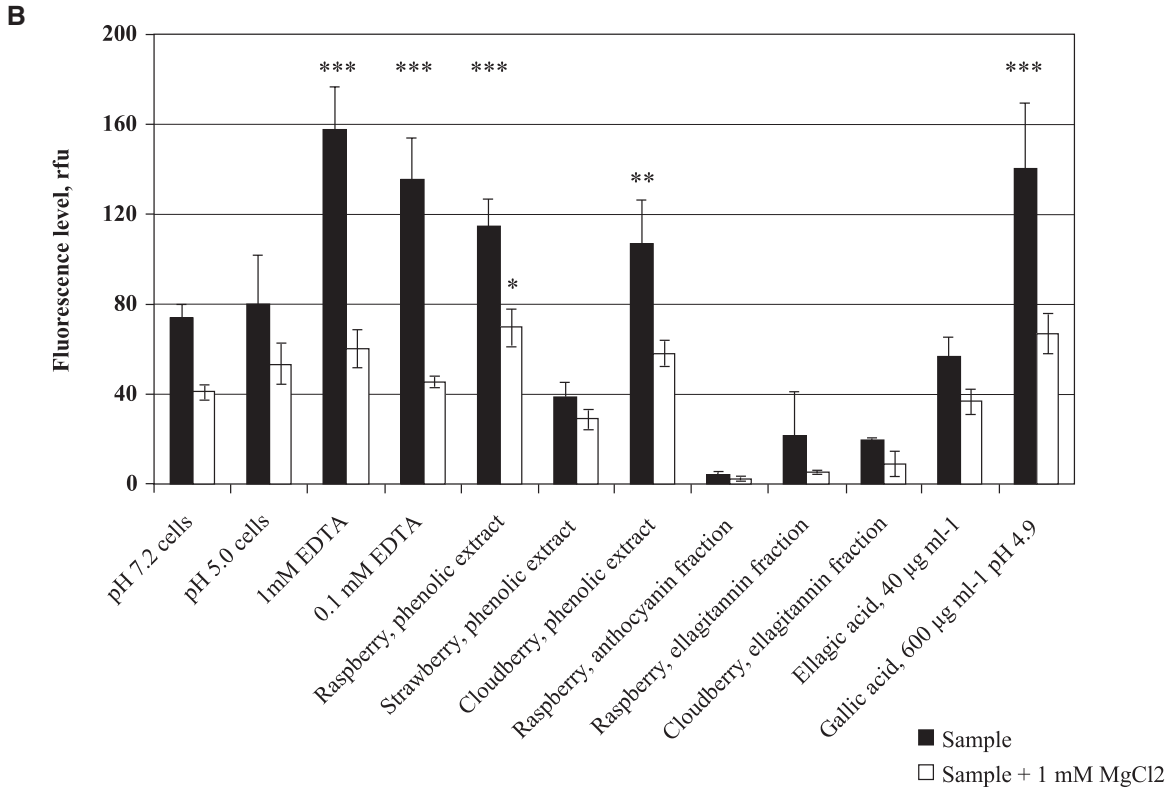


Figure 2. 1-*N*-Phenylnaphthylamine (NPN) uptake in *Salmonella enterica* serovar Typhimurium bacterial suspensions containing either buffer alone (pH 7.2), HCl (pH 5.0), ethylenediaminetetraacetic acid (0.1 and 1 mM), phenolic plant extracts (1 mg ml^{-1}), or ellagitannin of anthocyanin fractions (1 mg ml^{-1}) with or without 1 mM $MgCl_2$. NPN uptake levels for (A) *S. enterica* sv. Typhimurium VTT E-981151, (B) *S. enterica* sv. Infantis VTT E-97738, and (C) *S. enterica* sv. Typhimurium SH-5014 (lipopolysaccharide chemotype Rb2). Error bars present standard deviations. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ compared with the controls.

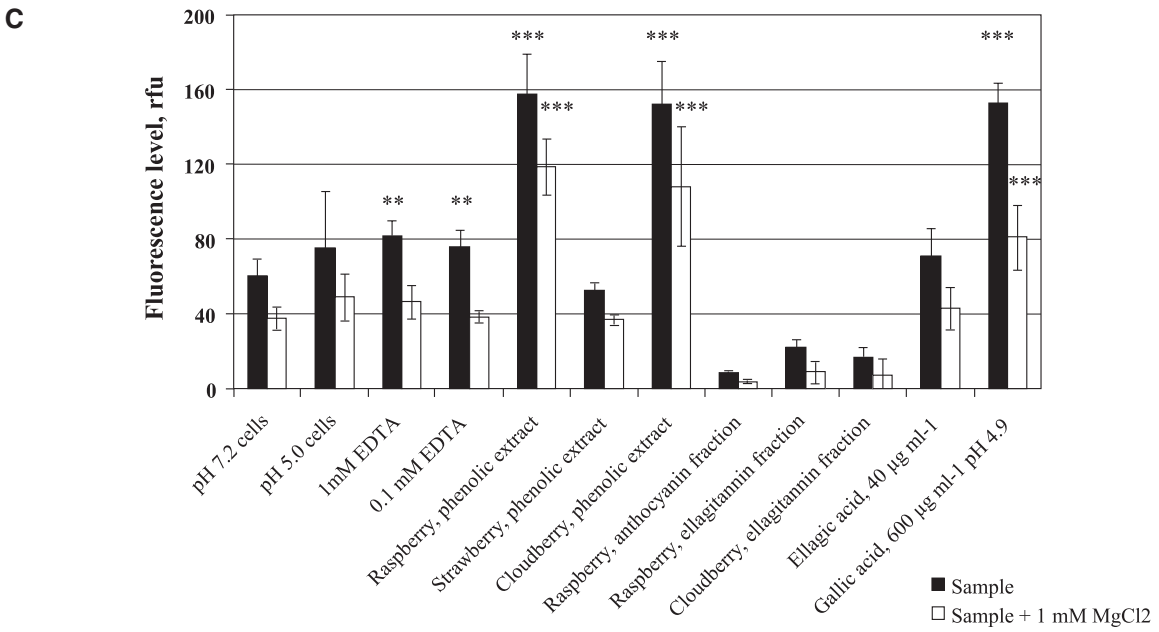


Figure 2 *(Continued)*

[^{14}C]Gal-LPS was not significantly different from the control treatment.

Discussion

Berries and especially their phenolics are reported to possess potential health-promoting effects, such as antioxidative, antitumorigenic, and anticarcinogenic (5,48–51). Our focus in this study was to complement the previous knowledge of antimicrobial activities and mechanisms of berry phenolics on human pathogenic microbes and to evaluate the effect of frozen storage on the stability and antimicrobial activity of phenolic berry extracts.

The antimicrobial effect of phenolic berry extracts varied depending on bacterial strain and berry tested. Phenolic extract of cloudberry had the strongest antimicrobial activity, followed by raspberry and strawberry, which was similar to the results reported earlier by us of *E. coli*, *Staphylococcus aureus*, *Salmonella* sv. Typhimurium, and *S.* sv. Infantis (P-P, 2001, P-P-2005b) (16,40). Rauha and coworkers (14) studied phenolic berry extracts using the agar diffusion method and reported antibacterial effects of cloudberry, raspberry, and crowberry against several bacterial strains. Antimicrobial activity of tannins toward microorganisms is well documented (52). Cloudberry, raspberry, and strawberry were the richest in ellagitannins of berries used in this study. Ellagitannin fraction in cloudberry and raspberry and ellagic acid in cran-

Table 5. EDTA, Phenolic Berry Extract, Ellagitannin, and Anthocyanin Fractions Induced [^{14}C] LPS Release From *Salmonella enterica* serovar Typhimurium VTT E-981151 and *S. enterica* sv. Infantis VTT E-97738[a]

		Release of LPS (%)	
Additive to Assay Buffer	pH	*S.* sv. Typhimurium E-981151	*S.* sv. Infantis E-97738
pH 7.2 cells	7.1 ± 0.1	7 ± 2	4 ± 1
EDTA, 1 mM	7.1 ± 0.1	26 ± 2	24 ± 2
EDTA, 0.1 mM	7.1 ± 0.1	7 ± 1	20 ± 0
Cloudberry, 1 mg ml^{-1} phenolic extract	3.8 ± 0.1	32 ± 0	38 ± 3
Black currant, 1 mg ml^{-1} phenolic extract	3.5 ± 0.1	35 ± 2	34 ± 6
Cranberry, 1 mg ml^{-1} phenolic extract	4.0 ± 0.4	27 ± 5	31 ± 7
Blueberry, 1 mg ml^{-1} phenolic extract	4.1 ± 0.2	38 ± 2	37 ± 1
Raspberry, 0.5 mg ml^{-1} ellagitannin fraction	6.5 ± 0.1	8 ± 3	Not determined
Raspberry, 0.5 mg ml^{-1} anthocyanin fraction	6.6 ± 0.1	10 ± 2	Not determined
Cloudberry, 0.5 mg ml^{-1} ellagitannin fraction	6.0 ± 0.2	8 ± 3	Not determined
HCl, pH 3.5	3.5 ± 0.1	35 ± 0	32 ± 1
HCl, pH 3.8	3.8 ± 0.3	35 ± 6	30 ± 6
HCl, pH 6.7	6.7 ± 0.1	10 ± 2	7 ± 0

a: Abbreviations are as follows: EDTA, ethylenediaminetetraacetic acid; LPS, lipopolysaccharide.

berry are presumed to be responsible for their strong antimicrobial activity (16,48). In the present study, *Candida albicans* NCPF 3179 and *Campylobacter jejuni* E-1008^T were sensitive only to cloudberry, raspberry, and strawberry extracts, suggesting that ellagitannins are the main antimicrobial compounds against these microbes.

Antimicrobial activity of cranberry has been of interest, especially against *H. pylori* (20–22) and *E. coli* causing urinary tract infections (24,29,53,54). The inhibitory agent of *H. pylori* was reported to be a high molecular weight constituent of cranberry with the main function of preventing adhesion of bacterial cells to human gastric mucus (21,55). Cunningham and coworkers (23) reported that cranberry proanthocyanidins are responsible for anti-adhesion of *H. pylori*. The same phenolics were shown to prevent adhesion of *E. coli* associated with urinary tract infections (23,24). In addition, as an anti-adhesive effect, consumption of cranberry juice may reduce the formation of biofilms by gram-positive and -negative bacteria on uroepithelial cells (25). In the present study, cranberry extract was strongly antimicrobial against gram-positive bacteria *B. cereus* E-727, *Clostridium perfringens* E-861^T, and *Staphylococcus epidermidis* E-768^T. Our earlier studies showed a bacteriocidic effect of lyophilized cranberry against *Salmonella* sv. Typhimurium, *Staphylococcus aureus*, and *Listeria monocytogenes*, and phenolic cranberry extract was strongly bacteriocidic against *S. aureus* (16). Phenolic extracts enriched in cranberry pomace showed antimicrobial activity against several pathogenic bacteria, such as *L. monocytogenes*, *Vibrio parahaemolyticus*, and *E. coli* O157:H7 (18,19). Part of the antibacterial effect of cranberry may be caused by the low pH of the berry (16), but phenolic compounds such as proanthocyanidins may also be involved. Cranberry extract was not tested in the present study with *H. pylori*, but this strain was extremely sensitive against all tested berry extracts in liquid culture. The studies with anti-adhesive and antimicrobial compounds in berry extracts against *H. pylori* will continue.

The antimicrobial effect observed by decreasing plate count numbers of *Salmonella* Typhimurium E-1151 resulted partly from immobilization of viable bacterial cells by the cloudberry, strawberry, and bilberry extracts. In *Staphylococcus aureus* E-045 cultures treated with cloudberry and strawberry extracts, there were only few viable cells detected by plate count after incubation of 24 h, and dead cells were observed attaching to berry material by fluorescence viability staining. Adhesion of microorganisms to biological and inert surfaces is a vital prerequisite for successful microbial colonization and infection (56–58). For example, *Staphylococcus* strains causing severe chronic infections via catheter materials develop resistance to antibiotics and immune systems in biofilms, in which they are nearly invulnerable against treatments (57). Our finding of the capability of phenolic strawberry and cloudberry extracts to immobilize and kill staphylococcal cells could be used in further studies and development of pharmaceuticals and treatments against staphylococcal infections. GI tract infections caused by salmonella are initiated by attachment of pathogenic bacterial cells to human intestinal mucus (59). Inactivation and even short-term immobilization of viable salmonella cells by berry extracts may prevent their adhesion in human gut.

According to Mullen and coworkers (60), freshly picked and frozen raspberries stored for a few days contain similar levels of phenolic compounds, including ellagitannins, anthocyanins, and flavonols. However, there are only a few reports of the behavior of berry phenolics during long-term frozen storage, which is relevant for industrial use and households. The effects seem to vary in different berries and different compounds, and both increase and decrease in contents of phenolic compounds have been reported during storage (10,11). In this study, the common trend was the decrease of phenolics in berry extracts during the test period of 12 mo (Table 3). Anthocyanins in bilberries (32% reduction) were better preserved than anthocyanins in red raspberries (43% reduction) during 12 mo of frozen storage. de Ancos and coworkers (61) studied the stability of raspberry anthocyanins during freezing and frozen storage. They found that the effects were cultivar dependent. Early cultivars were less affected by the freezing process and long-term frozen storage for 1 yr at –20°C compared with late cultivars. The total amount of anthocyanins showed a 5–17% increase in early cultivars, whereas, in the late cultivars, a decrease of the total anthocyanin content of 4–17.5% was measured (61). Cyanidin 3-glucoside most easily suffered the degradative reactions that took place during processing and the storage period (61).

Compared with the levels of anthocyanins as well as ellagitannins in some berries, the amount of flavonols, such as quercetin, was low in the berries investigated in the present study. However, no significant loss of flavonols was detected in red raspberries compared with bilberry flavonols (65% reduction) during 12 mo of storage. Häkkinen and coworkers (10) have studied the effects of freezing and freezer storage on flavonol contents of five berries. The effects varied among berries. In strawberry the amount of quercetin even increased during 9 mo of storage, and one explanation could be that in frozen strawberries quercetin is more easily extractable. This might be due to degradation of cell structures during storage. An increase in flavonol content for strawberries during storage in the refrigerator was reported by Gil and coworkers (9). Häkkinen and coworkers (10) found that quercetin was well preserved in frozen raspberries and black currants. Almost no changes were observed during 9 mo of storage, whereas quercetin contents in bilberries and lingonberries decreased by 40%. It was suggested that the high content of vitamin C in black currants and red raspberries might protect quercetin during storage in the freezer.

Berries, especially of the family Rosaceae, genus *Rubus* (red raspberry, arctic bramble, and cloudberry), are rich in ellagitannins. These berries and strawberry produce only ellagitannins based on stable glucose conformation. The ellagitannin monomers tend to form dimers, trimers, and even larger oligomers via dehydrogenation reactions. In raspberries (*R. idaeus* L.) the major ellagitannins have been

identified as dimeric sanquiin H-6 and trimeric lambertianin C (62–64). In the present study, the ellagitannins in cloudberries were better preserved during 12 mo of frozen storage compared with raspberry ellagitannins. However, this did not affect the antimicrobial activity of these berries, and both maintained their activity through the year. An unexpected increase in the total phenolic amount was detected in cloudberries, perhaps indicating structural changes during long-term storage in the ellagitannin molecule or in other phenolics present in the extract, with the nonspecific spectrophotometric assay responding to different amounts of hydroxyl groups available. The ellagitannins measured by HPLC were reduced by 25% in cloudberries and by 50% in red raspberries. Häkkinen et al. (11) reported that the content of ellagitannins measured as ellagic acid after hydrolysis was reduced by 40% in strawberries and by 30% in red raspberries after 9 mo of storage at –20°C, and, according to de Ancos et al. (65), the amount of ellagic acid in raspberries stored frozen decreased significantly (14–21%) during 1 yr. During freezer storage, ellagitannins may hydrolyze to form free ellagic acid. Thus, free ellagic acid may act as an antioxidant in berries due to its metal-chelating capacity and ability to react with free radicals (66), resulting in reduction in its total amount during storage. Bioprocessing of cranberry pomace using fungus mobilized especially ellagic acid, and the amount of soluble phenolics as well as antioxidant and antimicrobial activities in fermented pomace was increased (18,19). In this study, frozen storage did not have remarkable effects on antimicrobial activity of berry extracts, which is desirable from the point of view of the food and pharmaceutical industries.

Phenolic extracts of cloudberry and raspberry disintegrated the OM of examined *Salmonella* strains as indicated by NPN uptake increase and analysis of liberation of [^{14}C]Gal-LPS. Because $MgCl_2$ addition in the case of *S.* Typhimurium and *S.* Infantis abolished the majority of the OM-disintegrating activity of raspberry and cloudberry phenolic extracts in the NPN assay, part of the activity may occur by chelation of divalent cations from the OM or intercalation into the OM with the replacement of stabilizing cations. This activity is similar to the activity of EDTA (35,47). Puupponen-Pimiä and coworkers (16) reported that phenolic extracts of raspberry and cloudberry inhibited growth of *Salmonella* in the beginning of the incubation, and during prolonged incubation regrowth occurred. *Salmonella* Typhimurium SH5014 cells produce rough LPS of chemotype Rb2 and lack the O-specific chain and the outer-core oligosaccharides in their LPS (36). Phenolic extracts of cloudberry and raspberry and gallic acid treatments disintegrated the OM of *S.* Typhimurium SH5014 cells, and a significant NPN uptake was detected, suggesting that the outer core and the O-specific chain played no critical role in the effects caused by these substances.

Gallic acid effectively permeabilized the tested *Salmonella* strains, and a significant increase in the NPN uptake was recorded. The OM-disintegrating activity of gallic acid is suggested to be based on chelation of divalent cations from the OM because $MgCl_2$ addition decreased the NPN uptake. Vattem and coworkers (19) proposed that antimicrobial activity of cranberry pomace was mainly caused by gallic acid because there was a high correlation between gallic acid concentration and antimicrobial activity of cranberry pomace. They also suggested that partial hydrophobicity of the gallic acid would allow it to act efficiently on bacterial membranes destabilizing them. It has been reported that the phenolic essential oil compounds thymol and carvacrol have membrane-disturbing activities and cause significant liberation of OM components (38).

Phenolic extracts of black currant, cranberry, and bilberry did not increase the NPN uptake of tested *Salmonella* strains. However, in the LPS release assay, phenolic extract of black currant, cranberry, and bilberry efficiently released LPS from the *S.* Typhimurium E-1151 and *S.* Infantis E-738 strains. In this assay, the phenolic extracts caused a lower pH (pH 3.4–4.2) than that found in the NPN uptake assay (pH 5.5–5.8). Efficacy of the samples in the LPS release assay can partially be due to the weak organic acids present in the samples, which are known to be effective in an undissociated state (in pH below their pK_a values; between pH 3 and 5 for most weak organic acids) (67). Weak organic acids, such as citric and lactic acid, have been reported to permeabilize the OM of gram-negative bacteria (45,46,68). We have reported that phenolic extracts of black currant, lingonberry, cranberry, and buckthorn berry are not active against *Salmonella*, whereas corresponding lyophilized whole berries are effective and have growth-inhibiting activity (16). Because phenolic extracts of cloudberry and raspberry in the NPN assay destabilized the OM also at pH 5, these preparations are likely to contain other active compounds besides weak organic acids. The major phenolic class of berries of the *Rubus* genus (including raspberry and cloudberry) are hydrolyzable tannins (ellagitannins), with anthocyanins being the second most abundant. In addition, hydroxycinnamic acids and flavonols were minor phenolic classes (69).

Ellagitannin and anthocyanin fractions of raspberry and ellagitannin faction of cloudberry did not permeabilize or disintegrate the OM of the tested *Salmonella* strains because no NPN uptake increase or release of LPS was observed. Moreover, cloudberry ellagitannin fractions have only slight growth-inhibiting activity against *Salmonella* (16).

According to Viljakainen and coworkers (70), cultivar, seasonal, and geographic origin contribute to the characteristic sugar and acid composition of individual berries. The main acids of the northern region wild berry juices are invariably citric and malic acids, even though their concentrations varied widely from one berry to another. In addition, juices of lingonberry, cranberry, cloudberry, and black currant contained benzoic acid (70). Hydroxycinnamic acids, due to their propenoid side chain, are less polar than the corresponding hydroxybenzoic acids, and this property might facilitate the transport of these molecules across the cell membrane (71). The proposed mechanisms to explain the antimicrobial activity of tannin include inhibition of extracellular microbial enzymes, deprivation of the substrates required for mi-

crobial growth, or direct action on microbial metabolism through inhibition of oxidative phosphorylation or iron deprivation (52).

Conclusions

Phenolic berry extracts inhibit the growth of pathogens of the human GI tract and may therefore be used as therapeutic or disinfection compounds against these bacteria. Antimicrobial effects of phenolic extracts are stable during long-term frozen storage of berries, which is the prerequisite for industrial applications. Antimicrobial activity of berries is likely to be caused by multiple mechanisms and synergies because they contain various compounds, for example, weak organic acids, phenolic acids, and tannins and their mixtures of different chemical forms; therefore, antimicrobial effects of chemically complex compounds must be critically analyzed. However, information about antimicrobial mechanisms, such as disintegration of the OM, will aid the planned use of berry extracts in different applications. Further studies in conditions mimicking the food matrix or physiological conditions in humans and human clinical trials are needed to verify the mechanisms and antimicrobial activity of the compounds.

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