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# Weakening of *Salmonella* with Selected Microbial Metabolites of Berry-Derived Phenolic Compounds and Organic Acids

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Gram-negative bacteria are important food spoilage and pathogenic bacteria. Their unique outer membrane (OM) provides them with a hydrophilic surface structure, which makes them inherently resistant to many antimicrobial agents, thus hindering their control. However, with permeabilizers, compounds that disintegrate and weaken the OM, Gram-negative cells can be sensitized to several external agents. Although antimicrobial activity of plant-derived phenolic compounds has been widely reported, their mechanisms of action have not yet been well demonstrated. The aim of our study was to elucidate the role of selected colonic microbial metabolites of berry-derived phenolic compounds in the weakening of the Gram-negative OM. The effect of the agents on the OM permeability of *Salmonella* was studied utilizing a fluorescence probe uptake assay, sensitization to hydrophobic antibiotics, and lipopolysaccharide (LPS) release. Our results show that 3,4-dihydroxyphenylacetic acid, 3-hydroxyphenylacetic acid, 3-(3,4-dihydroxyphenyl)propionic acid (3,4-diHPP), 3-(4-hydroxyphenyl)propionic acid, 3-phenylpropionic acid, and 3-(3-hydroxyphenyl)propionic acid efficiently destabilized the OM of *Salmonella enterica* subsp. *enterica* serovar Typhimurium and *S. enterica* subsp. *enterica* serovar Infantis as indicated by an increase in the uptake of the fluorescent probe 1-*N*-phenylnaphthylamine (NPN). The OM-destabilizing activity of the compounds was partially abolished by $MgCl_2$ addition, indicating that part of their activity is based on removal of OM-stabilizing divalent cations. Furthermore, 3,4-dihydroxyphenylacetic acid, 3-hydroxyphenylacetic acid, and 3,4-diHPP increased the susceptibility of *S. enterica* subsp. *enterica* serovar Typhimurium strains for novobiocin. In addition, organic acids present in berries, such as malic acid, sorbic acid, and benzoic acid, were shown to be efficient permeabilizers of *Salmonella* as shown by an increase in the NPN uptake assay and by LPS release.

**KEYWORDS: Permeability; Gram-negative; *Salmonella*; phenolic compound; metabolite; organic acid**

## INTRODUCTION

Many difficult food contaminants and pathogens belong to the group of Gram-negative bacteria, some of these, especially members of the genus *Salmonella*, can cause serious gastrointestinal infections. Hence, their control in food production and food materials is important. Salmonellosis has been reported to be the most common food-borne bacterial disease in the world (*1*). In the United States it has been estimated that 1.4 million nontyphoidal *Salmonella* infections with 400 deaths occur annually (*2*). *Salmonella enterica* subsp. *enterica* serovar Typhimurium has been reported to be the serovar most frequently associated with human illness (*1*). *S. enterica* subsp. *enterica* serovar Infantis cases have been associated with the consumption of contaminated poultry meat (*1*, *2*). The emergence and spread of antimicrobial resistance among *Salmonella* serovars, including Typhimurium and Infantis, has become a serious health hazard globally (*1*, *3*). The cells of Gram-negative bacteria are surrounded by an additional membrane (outer membrane, OM), which provides the bacterium with a hydrophilic surface and functions as a barrier for many external agents (*4*−*7*). The effect is mainly caused by the presence and features of lipopolysaccharide (LPS) molecules in the outer leaflet of the membrane, resulting in an inherent resistance to hydrophobic antibiotics, detergents, and hydrophobic dyes in many Gram-negative bacteria (*6*, *8*). The LPS, the main virulence factor in Gram-negative bacteria, comprises three regions: lipid A (endotoxic principle and anchoring molecule in the OM), core oligosaccharide (heteropolysaccharide chain), and O-specific

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chain (highly variable O-antigen oligosaccharide consisting of repeating units) (*8*, *9*). The LPS can be separated by SDS−PAGE into distinct "ladder" patterns according to the number of repeating carbohydrate units (*10*). "Smooth-type" *Salmonella* express core LPS and long-chain LPS (S, smooth, derives from the colony morphology of the bacteria). Some bacteria inherently or as a result of mutation are called "rough" (R, derives from the rough colony morphology) and are called Ra to Re mutants according to the length of the remnant core region (*8*). Gram-negative bacteria regulate OM permeability characteristics with hydrophilic channels known as porins, which allow nutrients with relatively small molecular weight (<600) to enter the inner parts of the cell. These water-filled pores generally exclude the entry of hydrophobic substances (*4*, *8*). The influx of lipophilic compounds into Gram-negative 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 (*4*, *11*). 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 (e.g., permeabilizers) that disintegrate the LPS layer (*7*). Permeabilizers themselves may not be antimicrobial, but they can potentiate activity of other antimicrobial agents. The classical example is EDTA, which can disorganize and weaken the interactions between LPS molecules by chelating the OM-stabilizing divalent cations ($Ca^{2+}$ and $Mg^{2+}$) (*7*).

A group of secondary metabolites called phenolic compounds are abundant in fruit, vegetables, and berries. Several recent studies suggest that they have beneficial effects on human health (*12*−*18*). In addition, their antimicrobial activities are extensively studied (*19*−*21*). The daily intake of phenolic compounds has been estimated to be around 1 g (*12*). The main dietary sources of phenolic compounds are fruits, berries, and to a lesser extent vegetables and cereals (*12*, *14*). Berries are especially rich sources of phenolic compounds, which are classified into five main groups: flavonoids, phenolic acids, lignans, stilbenes, and polymeric tannins (*16*). Many berries, such as bilberry and black currant, are rich sources of anthocyanins, which give the dark red or blue color to the berry fruit. Some berries, such as cloudberry, raspberry, and strawberry, contain substantial amounts of polymeric ellagitannins. In addition, berries contain hydroxycinnamic acids (e.g., chlorogenic acid) and flavonol glyrosides (e.g., quercetin derivatives) (*15*, *22*−*25*).

A certain proportion of ingested secondary metabolites can escape absorption in the small intestine and undergo transformation by intestinal microbes (*12*−*14*, *26*, *27*). In vitro model studies with human fecal microbiota have identified several microbial metabolites, although the information is still limited. Gonthier et al. (*26*) identified 3-(3-hydroxyphenyl)propionic acid (3-HPP) and benzoic acid (BA) as major microbial metabolites of phenolic compounds (caffeic acid and its esters, chlorogenic acid, and caftaric acid). Several in vivo studies with isolated intestinal microbes have also identified microbial degradation products from phenolic compounds (*14*, *28*−*32*). A strictly anaerobic bacterium, *Eubacterium ramulus*, has been reported to cleave the ring system of several flavonols and flavones, generating the corresponding hydroxyphenylacetic and (hydroxyphenyl)propionic acids, respectively, as well as acetate and butyrate (*14*, *29*). Schoefer et al. (*31*) reported that a quercetin-degrading bacterium, *Clostridium orbiscindens*, converted quercetin and taxifolin to 3,4-dihydroxyphenylacetic acid. According to Schneider et al. (*33*), *Enterococcus casseliflavus* degraded quercetin 3-glucoside, although the importance of this species in flavonoid metabolism is presumably minimal due to the low incidence of this microbe in the human gastrointestinal tract (*29*).

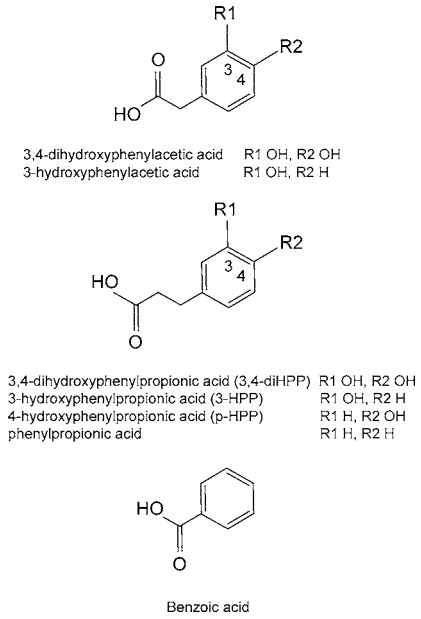



**Figure 1.** Chemical structures of microbial metabolites of phenolic compounds.

Certain small terpenoid and phenolic compounds found in herb plants (essential oil components) are known to disintegrate the OM of Gram-negative bacteria, releasing LPS and increasing the permeability for the cytoplasmic ATP (*34*, *35*). Relatively few data, however, are available concerning the antimicrobial mechanisms of berry-derived compounds or their microbial metabolites, although growth-inhibitory and antimicrobial activity of berries and phenolic berry extracts has been reported (*17*, *19*, *20*, *36*, *37*). In the present study our aim was to reveal some of the mechanisms involved in the antimicrobial activity of selected colonic microbial metabolites of berry-derived phenolic compounds and organic acids.

## MATERIALS AND METHODS

**Chemicals**. *n*-Heptadecanoic acid methyl ester (HEPES), 1-*N*-phenylnaphthylamine (NPN), lysozyme, sodium dodecyl sulfate (SDS), and Triton X-100 were from Sigma-Aldrich (Steinheim, Germany). D-[1-$^{14}$C]Galactose was from Amersham Pharmacia Biotech (Buckinghamshire, England) with a specific activity of 49.4 $\mu$Ci mmol$^{-1}$ and EDTA from Riedel-de-Haen (Seelze, Germany). A stock solution of NPN (0.5 M) was prepared in acetone and diluted to 40 $\mu$M in 5 mM HEPES (pH 7.2) for the fluorometric assays. Microbial metabolites 3,4-dihydroxyphenylacetic acid, 3-hydroxyphenylacetic acid, 3-(3,4-dihydroxyphenyl)propionic acid (synonyms 3,4-dihydroxyhydrocinnamic acid, hydrocaffeic acid, 3,4-diHPP), 3-(4-hydroxyphenyl)-propionic acid (pHPP), 3-phenylpropionic acid (synonyms hydrocinnamic acid, benzylacetic acid), and 2-hydroxyhexanoic acid (synonym 2-hydroxycaproic acid) were from Sigma-Aldrich, and 3-(3-hydroxyphenyl)-propionic acid (3-HPP) was purchased from AlfaAesar (Karlsruhe, Germany). See **Figure 1** for the structures of the metabolites. Organic acids, benzenecarboxylic acid (benzoic acid), 2,4-hexadienoic acid (sorbic acid), 2-hydroxypropionic acid (lactic acid), and *dl*-hydroxybutanedioic acid (malic acid) were from Sigma-Aldrich.

**Test Strain and Growth Conditions.** For the permeability studies *S. enterica* subsp. *enterica* serovar Typhimurium VTT E-981151 and VTT E-012041 (SH5014, an *rfaJ* mutant producing rough LPS of chemotype Rb2; *38*), and *S. enterica* subsp. *enterica* serovar Infantis VTT E-97738 strains were used. The working cultures were stored at −70 °C and cultured on trypticase soya agar (TSA; Oxoid, Bakingstoke,

U.K.) at 37 °C. For permeability assays cells were grown in Luria−Bertani (LB) broth as described by Helander et al. (*39*). Cultivations were carried out at 37 °C with shaking (200 rpm unless otherwise stated). Growth was monitored by measuring $A_{630}$ with a Multiskan MCC/340 spectrophotometer (ThermoLabSystems, Helsinki, Finland). Further details of cell treatments are given below under various experimental settings.

**Permeability Assays.** Two different methods were utilized to determine permeability properties of the OM: (i) NPN uptake and (ii) susceptibility to hydrophobic antibiotics.

*NPN Uptake.* NPN is a hydrophobic probe whose quantum yield is greatly enhanced in a glycerophospholipid environment compared with an aqueous environment. Uptake of NPN by bacterial membranes is manifested as fluorescence, and it indicates damage in the Gram-negative OM, which normally is able to exclude hydrophobic substances (*39*). The uptake of the hydrophobic fluorescent probe NPN by bacterial cells in buffer suspensions was measured using black fluorotiter plates (ThermoLabSystems) and the automated fluorometer Fluoroskan Ascent FL (ThermoLabSystems) as described earlier (*39*, *40*). Briefly, cells grown to $OD_{630} = 0.5 \pm 0.02$ were harvested by centrifugation (1000*g*, 10 min, room temperature) and suspended in a half-volume of 5 mM HEPES buffer (pH 7.2). Aliquots (100 $\mu$L) of this cell suspension were pipetted into fluoroplate wells, which contained NPN (10 $\mu$M) and as test substances EDTA (1.0 and 0.1 mM), HEPES buffer (control pH 7.2), microbial metabolites of phenolic compounds (10 mM, pH 4.0; 2.5 mM, pH 5.0), 2-hydroxyhexanoic acid (5 mM, pH 4.0), lactic acid (5 mM, pH 4.0), malic acid (10 mM, pH 4.0), benzoic acid (5 mM, pH 4.0), sorbic acid (5 mM, pH 4), or HCl (pH 4.0 and 5.0) to make up a total volume of 200 $\mu$L. If desired, $MgCl_2$ was added to the cell suspension before addition of NPN. 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.

*Antibiotic Susceptibility.* The susceptibility of bacterial cultures to hydrophobic antibiotics was tested with the agar diffusion method on Iso-Sensitest agar (Oxoid) with or without 3,4-dihydroxyphenylacetic acid, 3-hydroxyphenylacetic acid, 3,4-diHPP, or pHPP, using Neo-Sensitabs (clindamycin, 25 $\mu$g; fucidin, 100 $\mu$g; novobiocin, 100 $\mu$g; Rosco Diagnostica) according to Helander et al. (*41*). The diameters of inhibition zones were measured after incubation of the plates at 37 °C for 24 h. All determinations were performed in duplicate, and the results are presented as mean values.

**LPS Release.** The effect of phenolic metabolites on LPS release was studied using radiolabeled LPS. Smooth *S. enterica* subsp. *enterica* serovar Infantis VTT E-97738 cells were grown in LB 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]galactose (0.1 $\mu$Ci mL$^{-1}$) to label their LPS. Labeling with [$^{14}$C]galactose was performed for 10 min at 37 °C with shaking (200 rpm). The release of [$^{14}$C]Gal−LPS was monitored according to Alakomi et al. (*42*). Briefly, harvested and washed cells were resuspended in 10 mM Tris/HCl buffer (pH 7.2) at room temperature, divided into portions in Eppendorf tubes, and subjected to treatments with EDTA (1.0 mM), microbial metabolites of phenolic compounds (2.5 mM), organic acids (malic, lactic, and benzoic acids), or HCl (pH 4.0 or 5.0) for 10 min at 37 °C with shaking (100 rpm). Samples were taken for radioactivity measurements, and the suspensions were centrifuged (11000 rpm for 2 min) 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 to the radioactivity of a similar volume of untreated and uncentrifuged bacterial suspensions. Each assay was performed three times. In addition to the release of [$^{14}$C]Gal−LPS, the effect of phenolic metabolites, EDTA, and HCl (pH 5.0) on the LPS release was monitored with SDS−PAGE from cell-free supernatants of *S. enterica* subsp. *enterica* serovar Typhimurium E-981151 and *S. enterica* subsp. *enterica* serovar Infantis E-97738 strains according to Helander et al. (*34*).

**Bacteriolysis.** Sensitization of target cells to the lytic action of lysozyme and the detergents SDS and Triton X-100 by microbial metabolites of phenolic compounds was investigated according to the method described in detail by Helander et al. (*41*). Briefly, bacteria at standardized $OD_{630} = 0.5 \pm 0.02$ were subjected to treatments with microbial metabolites of phenolic compounds (2.5 mM) for 10 min at room temperature and added to microtiter plate wells which already contained lysozyme (10 $\mu$g mL$^{-1}$), Triton X-100 (0.1% and 1%), or SDS (0.1% and 1%). The turbidity of the cell suspensions was then monitored with the Multiskan MCC/340 spectrophotometer (Thermo-LabSystems). The value of the control without added lysozyme or detergent was taken as 100%; lower percentages indicate lytic action. Each assay was performed in quadruplicate. Three independent lysis tests were performed.

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

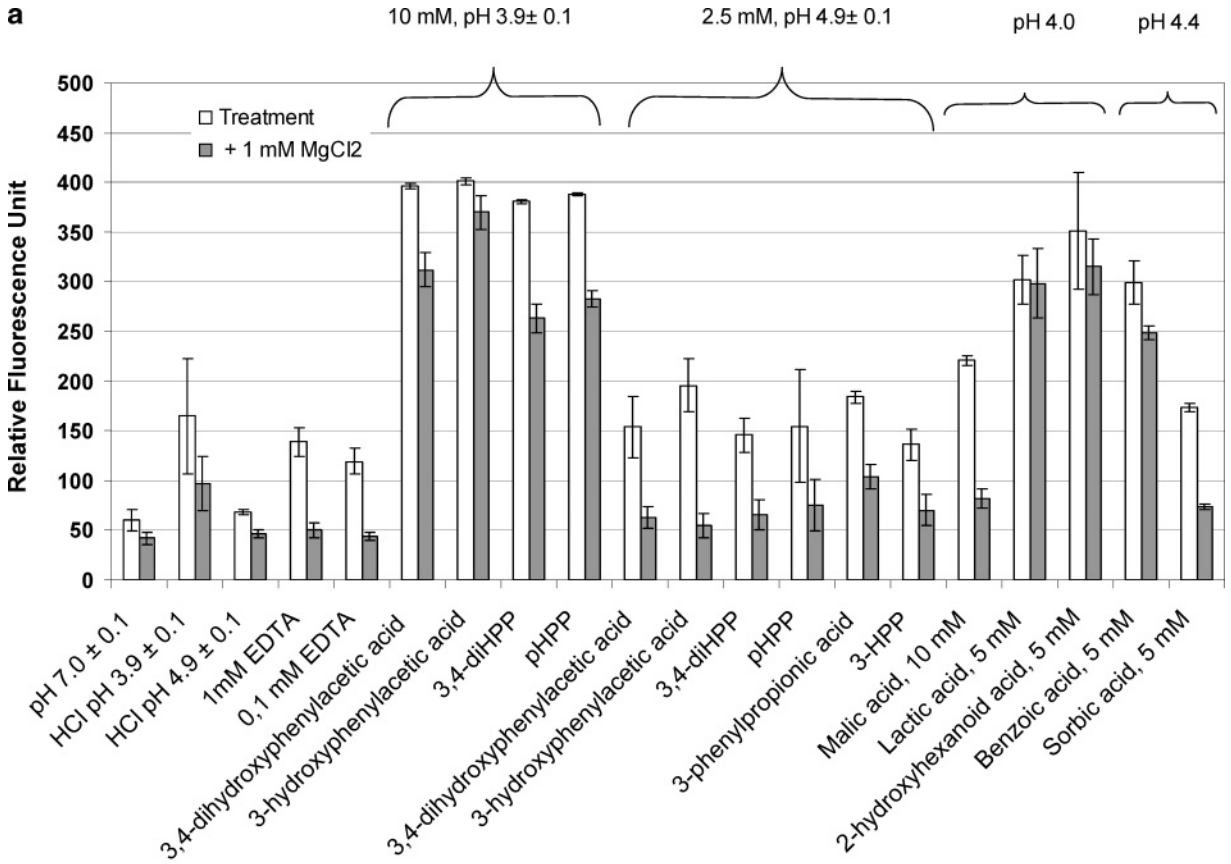
## RESULTS

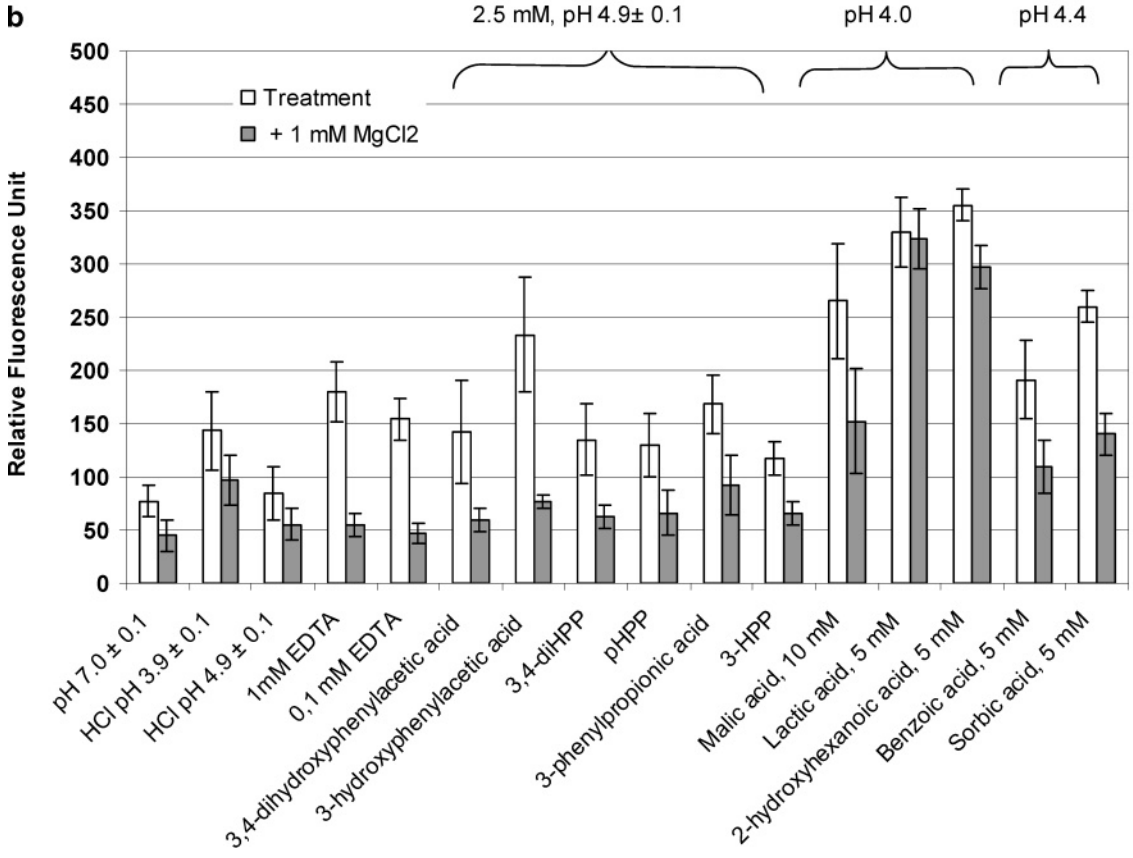
**Effect of Tested Compounds on the Uptake of NPN.** To reveal changes in OM permeability, two smooth-type *S. enterica* strains and one rough-type (LPS chemotype Rb2) *S. enterica* strain were selected for NPN uptake studies. The detailed results of the NPN uptake experiments with the treatments, including the effect of addition of $MgCl_2$ in the assay buffer, are shown in **Figure 2**. EDTA, the classical chelator, caused significant ($p < 0.001$) NPN uptake in the smooth *S. enterica* subsp. *enterica* serovar Typhimurium E-981151 and *S. enterica* subsp. *enterica* serovar Infantis E-97738 cells at concentrations of 0.1 and 1.0 mM. However, the NPN uptake of the rough mutant *S. enterica* subsp. *enterica* serovar Typhimurium E-012041 cells by EDTA was not increased compared with that of the control cells (**Figure 2c**).

3,4-Dihydroxyphenylacetic acid, 3-hydroxyphenylacetic acid, 3,4-diHPP, and pHPP (already at 2.5 mM (pH 5.0) test concentration) destabilized and disintegrated the target bacteria and brought about a significantly higher NPN uptake than hydrochloric acid for all target bacteria. 3-Phenylpropionic acid and 3-HPP also destabilized the cells and significantly increased the NPN uptake ($p < 0.001$) of the cells. Malic acid, lactic acid, and 2-hydroxyhexanoic acid at pH 4.0 significantly ($p < 0.001$) increased the NPN uptake of all tested bacteria, lactic acid and 2-hydroxyhexanoic acid being more effective than malic acid. The response caused by malic acid (pH 4.0) in the rough mutant *S. enterica* subsp. *enterica* serovar Typhimurium E-012041 cells was lower than in the smooth target strains. Benzoic acid and sorbic acid at pH 4.4 also significantly ($p < 0.001$) increased the NPN uptake of the cells.

Divalent cations are known to inhibit the action of many outer membrane permeabilizers which act by chelation or replacement of divalent cations in the OM. We therefore tested whether $Mg^{2+}$ could affect the activity of the tested agents. Parts **a** and **b** of **Figure 2** show that, for the smooth strains, 1 mM $MgCl_2$ addition almost totally abolished the activity of EDTA. In addition, $MgCl_2$ addition abolished the permeabilizing activity of 3,4-dihydroxyphenylacetic acid, 3-hydroxyphenylacetic acid, 3,4-diHPP, and pHPP at a concentration of 2.5 mM for all target strains. With higher (10 mM) test agent concentrations 1 mM $MgCl_2$ addition only slightly decreased the NPN uptake of the cells. In addition, $MgCl_2$ addition only slightly abolished the activity of lactic acid, benzoic acid, and 2-hydroxyhexanoic acid. NPN uptake inducing activity of sorbic acid (5 mM) was also abolished by $MgCl_2$ addition.

**Antibiotic Susceptibility.** A sensitizing effect to hydrophobic antibiotics is one of the indications of OM-permeabilizing action. We tested the susceptibility of the target strains to a set of


a
10 mM, pH 3.9± 0.1
2.5 mM, pH 4.9± 0.1
pH 4.0
pH 4.4
Treatment
+ 1 mM MgCl2
Relative Fluorescence Unit
0
50
100
150
200
250
300
350
400
450
500
pH 7.0 ± 0.1
HCl pH 3.9 ± 0.1
HCl pH 4.9 ± 0.1
1mM EDTA
0,1 mM EDTA
3,4-dihydroxyphenylacetic acid
3-hydroxyphenylacetic acid
3,4-diHPP
pHPP
3,4-dihydroxyphenylacetic acid
3-hydroxyphenylacetic acid
3,4-diHPP
pHPP
3-phenylpropionic acid
3-HPP
Malic acid, 10 mM
Lactic acid, 5 mM
2-hydroxyhexanoid acid, 5 mM
Benzoic acid, 5 mM
Sorbic acid, 5 mM


b
2.5 mM, pH 4.9± 0.1
pH 4.0
pH 4.4
Treatment
+ 1 mM MgCl2
Relative Fluorescence Unit
0
50
100
150
200
250
300
350
400
450
500
pH 7.0 ± 0.1
HCl pH 3.9 ± 0.1
HCl pH 4.9 ± 0.1
1mM EDTA
0,1 mM EDTA
3,4-dihydroxyphenylacetic acid
3-hydroxyphenylacetic acid
3,4-diHPP
pHPP
3-phenylpropionic acid
3-HPP
Malic acid, 10 mM
Lactic acid, 5 mM
2-hydroxyhexanoic acid, 5 mM
Benzoic acid, 5 mM
Sorbic acid, 5 mM

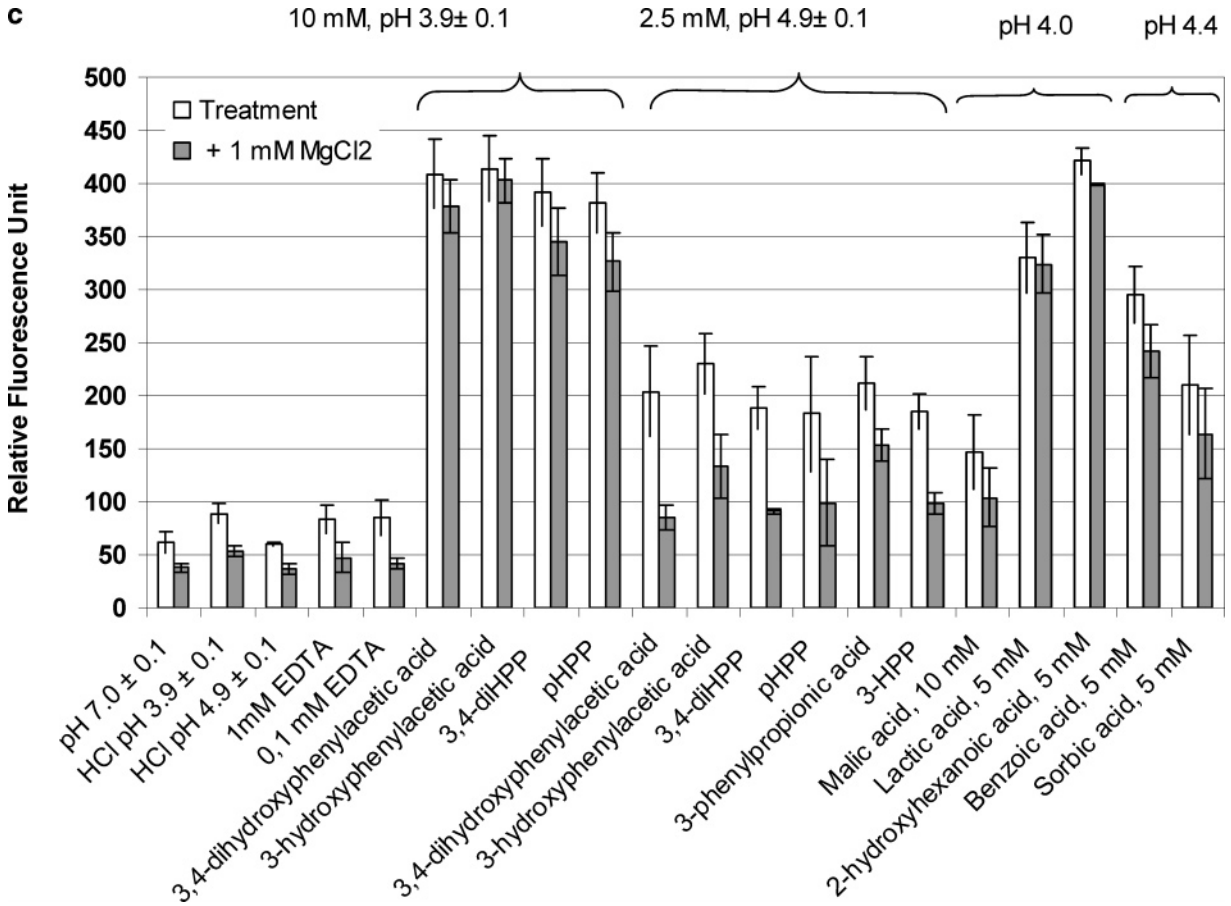



**Figure 2.** (**a**) NPN uptake of *S. enterica* subsp. *enterica* serovar Typhimurium E-981151. (**b**) NPN uptake of *S. enterica* subsp. *enterica* serovar Infantis E-97738. (**c**) NPN uptake of *S. enterica* subsp. *enterica* serovar Typhimurium E-012041.

hydrophobic antibiotics by the agar diffusion method on plates containing microbial metabolites of phenolic compounds. 3,4-Dihydroxyphenylacetic acid, 3-hydroxyphenylacetic acid, and 3,4-diHPP sensitized *S. enterica* subsp. *enterica* serovar Typhimurium E-981151 cells to novobiocin, the diameters of the inhibition zones being 12, 12, and 14 mm, respectively. *S. enterica* subsp. *enterica* serovar Infantis E-97738 cells were sensitized only by 3-hydroxyphenylacetic acid to novobiocin (diameter of the inhibition zone 11 mm). The rough *S. enterica* supsp. *enterica* serovar Typhimurium E-012041 cells were sensitized to novobiocin by 3,4-dihydroxyphenylacetic acid and 3,4-diHPP, the diameters of the inhibition zones being 12 and 11 mm, respectively. The susceptibility of target strains to clindamycin and fucidin was not enhanced.

**Release of LPS. Table 1** summarizes the results of specific labeling of [$^{14}$C]galactose−LPS and LPS release. EDTA-induced [$^{14}$C]Gal−LPS release in *S. enterica* subsp. *enterica* serovar Infantis E-97738 was 29 ± 8%. Treatment with 3,4-dihydroxyphenylacetic acid, 3-hydroxyphenylacetic acid, 3,4-diHPP, or pHPP (2.5 mM, pH 5.0) induced 10−13% release of [$^{14}$C]Gal−LPS from *S. enterica* subsp. *enterica* serovar Infantis E-97738, which was at the same level (10 ± 4 %) as in the treatment with hydrochloric acid (pH 5.0). Compared with the pH 7 treatment, benzoic acid, 2-hydroxyhexanoic acid, malic acid, lactic acid, and sorbic acid (pH 4.0) induced significant release of [$^{14}$C]Gal−LPS from *S. enterica* subsp. *enterica* serovar Infantis E-97738. The induced [$^{14}$C]Gal−LPS releases for the treatments were 32 ± 2%, 34 ± 4%, 39 ± 3%, 33 ± 1%, and 33 ± 2%, respectively. The amount of malic acid-induced [$^{14}$C]-Gal−LPS release was significantly higher ($p < 0.02$) compared with the hydrochloric acid (pH 4.0)-induced release. However, no significant difference for other organic acid treatments compared with the hydrochloric acid (pH 4.0) treatment was observed. SDS−PAGE analysis of cell-free culture supernatants of treated cells revealed a ladder pattern typical for smooth-type LPS for *S. enterica* subsp. *enterica* serovar Typhimurium E-981151 and *S. enterica* subsp. *enterica* serovar Infantis E-97738 strains. Supernatants of phenolic metabolite-treated cells revealed a ladder pattern that stained with intensity similar to that of the ladder pattern of the EDTA and HCl supernatants.

**Table 1.** [$^{14}$C]Gal–LPS Release from *S. enterica* Subsp. *enterica* Serovar Infantis VTT E-97738

| additive to assay buffer | pH | release of LPS (% ± SD) |
|---|---|---|
| pH 7.2 | 7.1 ± 0.1 | 4 ± 1 |
| EDTA, 1 mM | 7.1 ± 0.1 | 29 ± 8[a] |
| HCl, pH 5.0 | 4.9 ± 0.1 | 10 ± 4 |
| 3,4-dihydroxyphenylacetic acid, 2.5 mM | 4.9 ± 0.1 | 13 ± 2 |
| 3,4-diHPP, 2.5 mM | 4.9 ± 0.1 | 11 ± 3 |
| pHPP, 2.5 mM | 4.9 ± 0.1 | 11 ± 2 |
| 3-phenylpropionic acid, 2.5 mM | 4.9 ± 0.1 | 11 ± 4 |
| 3-HPP, 2.5 mM | 4.9 ± 0.1 | 10 ± 3 |
| HCl, pH 4.0 | 3.8 ± 0.2 | 28 ± 4 |
| benzoic acid, 5 mM | 4.0 ± 0.1 | 32 ± 2 |
| 2-hydroxyhexanoic acid, 5 mM | 3.9 ± 0.1 | 34 ± 4 |
| malic acid, 10 mM | 3.9 ± 0.1 | 39 ± 3[b] |
| lactic acid, 5 mM | 3.9 ± 0.1 | 33 ± 1 |
| sorbic acid, 5 mM | 4.0 ± 0.1 | 33 ± 2 |

[a] $p < 0.001$ compared to release from pH 7.2 cells. [b] $p < 0.02$ compared to release from pH 4.0 cells.

**Bacteriolysis.** To further investigate the permeabilization effect of microbiological metabolites of phenolic compounds,

their effect on the sensitivity of *Salmonella* toward lysozyme and the detergents SDS and Triton X-100 was measured. *S. enterica* subsp. *enterica* serovar Typhimurium E-981151 was not sensitized to the lytic activity of the tested compounds at a 2.5 mM concentration. No significant difference compared with the control treatment was observed. Relative turbidities in the lysozyme-treated control and metabolite-treated cells were 101 ± 2 and 103 ± 2, respectively. With Triton X-100 (1%) treatment the relative turbidities of the control and metabolite-treated cells were 108 ± 0 and 107 ± 2, respectively. Similar results were obtained with 1% SDS (relative turbidities in the control and metabolite-treated cells were 98 ± 2 and 99 ± 5, respectively).

## DISCUSSION

The results obtained in the NPN uptake assay demonstrate that 3,4-dihydroxyphenylacetic acid, 3-hydroxyphenylacetic acid, 3,4-diHPP, pHPP, 3-phenylpropionic acid, and 3-HPP, which are known colonic microbial metabolites of phenolic compounds, destabilized and disintegrated the target *Salmonella* strains. *S. enterica* subsp. *enterica* serovar Infantis E-97738 was less sensitive than *S. enterica* subsp. *enterica* serovar Typhimurium E-981151 cells, a phenomenon which possibly is related to differences in the respective LPS structures. Since $MgCl_2$ addition abolished the majority of the OM-disintegrating activity of 3,4-dihydroxyphenylacetic acid, 3-hydroxyphenylacetic acid, 3,4-diHPP, and pHPP at a 2.5 mM concentration, part of their activity may occur by chelation of divalent cations from the OM or intercalation into the OM with the replacement of stabilizing cations. Certain polyphenols (i.e., tannins and gallic acid) have been reported to form complexes with cations and to chelate ferrous ions (*12*, *43*, *44*). Since the *S. enterica* subsp. *enterica* serovar Typhimurium E-012041 (SH5014) cells, which produce rough LPS of chemotype Rb2 and lack the O-specific chain and most of the outer core oligosaccharides in their LPS, were also destabilized by these compounds, our results indicate that the outer core and the O-specific chain played no significant role in the effects caused by these substances. This is in accord with the general conclusion that charged regions in the lipid A and inner core oligosaccharide are chiefly responsible for maintenance of the barrier function in the OM (*4*, *7*, *8*).

3,4-Dihydroxyphenylacetic acid and 3,4-diHPP sensitized *S. enterica* subsp. *enterica* serovar Typhimurium cells for novobiocin, whereas only 3-hydroxyphenylacetic acid sensitized *S. enterica* subsp. *enterica* serovar Infantis for novobiocin. This suggests that dihydroxy acids can disintegrate the OM of *S. enterica* subsp. *enterica* serovar Typhimurium more strongly than acids with one hydroxyl group. However, with *S. enterica* subsp. *enterica* serovar Infantis this phenomenon was not observed either in the antibiotic sensitization or in the LPS release assay. We earlier reported that lactic acid (another hydroxy acid) acts as a permeabilizer of Gram-negative bacteria (*40*). Veldhuizen et al. (*45*) recently reported that the hydroxyl group of carvacrol affects its antimicrobial mode of action.

In the LPS release assay the microbial metabolites induced 10−13% release of [$^{14}$C]Gal−LPS from *S. enterica* subsp. *enterica* serovar Infantis E-97738, which was at a level (10 ± 4%) similar to that in the treatment with hydrochloric acid (pH 5.0). On the basis of the visual estimation of the intensity of the silver-stained SDS−PAGE gel, LPS release from *S. enterica* subsp. *enterica* serovar Infantis was evident. In fact, with *S. enterica* subsp. *enterica* serovar Typhimurium E-981151 visual inspection of the SDS−PAGE gels revealed higher amounts of LPS in the treatment supernatants compared with those of *S. enterica* subsp. *enterica* serovar Infantis. No sensitization to lysozyme was observed with the microbial metabolites at a 2.5 mM concentration. The permeation of lysozyme apparently requires extensive disorganization of the OM, such as that resulting from massive loss of LPS induced by EDTA (reviewed in refs *7* and *46*).

Phenolic extracts of berries have been shown to exhibit antimicrobial activity against Gram-negative bacteria (*19*, *20*). We earlier reported that phenolic extracts of cloudberry and raspberry disintegrated the OM of *Salmonella* strains as indicated by NPN uptake increase and analysis of liberation of [$^{14}$C]Gal−LPS (*29*). In addition, gallic acid disintegrated the OM of *Salmonella* (*37*).

Besides being rich in phenolic compounds, many berries also contain high amounts of organic acids (*47*). After studying six wild and five cultivated berries, Viljakainen et al. (*47*) reported that the main acids in juices from Nordic berries (e.g., bilberry, lingonberry, cranberry, cloudberry, red raspberry) were citric and malic acid, even though their concentrations varied widely from one berry to another (2.9−16.2 and 3.3−24.7 g $L^{-1}$, respectively). In addition, juices of lingonberry, cranberry, cloudberry, and black crowberry contained benzoic acid (0.1−0.7 g $L^{-1}$). Furthermore, sorbic acid has been detected from rowan berries (*48*). The amount of total organic acids in several Nordic berries has been reported to vary (1.4−3.1 g/100 g) (*49*). Earlier it was reported that a major part of the inhibitory effects of food preservative weak acids are related to the undissociated forms of acids which can penetrate cell membranes, causing perturbation of membrane function, acidification of the cytoplasm, and inhibition of acid-sensitive enzymes (*50*). Citric acid, an organic acid abundant in berries, was earlier shown to destabilize and permeabilize Gram-negative bacteria (*39*). The organic acids examined in this study, i.e., malic acid, lactic acid, benzoic acid, sorbic acid, and 2-hydroxyhexanoic acid, efficiently destabilized and disintegrated the OM of all target strains. Since $MgCl_2$ addition abolished the majority of the OM-disintegrating activity of malic acid, 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. However, besides weakening the OM, the examined organic acids also have other mechanisms of action, since destabilization was also observed with the rough LPS (type Rb2), producing an *S. enterica* subsp. *enterica* serovar Typhimurium mutant. In the LPS release assay malic acid induced slightly higher release of [$^{14}$C]Gal−LPS from *S. enterica* subsp. *enterica* serovar Infantis E-97738 compared with other organic acids. This observation further reinforces our earlier observation with lactic acid that hydroxy acids are effective permeabilizers of Gram-negative bacteria and may potentiate antimicrobial activity of other compouds (*40*). Recently, Zhao et al. (*51*) reported that various combinations of lactic acid, calcium sulfate, and sodium benzoate/butyric acid effectively inactivated enterohemorrhagic *Escherichia coli* in rumen content- or feces-contaminated drinking water for cattle. However, there have been reports that acid adaptation of pathogenic strains leads to increased resistance for antimicrobial agents and better survival in acidic conditions (*50*, *52*). Therefore, the effect of such adaptive responses on the permeability properties of Gram-negative bacteria should be studied.

The daily intake of berries and other plant materials containing phenolic compounds varies individually, causing variation in the dose of phenolic derivatives and their colonic microbial metabolites, but an estimate of about 1 g/day acquisition has

been made (*53*). In addition, the bioavailability of phenolic compounds varies greatly among various phenolic compounds (*13*, *53*). According to Manach et al., the plasma concentrations of total phenolic metabolites ranged from 0 to 4 $\mu$mol $L^{-1}$ with an intake of 50 mg of aglycon equivalents, and the relative urinary excretion ranged from 0.3% to 43% of the ingested dose, depending on the phenolic compound (*53*). Hence, a major part of the phenolic compounds ingested (75−99%) is not found in the urine (*12*, *53*), and phenolic compounds which are not absorbed in the small intestine will reach the colon (*13*). According to Scalbert and Williamson (*12*), the dilution of 500 mg of phenolic compounds with the digestive suspension in the colon would give a local concentration of 3 mM. Our data suggest (OM-destabilizing activity using a 2.5 mM test concentration) that microbial colonic metabolites of phenolic compounds may play a role in the defense of the GI tract against gastrointestinal pathogens. However, the physiological importance of this phenomenon requires further investigation. In addition, conditions in the lumen can affect the inhibitory activity of the compounds. The mild acidity conditions of the large intestine (pH 5.4; *54*) can be favorable for the permeabilizing action of phenolic metabolites. In addition, there may be microenvironments where the accumulation of colonic metabolites may be high enough for destabilization of the OM and potentiating activity of other antimicrobial agents.

## ABBREVIATIONS USED

EDTA, ethylenediaminetetraacetic acid; Gal, galactose; HEPES, *n*-heptadecanoic acid methyl ester; LPS, lipopolysaccharide; NPN, 1-*N*-phenylnaphthylamine; OM, outer membrane; SDS, sodium dodecyl sulfate; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; 3,4-diHPP, 3-(3,4-dihydroxyphenyl)propionic acid; pHPP, 3-(4-hydroxyphenyl)-propionic acid; 3-HPP, 3-(3-hydroxyphenyl)propionic acid.

## SAFETY

The work with pathogenic microbes was carried out in a biosafety laboratory (level 2) using good laboratory practice.

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## LITERATURE CITED

(1) Plym Forshell, L.; Wierup, M. *Salmonella* contamination: a significant challenge to the global marketing of animal food products. *Sci. Tech. Rev., OIE World Organ. Anim. Health* **2006**, *25*, 541−554.

(2) Voetsch, A. C.; Van Gilder, T. J.; Angulo, F. J.; Farley, M. M.; Shallow, S.; Marcus, R.; Cieslak, P. R.; Deneen, V. C.; Tauxe, R. V. FoodNet estimate of the burden of illness caused by nontyphoidal *Salmonella* infections in the United States. *Clin. Infect. Dis.* **2004**, *38*, S127−S134.

(3) Fonseca, E. L.; Mykytczuk, O. L.; Asensi, M. D.; Reis, E. M. F.; Ferraz, L. R.; Paula, F. L.; Ng, L. K.; Rodrigues, D. P. Clonality and antimicrobial resistance gene profiles of multidrug-resistant *Salmonella enterica* serovar *Infantis* isolates from four public hospitals in Rio de Janeiro, Brazil. *J. Clin. Microb.* **2006**, *44*, 2767−2772.

(4) Nikaido, H. Molecular basis of bacterial outer membrane permeability revisited. *Microbiol. Mol. Biol. Rev.* **2003**, *67*, 593−656.

(5) Nikaido, H. Multidrug efflux pumps of gram-negative bacteria. *J. Bacteriol.* **1996**, *178*, 5853−5859.

(6) Vaara, M.; Nurminen, M. Outer membrane permeability barrier in *Escherichia coli* mutants that are defective in the late acyltransferases of lipid A biosynthesis. *Antimicrob. Agents Chemother.* **1999**, *43*, 1459−1462.

(7) Vaara, M. Agents that increase the permeability of the outer membrane. *Microbiol. Rev.* **1992**, *56*, 395−411.

(8) Helander, I. M.; Mäkelä, P. H.; Westphal, O.; Rietschel, E. T. Lipopolysaccharides. In *Encyclopedia of molecular biology and molecular medicine*, 1st ed.; Meyers, R. A., Ed.; VCH: Weinheim, Germany, 1996; Vol. 3, pp 462−471.

(9) Trent, M. S.; Stead, C. M.; Tran, A. X.; Hankins, J. V. Diversity of endotoxin and its impact on pathogenesis. *J. Endotoxin Res.* **2006**, *12*, 205−223.

(10) Mansfield, L. P.; Forsythe, S. J. Demonstration of the Rb1 lipopolysaccharide core structure in *Salmonella* strains with the monoclonal antibody M105. *J. Med. Microbiol.* **2001**, *50*, 339−344.

(11) Tegos, G.; Stermitz, F. R.; Lomovskaya, O.; Lewis, K. Multidrug pump inhibitors uncover remarkable activity of plant antimicrobials. *Antimicrob. Agents Chemother.* **2002**, *46*, 3133−3141.

(12) Scalbert, A.; Williamson, G. Dietary intake and bioavailability of polyphenols. *J. Nutr.* **2000**, *130*, 2073S-2085S.

(13) Manach, C.; Scalbert, A.; Morand, C.; Rémésy, C.; Jiménez, L. Polyphenols: food sources and bioavailability. *Am. J. Clin. Nutr.* **2004**, *79*, 727−747.

(14) Blaut, M.; Schoefer, L.; Braune, A. Transformation of flavonoids by intestinal microorganisms. *Int. J. Vitam. Nutr. Res.* **2003**, *73*, 79−87.

(15) Häkkinen, S. H.; Karenlampi, S. O.; Heinonen, I. M.; Mykkänen, H. M.; Törrönen, A. R. Content of the flavonols quercetin, myricetin, and kaempferol in 25 edible berries. *J. Agric. Food Chem.* **1999**, *47*, 2274−2279.

(16) Puupponen-Pimiä, R.; Nohynek, L.; Alakomi, H. L.; Oksman-Caldentey, K. M. Bioactive berry compounds-novel tools against human pathogens. *Appl. Microbiol. Biotechnol.* **2005**, *67*, 8−18.

(17) Puupponen-Pimiä, R.; Nohynek, L.; Hartmann-Schmidlin, S.; Kähkönen, M.; Heinonen, M.; Määttä-Riihinen, K.; Oksman-Caldentey, K. M. Berry phenolics selectively inhibit the growth of intestinal pathogens. *J. Appl. Microbiol.* **2005**, *98*, 991−1000.

(18) Zadernowski, R.; Naczk, M.; Nesterowicz, J. Phenolic acid profiles in some small berries. *J. Agric. Food Chem.* **2005**, *53*, 2118−2124.

(19) Puupponen-Pimiä, R.; Nohynek, L.; Meier, C.; Kähkönen, M.; Heinonen, M.; Hopia, A.; Oksman-Caldentey, K. M. Antimicrobial properties of phenolic compounds from berries. *J. Appl. Microbiol.* **2001**, *90*, 494−507.

(20) Puupponen-Pimiä, R.; Nohynek, L.; Alakomi, H. L.; Oksman-Caldentey, K. M. The action of berry phenolics against human intestinal pathogens. *Biofactors* **2005**, *23*, 243−251.

(21) Scalbert, A. Antimicrobial properties of tannins. *Phytochemistry* **1991**, *30*, 3875−3883.

(22) Hukkanen, A. T.; Pölönen, S. S.; Kärenlampi, S. O.; Kokko, H. I. Antioxidant capacity and phenolic content of sweet rowanberries. *J. Agric. Food Chem.* **2006**, *54*, 112−119.

(23) Määttä-Riihinen, K. R.; Kamal-Eldin, A.; Törrönen, A. R. Identification and quantification of phenolic compounds in berries of *Fragaria* and *Rubus* species (family Rosaceae). *J. Agric. Food Chem.* **2004**, *52*, 6178−6187.

(24) Määttä-Riihinen, K. R.; Kamal-Eldin, A.; Mattila, P. H.; González-Paramás, A. M.; Törrönen, R. Distribution and contents of phenolic compounds in eighteen Scandinavian berry species. *J. Agric. Food Chem.* **2004**, *52*, 4477−4486.

(25) Määttä-Riihinen, K. R.; Kähkönen, M. P.; Törrönen, A. R.; Heinonen, I. M. Catechins and procyanidins in berries of vaccinium species and their antioxidant activity. *J. Agric. Food Chem.* **2005**, *53*, 8485−8491.

(26) Gonthier, M. P.; Remesy, C.; Scalbert, A.; Cheynier, V.; Souquet, J. M.; Poutanen, K.; Aura, A. M. Microbial metabolism of caffeic acid and its esters chlorogenic and caftaric acids by human faecal microbiota in vitro. *Biomed. Pharmacother.* **2006**, *60*, 536−540.

(27) Aura, A. M.; O'Leary, K. A.; Williamson, G.; Ojala, M.; Bailey, M.; Puupponen-Pimiä, R.; Nuutila, A. M.; Oksman-Caldentey, K. M.; Poutanen, K. Quercetin derivatives are deconjugated and converted to hydroxyphenylacetic acids but not methylated by human fecal flora in vitro. *J. Agric. Food Chem.* **2002**, *50*, 1725−1730.

(28) Braune, A.; Gutschow, M.; Engst, W.; Blaut, M. Degradation of quercetin and luteolin by *Eubacterium ramulus*. *Appl. Environ. Microbiol.* **2001**, *67*, 5558−5567.

(29) Schneider, H.; Blaut, M. Anaerobic degradation of flavonoids by *Eubacterium ramulus*. *Arch. Microbiol.* **2000**, *173*, 71−75.

(30) Schneider, H.; Simmering, R.; Hartmann, L.; Pforte, H.; Blaut, M. Degradation of quercetin-3-glucoside in gnotobiotic rats associated with human intestinal bacteria. *J. Appl. Microbiol.* **2000**, *89*, 1027−1037.

(31) Schoefer, L.; Mohan, R.; Schwiertz, A.; Braune, A.; Blaut, M. Anaerobic degradation of flavonoids by *Clostridium orbiscindens*. *Appl. Environ. Microbiol.* **2003**, *69*, 5849−5854.

(32) Schoefer, L.; Braune, A.; Blaut, M. Cloning and expression of a phloretin hydrolase gene from *Eubacterium ramulus* and characterization of the recombinant enzyme. *Appl. Environ. Microbiol.* **2004**, *70*, 6131−6137.

(33) Schneider, H.; Schwiertz, A.; Collins, M. D.; Blaut, M. Anaerobic transformation of quercetin-3-glucoside by bacteria from the human intestinal tract. *Arch. Microbiol.* **1999**, *171*, 81−91.

(34) Helander, I. M.; Alakomi, H. L.; Latva-Kala, K.; Mattila-Sandholm, T.; Pol, I.; Smid, E. J.; Gorris, L. G. M.; Wright, A. von Characterization of the action of selected essential oil components on Gram-negative bacteria. *J. Agric. Food Chem.* **1998**, *46*, 3590−3595.

(35) Burt, S. Essential oils: their antibacterial properties and potential applications in foods—a review. *Int. J. Food Microbiol.* **2004**, *94*, 223−253.

(36) Rauha, J. P.; Remes, S.; Heinonen, M.; Hopia, A.; Kähkonen, M.; Kujala, T.; Pihlaja, K.; Vuorela, H.; Vuorela, P. Antimicrobial effects of Finnish plant extracts containing flavonoids and other phenolic compounds. *Int. J. Food Microbiol.* **2000**, *56*, 3−12.

(37) Nohynek, L. J.; Alakomi, H. L.; Kähkönen, M. P.; Heinonen, M.; Helander, I. M.; Oksman-Caldentey, K. M.; Puupponen-Pimiä, R. H. Berry phenolics: antimicrobial properties and mechanisms of action against severe human pathogens. *Nutr. Cancer* **2006**, *54*, 18−32.

(38) Helander, I. M.; Kilpeläinen, I.; Vaara, M. Increased substitution of phosphate groups in lipopolysaccharides and lipid A of the polymyxin-resistant pmrA mutants of *Salmonella typhimurium*: a 31P-NMR study. *Mol. Microbiol.* **1994**, *11*, 481−487.

(39) Helander, I. M.; Mattila-Sandholm, T. Fluorometric assessment of gram-negative bacterial permeabilization. *J. Appl. Microbiol.* **2000**, *88*, 213−219.

(40) Alakomi, H. L.; Skyttä, E.; Saarela, M.; Mattila-Sandholm, T.; Latva-Kala, K.; Helander, I. M. Lactic acid permeabilizes gram-negative bacteria by disrupting the outer membrane. *Appl. Environ. Microbiol.* **2000**, *66*, 2001−2005.

(41) Helander, I. M.; Alakomi, H. L.; Latva-Kala, K.; Koski, P. Polyethyleneimine is an effective permeabilizer of gram-negative bacteria. *Microbiology* **1997**, *143*, 3193−3199.

(42) Alakomi, H. L.; Saarela, M.; Helander, I. M. Effect of EDTA on *Salmonella enterica* serovar Typhimurium involves a component not assignable to lipopolysaccharide release. *Microbiology* **2003**, *149*, 2015−2021.

(43) Lopes, G. K. B.; Schulman, H. M.; Hermes-Lima, M. Polyphenol tannic acid inhibits hydroxyl radical formation from Fenton reaction by complexing ferrous ions. *Biochim. Biochys. Acta* **1999**, *1472*, 142−152.

(44) Iwahashi, H. Some polyphenols inhibit the formation of pentyl radical and octanoic acid radical in the reaction mixture of linoleic acid hydroperoxide with ferrous iron. *Biochem. J.* **2000**, *346*, 265−273.

(45) Veldhuizen, E. J.; Tjeerdsma-van Bokhoven, J. L.; Zweijtzer, C.; Burt, S. A.; Haagsman, H. P. Structural requirements for the antimicrobial activity of carvacrol. *J. Agric. Food Chem.* **2006**, *54*, 1874−1879.

(46) Helander, I. M.; Mattila-Sandholm, T. Permeability barrier of the gram-negative bacterial outer membrane with special reference to nisin. *Int. J. Food Microbiol.* **2000**, *60*, 153−161.

(47) Viljakainen, S.; Visti, A.; Laakso, S. Concentrations of organic acids and soluble sugars in juices from Nordic berries. *Acta Agric. Scand., Sect. B* **2002**, *52*, 101−109.

(48) Sofos, J. N.; Busta, F. F. Sorbic acid and sorbates. In *Antimicrobials in Foods*; 2nd ed.; Davidson, P. M., Branen, A. L., Eds.; Marcel Dekker, Inc.: New York, 1993; Chapter 3, pp 49−94.

(49) Anonymous, Nutrition Unit, Finnish National Public Health Institute, Helsinki. Fineli. Finnish food composition database, release 7, 2007. http://www.ktl.fi/fineli/.

(50) Hirshfield, I. N.; Terzulli, S.; O'Byrne, C. Weak organic acids: a panoply of effects on bacteria. *Sci. Prog.* **2003**, *86*, 245−269.

(51) Zhao, T.; Zhao, P.; West, J. W.; Bernard, J. K.; Cross, H. G.; Doyle, M. P. Inactivation of enterohemorrhagic Escherichia coli in rumen content- or faeces-contaminated drinking water for cattle. *Appl. Environ. Microbiol.* **2006**, *72*, 3268−3273.

(52) Bjornsdottir, K.; Breidt, F., Jr.; McFeeters, R. F. Protective effects of organic acids on survival of *Escherichia coli* O157:H7 in acidic environments. *Appl. Environ. Microbiol.* **2006**, *72*, 660−664.

(53) Manach, C.; Williamson, G.; Morand, C.; Scalbert, A.; Rémésy, C. Bioavailability and bioefficacy of polyphenols in humans. I. Review of 97 bioavailability studies. *Am. J. Clin. Nutr.* **2005**, *81*, 230S-242S.

(54) Gee, J. M.; Wortley, G. M.; Price, K. R.; Johnson, L. T. Use of an in vitro model to assess factors influencing the effect of legume saponins on the intestinal epithelium. *COST 98. Proceedings of the sixth scientific workshop in Rome*, May 8−10, 1997; Carnovale, E., Ed.; European Commission: Brussels, 1999; pp 139−144.

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