Effects of Iron and Phytic Acid on Production of Extracellular Radicals by *Enterococcus faecalis*

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Enterococcus faecalis **is a human intestinal commensal that produces extracellular superoxide, hydrogen peroxide, and hydroxyl radical while colonizing the intestinal tract. To determine whether dietary factors implicated in colorectal cancer affect oxidant production by *E. faecalis*, radicals were measured in rats colonized with this microorganism while on diets supplemented with iron or phytic acid. Hydroxyl radical activity was measured by assaying for aromatic hydroxylation products of D-phenylalanine using reverse-phase high-performance liquid chromatography and electrochemical detection. *In vitro*, as expected, iron enhanced, and phytic acid decreased, hydroxyl radical formation by *E. faecalis*. For rats colonized with *E. faecalis* given supplemental dietary iron (740 mg elemental iron as ferric phosphate per kg diet) or phytic acid (1.2% w/w), no differences were found in concentrations of urinary *ortho*- or *meta*- isomers of D-phenylalanine compared to rats on a basal diet. Aqueous radicals in colonic contents were further assessed *ex vivo* by electron spin resonance using 5,5-dimethyl-1-pyrroline-*N*-oxide as a spin trap. Mixtures of thiyl (sulfur-centered) and oxygen-centered radicals were detected across all diets. *In vitro*, similar spectra were observed when *E. faecalis* was incubated with hydrogen sulfide, air-oxidized cysteine, or an alkylsulfide, as typical sulfur-containing compounds that might occur in colonic contents. In conclusion, intestinal colonization with *E. faecalis* in a rat model generates both thiyl and oxygen-centered radicals in colonic contents. Radical formation, however, was not significantly altered by short-term dietary supplementation with iron or phytic acid.** Exp Biol Med 229:1186–1195, 2004

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Received June 16, 2004.
Accepted September 13, 2004.

1535-3702/04/22911-1186$15.00

Key words: *Enterococcus faecalis;* free radicals; dietary iron; dietary phytic acid; aromatic hydroxylation; electron spin resonance spectroscopy

Each year on a global basis nearly one million persons are diagnosed with colorectal cancer (CRC), and of these more than half die from complications (1). The role of intestinal commensal flora in the etiology of CRC has gained increasing attention in recent years (2). One bacterium in particular, *Enterococcus faecalis*, has been suggested as a potential cause of CRC through the production of extracellular superoxide (2, 3). This anionic radical leads to other potent oxidants such as hydrogen peroxide and hydroxyl radical. These reactive oxygen species have been detected in the colonic contents of animals colonized by *E. faecalis* (4, 5) and may promote epithelial cell transformation through oxidative damage to DNA (3). However, it remains to be determined whether bacterially mediated oxidative stress on the intestinal epithelium plays a role in colorectal carcinogenesis.

This unique bacterial phenotype, *viz*, the production of extracellular radicals, is expressed by a majority of *E. faecalis* isolates from normal stool but few other intestinal commensals (6). *Enterococcus faecalis* strains that produce extracellular superoxide commonly colonize the human intestinal tract (6), although, overall, enterococci comprise <1% of the total fecal flora (7–9) and are not typically a predominant genus. *In vitro* and *in vivo* formation of extracellular superoxide by *E. faecalis* has been shown to damage colonic epithelial cell DNA, presumably *via* derivative hydrogen peroxide that diffuses into cells (10). DNA is readily oxidized by hydrogen peroxide through hydroxyl radical generation at sites where DNA is associated with iron (11):

$$Fe^{2+} + H^+ + H_2O_2 \rightarrow Fe^{3+} + {}^{\bullet}OH + H_2O$$

Other biologically damaging events, in addition to the Fenton reaction shown here, can be initiated by superoxide. For example, superoxide is known to cause peroxidation of polyunsaturated fatty acids in epithelial cell membranes. This, in turn, can lead to the formation of breakdown products like 4-hydroxynonenal and malondialdehyde that are freely diffusible and highly mutagenic (12).

Iron is an important catalyst in redox reactions and therefore a potential risk factor for CRC through pro-oxidant mechanisms (13–16). Dietary iron or iron taken therapeutically is largely unabsorbed by the intestine. Most passes into the colon, where, in theory, it could generate hydroxyl radical in the presence of superoxide or hydrogen peroxide. In models where animals are chronically exposed to supplemental iron, the peroxidation of intestinal epithelial cell lipid increases, and crypt cells show hyperproliferative changes (17, 18). Finally, epidemiological studies indicate, although not consistently, a greater risk for CRC with increasing dietary iron or total body iron (15). In contrast to iron as a potential risk factor for CRC, phytic acid or *myo*-inositol hexakisphosphate is a potential anticancer agent (19, 20). Although the effects of phytic acid are pleiotrophic, one prominent feature is its ability to chelate iron and inhibit the Fenton reaction (21). Ingested phytic acid may limit oxidant damage to the colonic epithelium by this mechanism.

In this study, we assessed the effects of iron and phytic acid on radical production by *E. faecalis in vitro* and as dietary supplements in colonized rats. Intestinal radicals were detected by aromatic hydroxylation of D-phenylalanine and through electron spin resonance (ESR) spin trapping. Our results indicate that iron and phytic acid, despite having significant *in vitro* effects on radical formation, showed minimal effects in colonized rats. Surprisingly, thiyl (or sulfur-centered) radicals, along with oxygen-centered species, were commonly observed in colonic contents, indicating that mixtures of radicals are formed by *E. faecalis* in the intestine.

Materials and Methods

Chemicals, Bacteria, Media, and Production of Extracellular Superoxide. Methanol, acetonitrile, and tetrahydrofuran were purchased from Fisher Scientific (Pittsburgh, PA). All other chemicals were of analytical reagent grade from Sigma (St. Louis, MO). *Enterococcus faecalis* strain OG1RFSS is a derivative of a human oral isolate (22) with spontaneous resistance to rifampin, fusidic acid, streptomycin, and spectinomycin (4, 23) and otherwise similar to members of this species capable of producing extracellular superoxide (6). Enterococci from intestinal contents were enumerated using bile-esculin-azide agar plates (Difco) as previously described (24). *In vitro* rates of extracellular superoxide production by *E. faecalis* were determined using the ferricytochrome *c* assay as previously described (4).

Intestinal Colonization Model. Young male Wistar rats in groups of 13–15 animals were fed *ad libitum* either basal (AIN-93M in pellet form with 34 mg iron per kg diet, ICN, Los Angeles, CA), iron supplemented (740 mg elemental iron [as ferric phosphate] per kg diet), or phytic acid supplemented (1.2% w/w) diets. Streptomycin and spectinomycin were added to drinking water (500 mg/L) following 11 days on basal or supplemental diets. Three days later, at Day 14, intestinal tracts were colonized with OG1RFSS *via* gastric intubation as previously described (3). Five days following colonization, rats were administered D-phenylalanine (150 mg/kg) by intraperitoneal injection. Sixty minutes later, urine samples were collected, pelleted to remove sediment, and injected onto a column for high-performance liquid chromatography (HPLC) analysis (see the following discussion). On the following day, rats were anesthetized using inhaled isoflurane and colons excised. Samples of colonic contents were assayed by ESR spin trapping, cultured to determine enterococcal concentrations, and tested for iron and peroxides.

Iron and Peroxide Assays. Plasma iron and iron in colonic contents were assayed using a minor modification of the method of Kok and Wild (25). Samples were initially acidified with concentrated thioglycolic acid and then 2 *M* HCl and diluted 1:2 (v/v) in saturated sodium acetate. Supernatants were diluted in distilled water 1:3.5 (v/v) and bathophenanthroline disulfonic acid added to 3 m*M*. Absorbance was measured at 536 nm after 30 secs. Hydrogen peroxide and hydroperoxides in colonic contents were determined by a glutathione peroxidase assay as previously described (3). In this assay glutathione peroxidase catalyses the reduction of peroxides using glutathione as a reductant (26). The assay couples recycling of glutathione by glutathione reductase to the oxidation of NADPH. Decreases in NADPH are followed spectrophotometrically at 340 nm using a molar extinction coefficient of 6.2×10^3 L/mol/cm. Azide was added to mixtures to inhibit catalase that can contaminate commercial preparations of glutathione peroxidase. Iron and peroxide concentrations for colonic contents were normalized to the gram wet weight of stool.

Aromatic Hydroxylation of D-Phenylalanine. Hydroxyl radical attack on D-phenylalanine can generate one of three isomers of tyrosine (*ortho*-, *meta*-, or *para*-). These products form under aerobic conditions when hydroxyl radical abstracts a hydrogen from the aromatic ring (27). Identification of these isomers can be used as a surrogate marker for hydroxyl radical production in cells, tissues, and animals (28). Because D-phenylalanine is not a substrate for phenylalanine hydroxylase, which is ordinarily found in tissues, it is considered a more specific target than L-phenylalanine. The assay, however, could still be confounded by racemases that convert chiral phenylalanine from the D- to L-enantiomer, with phenylalanine hydroxylase then producing *para*-tyrosine. The *para*- isomer is, therefore, of limited value in this assay. *Ortho*- and *meta*-

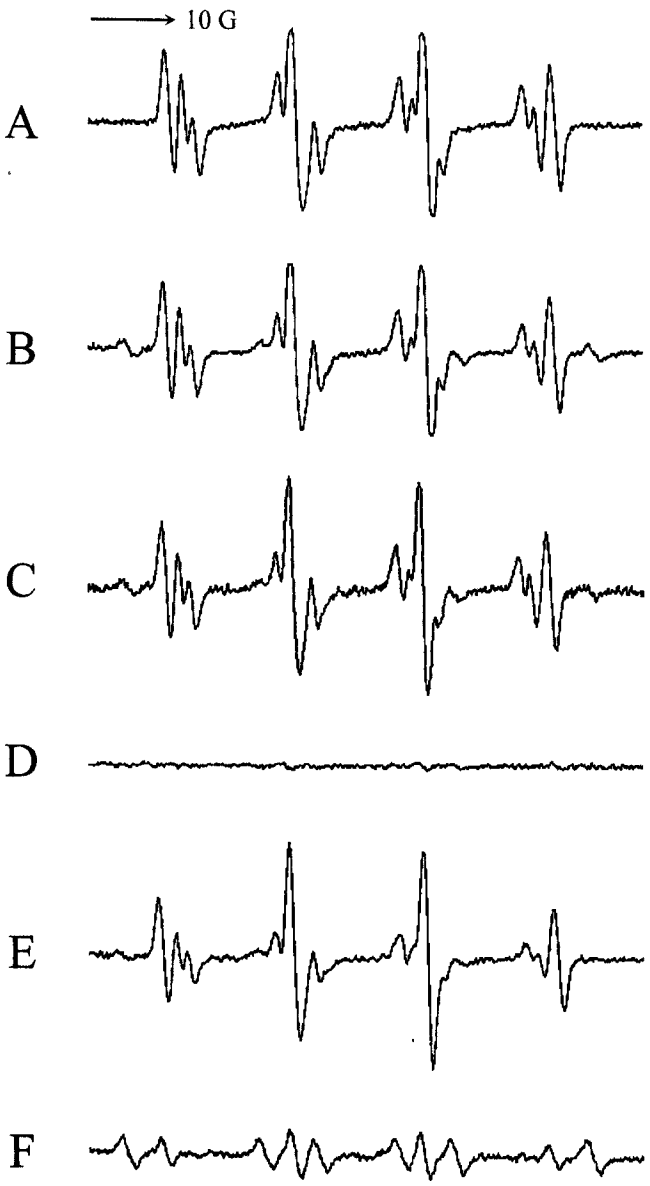

Figure 1. Electron spin resonance (ESR) spin trapping of superoxide-producing *Enterococcus faecalis*. No significant differences in spectra were noted for bacteria in (A) buffer containing 5 m*M* glucose, (B) 1000 μ*M* ferrous sulfate, or (C) 1000 μ*M* phytic acid. The typical 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) adduct for superoxide was confirmed by addition of 1200 units/ml of manganese-superoxide dismutase (D). Superoxide production was maintained for 60 mins (E) but not 150 mins (F). Instrument settings: 100-kHz field modulation, microwave power 20 mW, modulation amplitude 1.0 Gauss, sweep width 100 Gauss per 84 secs, and time constant 164 msecs. Arrow points upfield.

isomers do not occur in biological systems and should serve as specific markers for hydroxyl radical. Isomeric tyrosines in rat urine were separated by HPLC using a Microsorb C_{18} reverse-phase column (Varian, Inc., Palo Alto, CA). The mobile phase consisted of 6% tetrahydrofuran, 5% methanol, and 50 mmol lithium perchlorate at pH 3.5. Column effluent was run at 1.0 ml/min and analyzed using a Coularray electrochemical detection system (ESA, Chelmsford, MA) with the first detector set at 0.600 V. For tyrosine standards, integrated areas under peaks increased linearly up to 50 m*M*.

Table 1. *In Vitro* Hydroxylation of D-Phenylalanine by *Enterococcus faecalis*

	Hydroxylated D-phenylalanine (μ*M*)[a]		
Condition (μ*M*)[b]	*Ortho*-tyrosine	*Meta*-tyrosine	*Para*-tyrosine
Control	22 ± 2.6^{A,E}	5.6 ± 1.4^{A,E}	6.7 ± 1.3^{A,E}
Ferrous sulfate			
10	57 ± 2.3^{B}	27 ± 4.4^{B}	21 ± 1.9^{B}
50	88 ± 17^{C}	54 ± 5.4^{C}	28 ± 3.6^{B}
200	148 ± 39^{D}	131 ± 26^{D}	53 ± 12^{C}
P value[c]	<0.001	<0.001	<0.001
Phytic acid			
50	6.7 ± 0.5^{F}	3.0 ± 0.3^{F}	2.9 ± 0.3^{F}
200	4.1 ± 0.6^{G}	1.8 ± 0.3^{G}	1.8 ± 0.2^{G}
1000	4.8 ± 0.3^{G}	2.2 ± 0.2^{F,G}	2.0 ± 0.1^{G}
P value[d]	0.02	0.02	0.01

[a] Mean ± standard deviation (SD) for six independent experiments; for ferrous sulfate (*A–D*) and phytic acid (*E–G*), means within the same column without a common superscript are significantly different ($P < 0.05$).
[b] Washed *E. faecalis* resuspended at 10^9 cfu/ml in phosphate-buffered saline (pH 7.4) with 5 m*M* D-glucose and 50 m*M* D-phenylalanine; mixture incubated at 37°C for 60 mins.
[c] Comparisons by analysis of variance; for linear trends: *ortho*-, $R^2 = 0.76$; *meta*-, $R^2 = 0.92$; *para*-, $R^2 = 0.82$.
[d] Comparisons by analysis of variance.

ESR Spin Trapping. ESR spin trapping of live bacteria was performed as previously described using 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) as a spin trap (Aldrich, Milwaukee, WI; Ref. 4). The H_2S was generated by reacting 6 *M* HCl with ferrous sulfide pellets. A saturated solution (~6 m*M*) in Tris at pH 7.4 was prepared by bubbling H_2S through buffer. Similarly, the alkylsulfide 2-methyl-1-propanethiol was prepared by bubbling this volatile gas through buffer. An oxidized cysteine solution was prepared by exposing reduced cysteine to air for 48 hrs. The ESR spin trapping of colonic contents was performed by suspending 50–100 mg in 200 μl of 25 m*M* Tris at pH 7.4, clarifying by centrifugation, and mixing with DMPO to 40 m*M*. Spectra were recorded at ambient temperature (22°C) in a quartz flat cell using a Bruker

Table 2. Short-Term Intestinal Colonization with *Enterococcus faecalis*

	Colonic contents[a]		
Diet (no. of rats)	Enterococci ($\log_{10}$[cfu/g])	Peroxide (μ*M*)	Iron (μ*M*)
Basal (13)	9.25 ± 0.30	1.4 ± 0.6	510 ± 290^{A}
Iron (15)	9.75 ± 1.10	2.1 ± 0.9	1490 ± 1070^{B}
Phytic acid (15)	9.71 ± 1.00	1.6 ± 0.9	200 ± 60^{A}
P value	>0.10	>0.10	<0.0001

[a] Mean ± standard deviation (SD), comparisons by analysis of variance; means within the iron data without a common superscript are significantly different ($P < 0.05$).

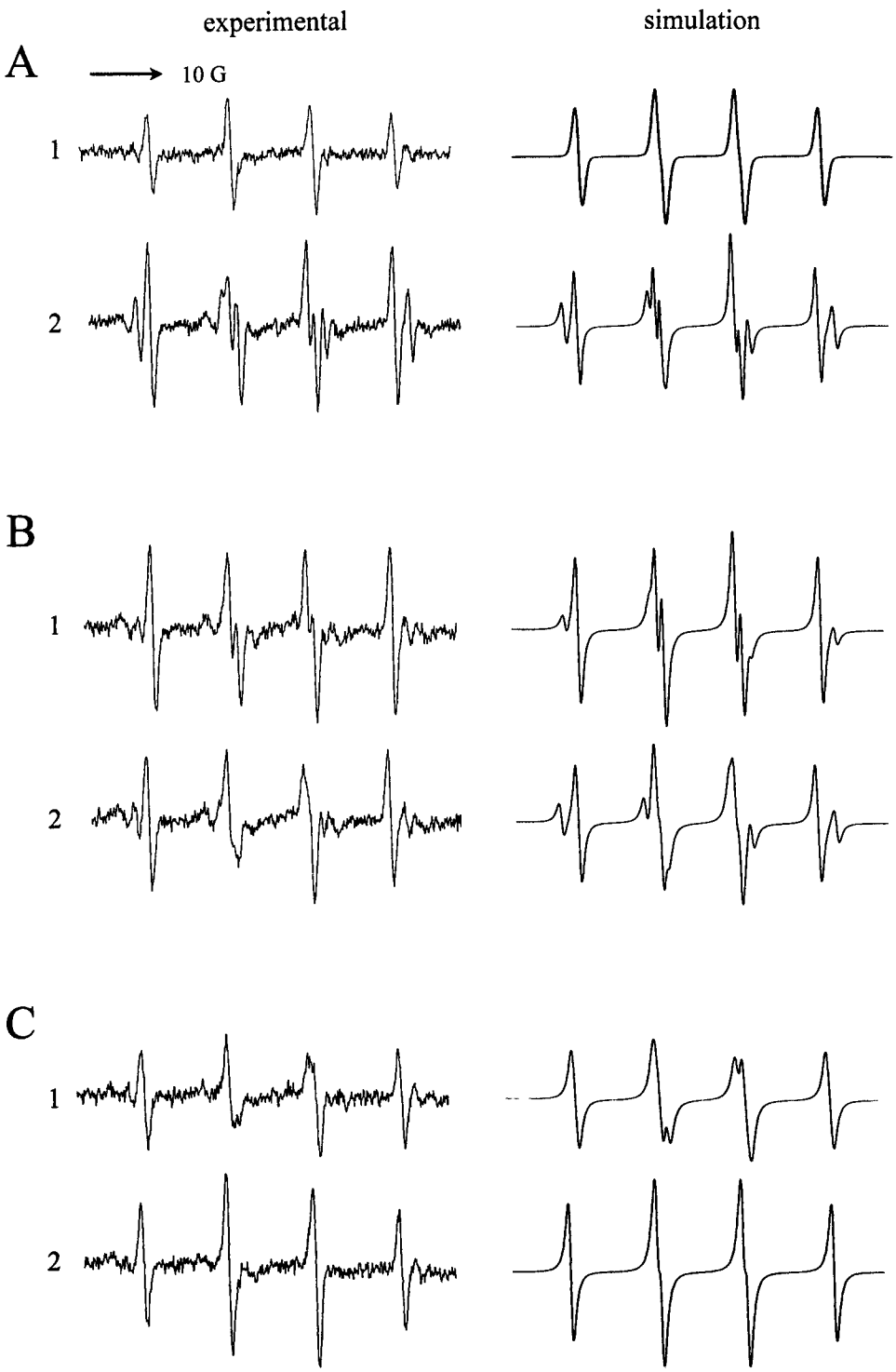

Figure 2. Electron spin resonance (ESR) spin trapping spectra of colonic contents from rats colonized with superoxide-producing *Enterococcus faecalis* showing oxygen-centered and thiyl radicals. Each panel shows two representative experimental spectra with corresponding computer simulations. In general, no differences were noted among spectra based on (A) basal, (B) iron-supplemented, or (C) phytic acid–supplemented diets. Hyperfine splitting constants for 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) adducts are consistent with mixtures (shown as %) of hydroxyl and thiyl radicals: A.1, Species 1 (87%): a_N = 15.0 G, a_H = 15.9 G and Species 2 (13%) a_N = 14.9 G, a_H = 14.9 G, g = 0.700; A.2, Species 1 (67%): a_N = 15.0 G, a_H = 16.0 G and Species 2 (33%) a_N = 16.3 G, a_H = 19.0 G, g = 0.350; B.1, Species 1 (12%): a_N = 16.3 G, a_H = 19.0 G, g = 0.150 and Species 2 (88%) a_N = 15.0 G, a_H = 16.3 G; B.2, Species 1 (22%): a_N = 16.2 G, a_H = 20.2 G, g = 0.150 and Species 2 (78%) a_N = 15.0 G, a_H = 15.9 G; C.1, Species 1 (50%): a_N = 15.8 G, a_H = 15.8 G, and Species 2 (50%) a_N = 15.2 G, a_H = 17.2 G; C.2, Species 1 (80%): a_N = 15.0 G, a_H = 15.9 G, and Species 2 (20%) a_N = 14.9 G, a_H = 14.9 G. Instrument settings: 100-kHz field modulation, microwave power 20 mW, modulation amplitude 1.0 Gauss, sweep width 100 Gauss per 84 secs, and time constant 164 msecs. Arrow points upfield.

ER300E X-band ESR spectrometer (Bruker Analytische Messtechnik GMBH, Rheinstetten, Germany) with the following parameters: 100-kHz field modulation, microwave power 20 mW, modulation amplitude 1.0 Gauss, sweep width 100 Gauss per 84 sec, and time constant 164 msecs. Spectra were simulated using WinSim software (http://epr.niehs.nih.gov/pest) and comparisons made through the NIEHS ESR spin-trap database (http://epr.niehs.nih.gov/stdb).

Statistical Analyses. Data are presented as the mean ± standard deviation (SD). Group comparisons were performed by analysis of variance and pairwise comparisons by the two-tailed Student *t* test (JMP Statistical Software, Version 5.0.1a, Cary, NC). *P* values <0.05 were considered significant.

Results

Enterococcus faecalis strain OG1RFSS produces copious extracellular superoxide, hydrogen peroxide, and hydroxyl radical (3, 5). The rate of superoxide production, as measured by the ferricytochrome *c* assay using glucose as a fermentable sugar, was 31 ± 1.3 μmol/min/10^9 cfu. At 15 and 30 mins, the rate decreased to 20 ± 0.1 and 13 ± 1.4 μmol/min/10^9 cfu, respectively. By 60 mins, despite ample glucose still remaining in the buffer, superoxide was no longer detectable. Similarly, ESR spin trapping detected DMPO adducts consistent with superoxide and hydroxyl radical. As with the ferricytochrome *c* assay, ESR signals were found for bacteria incubated with glucose for up to 1 hr but not beyond that time point (Fig. 1). Manganese superoxide dismutase eliminated adduct signals, confirming their origin from superoxide.

When iron or phytic acid was added to *E. faecalis*, no significant changes were observed in DMPO adducts or ESR signal strength (Fig. 1). Although ESR spin trapping is more sensitive for detecting superoxide than the ferricytochrome *c* assay (29), we were unable to measure kinetics by spin trapping because increasing signal intensity, which would have indicated ongoing superoxide production, was not seen. Radicals were readily detected immediately after addition of the spin trap, but increasing signal strength was not observed despite evidence for continuing superoxide production by the ferricytochrome *c* assay. Inhibition of spin trapping was not due to a soluble by-product since addition of supernatants from bacteria incubated with glucose did not affect spectra for freshly washed bacteria. Similarly, bacteria did not appear to degrade DMPO since spin trap incubated with bacteria still produced spectra when added to freshly washed bacteria. Furthermore, oxygen limitation did not explain these observations since spin trapping in an open flat cell, with supplemental oxygen, failed to alter adduct signals compared to spectra from closed flat cells. Superoxide and hydroxyl adducts of DMPO, however, are susceptible to decay that is facilitated by superoxide anions (30). This interplay may explain, in part, these findings since under these *in vitro* conditions there would be continued production of superoxide by *E. faecalis* for a period much longer than the duration of the spin-trapping experiments.

Because of these difficulties, we decided to examine the effects of iron and phytic on radical production by measuring the hydroxylation of D-phenylalanine (5). Initially, any potential toxicity of iron or phytic acid on *E. faecalis* strain OG1RFSS was assessed during overnight growth in BHI with 2000 μ*M* iron or 1000 μ*M* phytic acid. Growth inhibition was not observed, nor was superoxide production altered as measured by the ferricytochrome *c* assay (data not shown). Bacteria grown in BHI alone and subsequently washed were incubated in glucose-containing buffer with 0, 10, 50, or 200 μ*M* ferrous sulfate and 5 m*M* D-phenylalanine. After 60 mins, significant linear increases in isomeric tyrosines were observed (7- to 23-fold) compared to controls without added iron (Table 1). These results suggested that supplemental iron enhanced production of hydroxyl radical by *E. faecalis*, presumably *via* the Fenton reaction. Conversely, 50, 200, and 1000 μ*M* phytic acid significantly decreased concentrations of isomeric tyrosines at 60 mins (by approximately 2- to 4-fold), presumably by chelating trace metals in buffer and inhibiting the Fenton reaction (21). Trends for decreases in isomeric tyrosines, however, were not highly linear as were increases in these products for ferrous sulfate.

Next, the effect of short-term dietary supplementation with iron or phytic acid on free radical production by *E. faecalis* was examined using a rat colonization model. Neither iron- nor phytic acid–supplemented diets had any apparent effect on the density of enterococcal colonization or concentration of peroxide in colonic contents (Table 2). As expected, the iron-supplemented diet increased the concentration of iron in colonic contents compared to rats fed either the basal or the phytic acid–supplemented diets (Table 2). No statistically significant differences were noted in the average colonic iron content for phytic acid–supplemented compared to basal diets. To determine whether iron estimation may have been masked, in part, by an inability of the Kok and Wild assay to detect iron bound to phytic acid, phytic acid (50, 100, 200, or 1000 μ*M*) was added to a solution of 80 μ*M* ferrous sulfate and the iron assayed. Detectable iron was found to decrease 14%, 25%, 29%, and 36%, respectively, for these concentrations of phytic acid, indicating only a limited potential for interference. The average concentration of plasma iron for rats on the iron-supplemented diet was elevated (11.7 ± 2.8 μ*M*), as expected, compared to rats on the basal or phytic acid–supplemented diets (8.4 ± 1.3 and 9.2 ± 2.8 μ*M*, respectively; $P = 0.02$ by analysis of variance).

Ex vivo ESR analysis of free radical production in colonic contents was performed using DMPO as an aqueous spin trap. Although some degree of variability was observed in DMPO adducts across diets (Fig. 2), computer simulations

showed nearly all specimens contained mixtures of hydroxyl (a_N = 14.9 Gauss and a_H = 14.9 Gauss) and thiyl (or sulfur-centered) radicals (a_N = 15.0–16.3 Gauss and a_H = 15.9–20.2 Gauss), with thiyl radicals appearing as the predominant species. The spectrum center for thiyl adducts was located at slightly lower field strengths than for carbon-centered radical adducts, indicating that these species had larger *g* values. Since sulfur-centered radicals typically have larger *g* values than carbon-centered adducts, this finding is additional positive evidence for the correct assignment of these adducts.

Because thiyl radicals were not observed *in vitro* when *E. faecalis* was spin trapped in glucose-containing buffer, we decided to explore whether OG1RFSS could generate thiyl radicals when exposed to sulfur-containing compounds, as might be found in the colon. Bacteria were spin trapped in glucose-containing buffer saturated with 2-methyl-1-propanethiol (an alkylsulfide), air-oxidized cysteine, or hydrogen sulfide. Each experiment resulted in DMPO adducts that had hyperfine splitting constants similar to those detected in colonic contents (Fig. 3). Spin trapping these solutions without adding *E. faecalis* produced no detectable radicals. Not all sulfur-containing compounds, however, generated radicals. Several attempts were made to generate radicals using hog gastric mucin (which contains a significant proportion of cysteine residues) and cysteine alone, but for unclear reasons, each failed. These findings suggested that *E. faecalis* colonizing the colon forms not only oxygen-centered radicals but also thiyl radicals.

We decided next to assess the *in vivo* production of radicals by *E. faecalis* using the aromatic hydroxylation assay. Again, this method was selected because of limitations quantifying radicals by ESR spin trapping. Rats were colonized with *E. faecalis* and administered D-phenylalanine by intraperitoneal injection. After 1 hr, *ortho-*, *meta-*, and *para-* isomers of tyrosine were quantified in urine (Fig. 4). Supplemental dietary iron or phytic acid had no apparent effect on hydroxyl radical production, as no significant differences were noted in concentrations of *ortho-* or *meta-* isomers for these diets compared to the basal diet (Table 3). Concentrations of the *para-* isomer, which is potentially formed by endogenous phenylalanine racemase and hydroxylase, were decreased for iron- and phytic acid–supplemented diets compared to the basal diet. This finding, however, should be cautiously interpreted since the effect of iron or

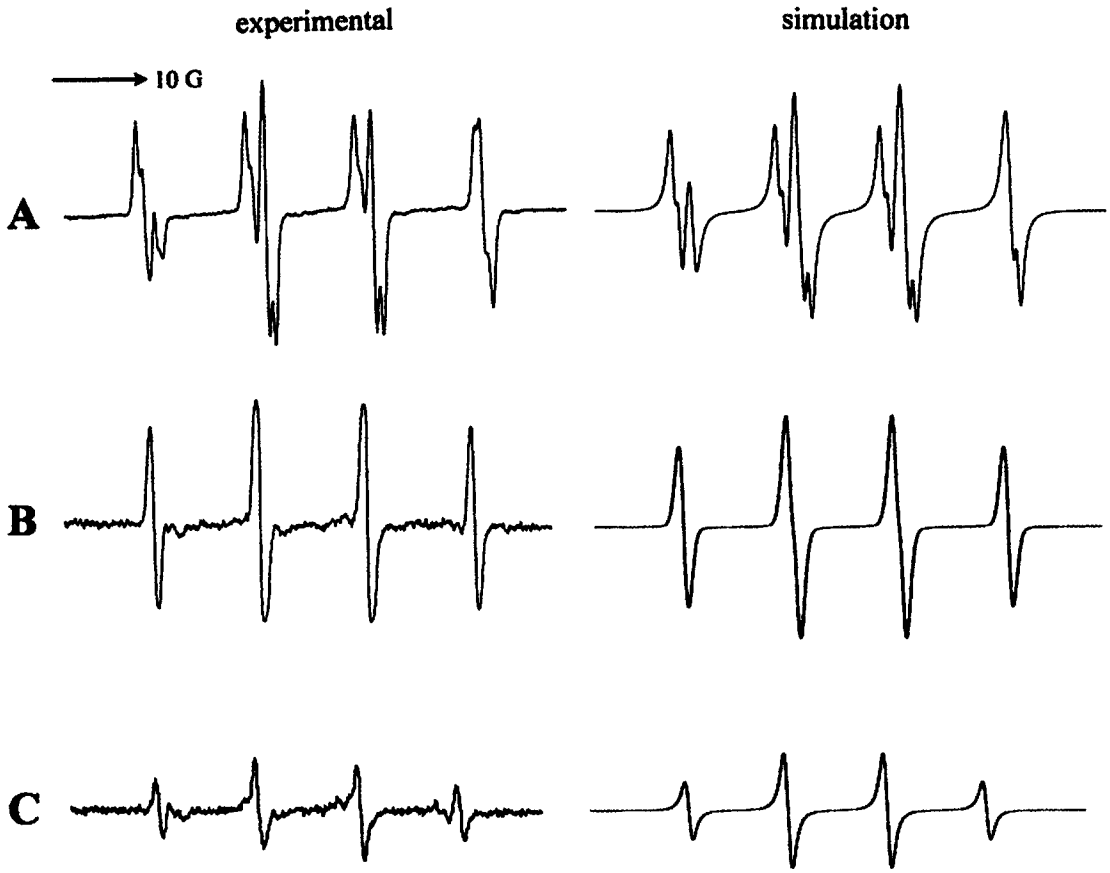

Figure 3. Thiyl radicals generated by superoxide-producing *Enterococcus faecalis*. Bacteria at 10^9 cfu/ml in Tris buffer (pH 7.4) with 5 m*M* glucose were mixed (v/v) with saturated solutions of (A) oxidized cysteine (1:2), (B) 2-methyl-1-propanethiol (1:30), or (C) H_2S (1:3) and trapped using 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO). Computer-simulated spectra showed hyperfine splitting constants consistent with thiyl and/or hydroxyl radicals for (A) with Species 1 (42%), a_N = 15.1 G, a_H = 15.1 G, g = 0.700; and Species 2 (58%), a_N = 15.0 G, a_H = 18.5 G; and (B) with a_N = 15.2 G and a_H = 16.2 G; and (C) with a_N = 15.8 G and a_H = 15.8 G. Instrument settings: 100-kHz field modulation, microwave power 20 mW, modulation amplitude 1.0 Gauss, sweep width 100 Gauss per 84 secs, and time constant 164 msecs. Arrow points upfield.

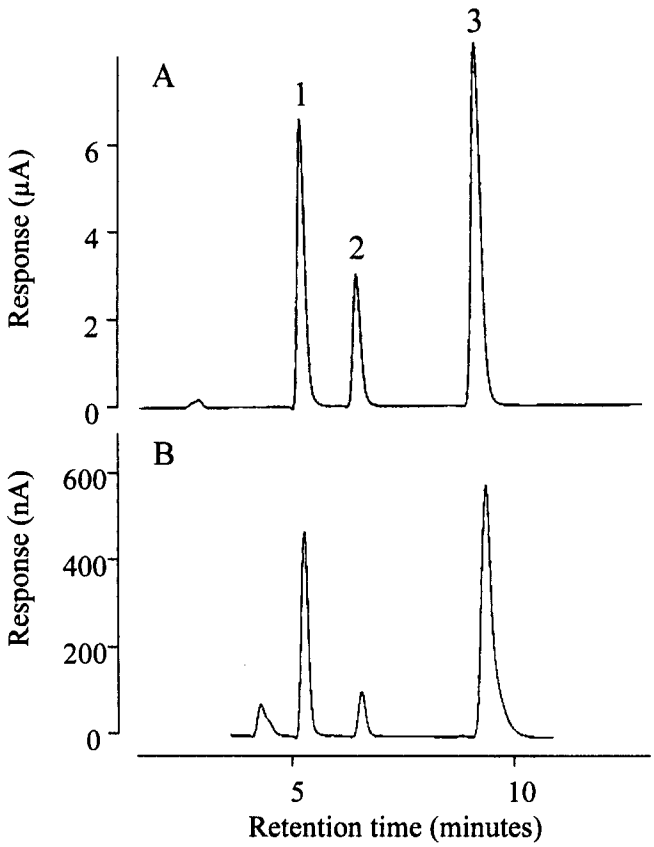

Figure 4. High-performance liquid chromatography (HPLC) separation and electrochemical detection of hydroxylation products of D-phenylalanine for (A) standard showing peaks for (1) *para*-, (2) *meta*-, and (3) *ortho*-tyrosine and (B) a typical urine sample from a rat colonized with *Enterococcus faecalis* strain OG1RFSS. See *Materials and Methods* for details.

phytic acid on the enzymatic metabolism of phenylalanine is unknown. In aggregate, these data fail to support the hypothesis that short-term dietary supplementation with iron or phytic acid significantly alters the production of hydroxyl radical by *E. faecalis* colonizing the intestine.

Discussion

Theories for the etiology of sporadic CRC should explain, at least in part, why certain dietary factors are positively (or negatively) associated with these tumors (31, 32). Oxidative stress from colonic flora is an attractive hypothesis in this respect. Diets rich in meat, as a marker for increased iron consumption, or iron-fortified foods might promote tumorigenesis through an accelerated generation of hydroxyl radical from superoxide produced by bacterial metabolism in the colon (13, 15, 33, 34). Conversely, phytic acid might chelate iron and potentially prevent oxidant damage by inhibiting hydroxyl radical formation (14).

Table 3. *In Vivo* Hydroxylation of D-Phenylalanine by *Enterococcus faecalis* Colonizing the Rat Intestinal Tract

Diet (no. of samples)	Hydroxylated D-phenylalanine(nmol/ml urine)[a]		
	Ortho-tyrosine	*Meta*-tyrosine	*Para*-tyrosine
Basal (13)	24 ± 21	218 ± 223	115 ± 76[A]
Iron (15)	26 ± 17	250 ± 321	53 ± 50[B]
Phytic acid (15)	42 ± 35	89 ± 128	43 ± 42[B]
P value	0.14	0.16	0.004

[a] Mean ± standard deviation (SD), comparisons by analysis of variance; means within the *para*-tyrosine data without a common superscript are significantly different ($P < 0.05$).

To determine whether radical production by *E. faecalis* colonizing the intestine could be altered by dietary iron or phytic acid, aqueous radicals in colonic contents of rats were spin trapped and detected by ESR. Hydroxyl radical formation was also assessed in colonized animals by aromatic hydroxylation using D-phenylalanine as a target molecule. Neither supplemented diet, however, showed any effect on radical production compared to a basal diet. This finding contrasted sharply with *in vitro* results that showed iron promoted, and phytic acid inhibited, hydroxyl radical formation by *E. faecalis*. Reasons for the apparent discrepancy may be dose or time dependent or simply due to the natural intestinal milieu that contains diverse mixtures of microorganisms, undigested food, desquamated epithelial cells, bile, mucus, fermentation products, metals, salts, and water (35). The effects of complex fecal constituents on radical formation (or scavenging) are largely unknown. Potentially, colonic iron is bound to one or more luminal components and unable to participate in Fenton reactions.

Similarly, phytic acid supplementation, like iron, appeared to have little effect on hydroxyl radical production in our model. Although the amount of phytic acid added to the rat diet was consistent with other animal studies reporting significant anticancer effects (20), we did not measure the concentration of phytic acid in colonic contents. The rat intestinal mucosa is not known to contain significant endogenous phytase activity, but these animals can, on occasion, be colonized with bacterial flora capable of degrading phytic acid (36, 37). Thus, it remains to be determined whether dietary supplementation with phytic acid can increase colonic concentrations to levels capable of inhibiting hydroxyl radical formation.

The extent to which the negative findings observed *in vivo* were dependent on the duration of dietary supplementation is likewise difficult to ascertain. A similar degree of iron loading in one pig model (750 mg iron per kg diet), when combined with degradation of dietary phytic acid by phytase-producing bacteria, increased indirect markers of colonic lipid peroxidation, but colonic radicals were not measured (38). The duration of treatments in this study was 5 months compared to slightly less than 3 weeks in our model. Perhaps a longer period of dietary supplementation might have led to different results. We would expect, however, the ability of iron or phytic acid to form or inhibit radical production to be apparent once steady-state concen-

trations of these compounds and *E. faecalis* are achieved in the intestine. This should have been the case in our model.

Previous studies indicate dietary supplementation with iron can increase hydroxyl radical production in feces (34, 39). These reports, unlike this study, measured hydroxyl radical through a reduction of dimethylsulfoxide to methane sulfinic acid (40). In this assay, samples are typically incubated >12 hrs under conditions that would kill and lyse intestinal bacteria. Radical formation by extracellular components of fecal matter would thus be augmented by release of redox active cytosolic constituents. Furthermore, high concentrations of EDTA in the assay (500 μM) would chelate transition metals and amplify, not inhibit, the Fenton reaction (21). Thus, the methane sulfinic acid assay is more an estimate of transition metal availability in feces for hydroxyl radical generation than a measure of radical production by metabolically active commensal bacteria.

This study was designed to overcome the limitations of this technique and directly measure radicals in colonic contents using ESR spin trapping and aromatic hydroxylation. Although spin trapping requires mixing samples to add a trap, Tris was used as a buffer instead of EDTA (21) or phosphate (41) to limit the artifactual generation of radicals. Surprisingly, the predominant radicals that were found using DMPO as the spin trap were sulfur- and not oxygen-centered species. Although hyperfine splitting constants for thiyl radicals varied from sample to sample, suggesting different species or a single species sensitive to minor changes in the colonic milieu, more similarities than differences were observed, regardless of diet.

These thiyl radicals may have originated from mucin which contains numerous cysteine-rich subdomains (42), glutathione from epithelial cells shed into the intestinal lumen (43), or cysteine-rich compounds (e.g., mycothiol) synthesized by intestinal microorganisms (44). Finally, colonization by bacteria that use sulfate as an electron acceptor may produce colonic concentrations of H_2S in excess of 1 mM (45, 46). *In vitro, E. faecalis* was found to readily produce thiyl radicals in buffers saturated with H_2S, air-oxidized cysteine, and the alkylsulfide 2-methyl-1-propanethiol. These DMPO adducts had hyperfine splitting constants similar to those detected *in vivo* (Fig. 3). Thus, a variety of endogenous intestinal compounds might serve as potential sources for thiyl radicals found in colonic contents.

Thiyl radicals have been described following cysteine oxidation (47), in irradiated aqueous thiol solutions (48–50), and from glutathione incubated with xanthine oxidase or peroxidase (51–53). As with hydroxyl radical, thiyl radicals are highly reactive with rate constants for oxygen that exceed 10^9 M/sec (47). This high reactivity limits the diffusion of these radicals to regions not far beyond their site of generation. Although thiyl radicals are most often considered antioxidants in biological systems (54–56), their potent reactivity could easily damage molecules like DNA (11, 12, 57–59). In addition, these radicals may have other subtle effects such as the cis-trans isomerization of monounsaturated fatty acid esters to alter cell membrane fluidity (60). The significance of thiyl radicals in carcinogenesis, however, remains largely uninvestigated.

In conclusion, our findings show that *E. faecalis* colonizing the rat colon produces mixtures of thiyl and oxygen-centered radicals. A role for reactive oxygen species in DNA damage has been partially formulated (3, 59), but what significance, if any, these or other radicals have in carcinogenesis merits further investigation. It does not appear, however, that iron supplementation as reported in this study (~20 mg/day elemental iron per 250 g rat) or the increased consumption of phytic acid significantly alters free radical production within the colonic lumen for rats colonized with this superoxide-producing bacterium.

This study was supported by a Merit Review Program from the Department of Veterans Affairs (MMH) and the Frances Duffy Endowment.

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