The quantitative determination of hydrogen peroxide (H2O2) produced by *Enterococcus faecalis* isolated from Colonic Cancer

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Abstract

The concentration of H_2O_2 produced from *E.faecalis* was measured directly by using glucose kit. The results showed that 48.21 nmol/ml of H_2O_2 produced from *E.faecalis* that isolated from the stool samples of patients suffering from colonic cancer (EFCS) , and 27.55 nmol/ml of H_2O_2 produced from *E.faecalis* that isolated from the stool samples of healthy individuals (EFHS).The results also showed that 60.613 nmol/ml of H_2O_2 produced from (EFHS) incubated with 50 µM ferrous sulphate ,and 71.289 nmol/ml of H_2O_2 from (EFHS), incubated with 200 µM ferrous sulphate. Using high-performance liquid chromatography, the role of iron in increasing the Hydroxyl radical ($^{\cdot}OH$), released from *E.faecalis* was studied. The results showed that *E.faecalis* has the ability to produce ($^{\cdot}OH$) and to generate one isomer of tyrosine (Para- tyrosine) under aerobic conditions due to abstracting hydroxyl from the aromatic ring of phenylalanine. These results were compared with other isolates that grew in iron free media. As was expected, iron enhanced hydroxyl radical, produced by *E.faecalis* and increased the concentration of para- tyrosine.

KEYWORDS: Hydrogen peroxide; *E.faecalis*, Colonic cancer, Para-tyrosine; Hydroxyl radical

INTRODUCTION

Each year, on a global basis, nearly one million persons are diagnosed with colorectal cancer (CRC) ; and these more than the half die from complications (Huycke *et al*., 2001). This disease represents the seventh of the top ten fetal diseases in Iraq (M.O.H.,2004).The role of intestinal flora in the etiology of CRC has recently gained an increasing attention.

One bacterium in particular, *Enterococcus faecalis*, has been considered a potential cause of CRC through the production of reactive oxygen species (ROS) (Huycke and Gaskins ,2004). These reactive oxygen species have been detected in the colonic contents of animals, colonized with *E. faecalis* and it was found that they promote epithelial cell transformation through oxidative damage of DNA (Huycke and Gaskins ,2004).

E. faecalis is unusual among prokaryotic organisms in its ability to produce substantial extracellular superoxide as a by-product of their normal aerobic metabolism. $O^{\cdot-}_2$ have been increasingly implicated in the clinical infections specially their role in causing cancer, more specifically superoxide ($O^{\cdot-}_2$) and hydroxyl radical ($^{\cdot}OH$) (Moore *et al*.,2004). The mechanism of $O^{\cdot-}_2$ generation is unknown but different investigations describe how bacterial respiration is essential for generation of $O^{\cdot-}_2$ by *E.faecalis* (Huycke *et al*., 2002). Moreover, growth conditions that favor $O^{\cdot-}_2$ production appear to be present in the mammalian intestinal tract, suggesting that this microorganism may be an

important source of oxidative stress on the epithelium and other components of intestinal ecology (Huycke *et al*.,2001).

Reactive oxygen species, including superoxide anion (O^{-}_{2}), hydrogen peroxide (H_2O_2) and hydroxyl radical ($^{\cdot}OH$) were generated by *E.faecalis* as a by-product of normal cellular metabolism (Owen *et al*., 2000).

Although oxygen is a powerful oxidant that reacts with many molecules such as lipids, proteins, and DNA, over production of ROS from cells were referred to as oxidative stress, which may cause DNA damage and mutations that lead to cancer (Gasche *et al*., 2001; Owen *et al*., 2000). This study aims to measuring hydroxyl radical ($^{\cdot}OH$) level and hydrogen peroxide (H_2O_2) level Which produced by *E.faecalis (in vivo* and *in vitro* assay),.

MATERIALS AND METHODS

A number of samples (98 sample - stool and tissue samples) were collected from different sources; patients [77 member(34 female and 43 male)] and healthy people [21 member (12 female and 9 male)].Their ages ranged between (38-83) years. Those samples were collected from different hospitals in Baghdad including Teaching Baghdad Hospital, Gastroenterology and Hepetology Teaching Hospital and Al-Kendi Hospital, starting from 25th of October 2007 to the 31st of December 2007. These samples included: Stool samples [63 stool sample (14 samples from healthy people and 49 samples from patients)] were put in sterile container that contained 3 ml of normal saline .

Tissue Samples

Tumor tissue samples [35 Tissue/ Biopsy/Autopsy (7 samples from healthy people and 28 sample from patients)] were collected in sterile tubes that contained a transport medium (Modified McCoy medium) . These samples were put in pre cleaned Petri dish, washed several times with normal saline ,then cut to small pieces. These pieces were transferred to a glass tube that contained normal saline ,mixed by vortex to obtain cells suspension (AL- Mahdawii, 2007)

Isolation and Identification of *E.faecalis*

After samples collection,1oopful was taken and cultured on Enterococci agar that was prepared according to (MacFadden, 1985) item. Then incubated at 37°C for 24-48 hrs. Later a single colony was selected to be cultured on Bile esculine azid agar medium and incubated at 45°C for 24 hrs, then sub cultured on the MacConky agar medium incubated at 37°C for 24 hr. (MacFadden, 2000).

Diagnosis of *E. faecalis*

Microscopic Examination

Microscopic Examination of *E. faecalis* isolates was made to define the shape of *E. faecalis* cells, gram stain reaction (Forbes *et al*., 2002).

Serological Identification

Latex D-Kit was used to examine which group these isolates belonged to. This test was applied according to the manufacture company. This test was performed by transferring single colony grown on blood agar to a reaction mixture were the agglutination indicated positive reaction (Collee *et al*., 1996).

Biochemical tests were applied according to (Berti *et al*.,1998; MacFadden, 2000, and Forbes *et al* ., 2002)

A Direct Modification Method for the Quantitative Determination of H_2O_2

The glucose kit was used to measure the quantity of H_2O_2. The procedure of the kit was modified here where the glucose was not included in the growth media.

A) Procedure of Assay

The procedure of assay includes the following:

1. Growing different isolates of *E.faecalis* in peptone broth for 2 hrs.

2. Assay conditions: Wavelength at 505 nm.

Pipetting into a cuvette as shown below:

Blank: [Tris pH7.4;Phenol;Glucose oxidase (GOD); peroxide (POD) and 4 - Aminophenazone(4-AP)].

Standard: Glucose aqueous solution (100 mg/ml).

Sample (A): 9×10^8 C.F.U. *E.faecalis* from stool samples of patients suffering from colon cancer (EFCS)/ ml of PBS.

Sample (B): 9×10^8 C.F.U. *E.faecalis* from stool samples of healthy persons (EFHS)/ ml of PBS.

Sample (C): 9×10^8 C.F.U. *E.faecalis*(EFHS) /ml of PBS with 50 µM ferrous sulphate.

Sample (D): 9×10^8 C.F.U. *E.faecalis* (EFHS) /ml of PBS that contain 200 µM of ferrous sulphate.

3. Samples were mixed and incubated for 10 min at 37ºC or 30 min at room temperature.

4. Absorbance (A) of samples and the standard were measured at 505nm.

5. Different concentration of H_2O_2 (0, 20,40,60,80,100 nmol/ml) were measured to obtain the standard curve.

The principle of this method depends on detecting H_2O_2 released from *E.faecalis* by a chromogenic oxygen accepter [phenol , 4-aminophenazone (4-AP)] in the presence of POD., as illustrated in the following reaction :

H_2O_2 +Phenol + 4-AP $\xrightarrow{\text{POD}}$ **Quinone + H_2O.**

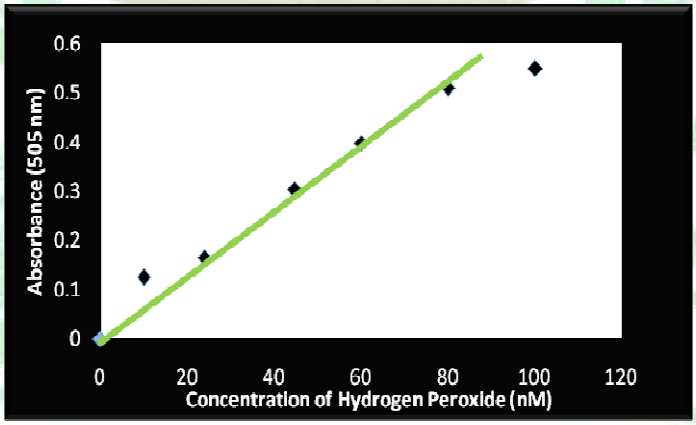


Figure (1) Standard Curve of H_2O_2 Concentration (nmol/ml).

Method for Measuring the Hydroxyl Radical Activity by Aromatic Hydroxylation (*in vitro*) Assay:

In order to estimate the production level of hydroxyl radical from *E.faecalis* and to study the effects of iron on radical production, hydroxylation of D-phenylalanine was measured according to Huycke and Moore (2002).

Initially, any potential toxicity of iron on *E. faecalis* isolates (EFCS) were assessed during overnight growth in BHI broth with 2000 µM iron. Bacteria that were grown in BHI alone were washed and re-suspended at 9×10^8 C.F.U./ ml in PBS with 5 mM D-glucose and different concentrations of (0,50,200 µM) ferrous sulfate and 5 mM D - phenylalanine. After 60 mins, HPLC was used to measure the hydroxylation of D-phenylalanine. The mobile phase composed of 5% methanol, and 50 mM of lithium perchlorate at pH 3.5 and flow rate was 0.7 ml/min, the type of column was C_{18}, length of λ was 280 nm.

Detection of Free Radical Production in Fecal Samples (*in vivo*) Assay:

The effect of dietary iron on free radical production in human stool was explored by using an assay according to (Babbs , 1990; Stevens *et al* ., 2002). By using 1-2 gm stool samples were incubated overnight at 37ºC in Tris buffered saline (pH 7.0) . Samples were centrifuged at 15,200 g for 10 mins at room temperature . Proteins were precipitated by lowering the pH to 1.0 for 10 mins. The pH was then returned to 7.4, samples were centrifuged at 15,200 xg for 10 mins. and the supernatants from each samples were stored at -20ºC. Two ml of supernatant was mixed with 0.2 ml H_2SO_4 (1%) and then with 4 ml of 1-butanol .The upper phase was mixed with 2 ml sodium acetate buffer (0.5 M), pH (5.0) and centrifuged at 3,200 xg for 3 mins. The lower aqueous phase was then adjusted to pH (2.5) before the addition of Fast Blue BB Salt (0.03 M) to produce the colored product (diazosulfone acid). Once the color reaction reached a plateau, after 10 min in the dark, 1.5 ml toluene :1-butanol (3:1), mixture was added and the sample was mixed for 2 min. before separation of the phases by centrifugation at 10,200 xg for 3 min. The upper phase was then removed, washed with 1-butanol-saturated water, and measured by scanning spectrophotometer at a peak of absorbance between 340 and 520 nm.

Results and Discussion

From all the diagnosed species of intestinal Enterococci isolates ,it was shown that 52 of *E.faecalis* isolates were from different sources (Table 1). The table showed the number of samples, percentages of *E.faecalis* isolates from healthy people and others suffering from colorectal cancer .

From Table (1) was shows is a difference in the number of *E.faecalis* isolates according to the healthy conditions. It was found that a high percent of isolates was evident in the stool and tissue samples of CRC patients (29.95% and 13.26 %) respectively.

These results were in agreement with the study of Al- Mahdawii,(2007) which indicated that the percentage of gram positive Enteroococci from colorectal tumor tissues was 56.8%and 18.9% of that due to *E.faecalis*. Table (1) also showed a difference in the percentage *E.faecalis* isolates from the total number of the isolates. It was found that the high percentage of *E.faecalis* isolates (about 15.4%) was in stool samples of healthy persons and about 55.76% in stool samples of patients.

Table (1): Number of Strains Isolated from Different Sources.

Individual case	Source of isolation	Number of Samples	Number of isolates	* % From total samples	** % from total isolates	% of isolates from each source
Healthy	Stool	19	8	8.16	15.40	42.10
	Tissue	7	2	2.04	3.84	28.57
Patient	Stool	52	29	29.95	55.76	55.76
	Tissue	20	13	13.26	25	65

*percentage of isolates from total number of samples(98).
**percentage of isolates from total number of isolates (52).

The concentration of free radicals produced from *E.faecalis* isolates were estimated by using a standardized curve. It was observed that *E. faecalis* isolates from stool samples of patients suffering from colonic cancer (EFCS) generated 48.215 nmol/ml of H_2O_2, when grown aerobically in PBS. The concentration was compared with 27.551 nmol/ml of H_2O_2 obtained from a *E.faecalis* isolated from stool samples of healthy individuals (EFHS) at the same conditions. The concentration of H_2O_2 released from (EFHS) increased to 60.613 nmol/ml of H_2O_2 and 71.289 nmol/ml of H_2O_2in media containing iron 50 and 200µM respectively.

The reason of this state depends on several reactions such as:

A: $O_2 \xrightarrow{e^-} O_2^{\cdot -} \xrightarrow{e^-,2H^+} H_2O_2 \xrightarrow{e^-,H^+} {}^{\cdot}OH + H_2O \xrightarrow{e^-,H^+} 2H_2O$ **…….....(1)**

These reactions show the unpaired orbitals the O_2 / O_2 could sequentially accommodate single electrons to yield $O_2^{\cdot -}$, H_2O_2 ,the reactive ${}^{\cdot}OH$, and H_2O (Korshunov and Imlay,2006).

B: $Fe^{2+} + H^+ + H_2O_2 \longrightarrow Fe^{3+} + H_2O + {}^{\cdot}OH$**……..(2)**

Fe^{2+} released from iron –sulphur cluster in the iron containing media ,could react with H_2O_2 generated from reaction (1) .Thus, the subsequently very reactive oxygen species (${}^{\cdot}OH$) could be formed via Fenton reaction . $O_2^{\cdot -}$ released from *E.faecalis* increased production of free radicals by using the Fenton reaction, this in turn led to a cellular and genomic damage. (Henle and Linn,1997) .

Quantitative Determination of ${}^{\cdot}OH$ Produced by *E.faecalis* (*in vitro*)

The production activity of ${}^{\cdot}OH$ resulted from *E.faecalis* isolates and the effects of iron in form of ferrous sulphate on radicals production by *E.faecalis* were determined by using *in vitro* hydroxylation of D-phenyl alanine (Huycke and Moore , 2002) . In the start point of the experiment, growth inhibition by *E.faecalis* was not observed after over night growth in BHI agar with 2000 µM iron as Fe SO_4 .

The hydroxylation of D-phenylalanine was observed by significant linear increases in isomeric tyrosine (Para tyrosine) (Table 2), the results showed the hydroxylation of D-

phenylalanine to the para tyrosine was high in 200 µM of ferrous sulphate (Figure 1), and less in both 50 µM ferrous sulphate (Figure 2), and the control sample (Figure 3).The different concentrations of tyrosine depends on the level of the free radicals released from *E.faecalis* which increased with the increased of ferrous sulphate concentration in the culture media .These results were compared with the standard curve of para tyrosine shown in figure (4).These results are in agreement with Moore *et al* .(2004).

Table (2) *in vitro* Hydroxylation of D-Phenylalanine by *E.faecalis*.

Condition	Iron Concentration (µM)	Retention Time of *in vitro* Hydroxylation of D-Phenylalanine by *E.faecalis*	Area% from HPLC run
Standard (Tyrosine)	-	7.705	65.85
Control	0	7.774	0.573
Sample 1	50	7.743	0.517
Sample 2	200	7.707	71.82

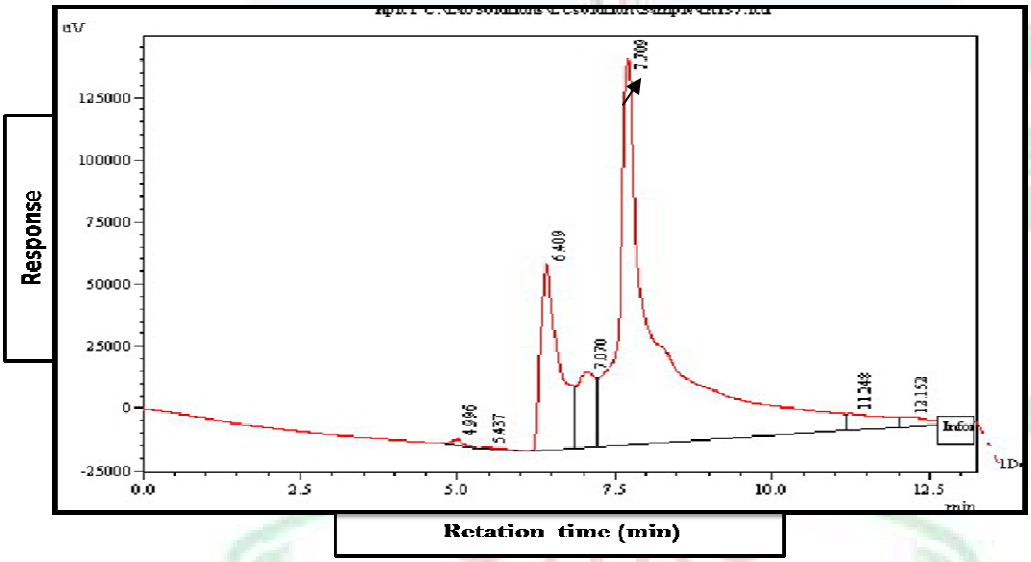


Figure (2): HPLC Separation of the Hydroxylation of D-Phenylalanine with 200 µM Ferrous Sulphate .

The mobile phase composed of 5% methanol, and 50 mM of lithium perchlorate at pH 3.5 and 37 ºC. Column effluent was run at 0.7 ml/min , the type of colum was C_{18}, length of λ was 280 nm.

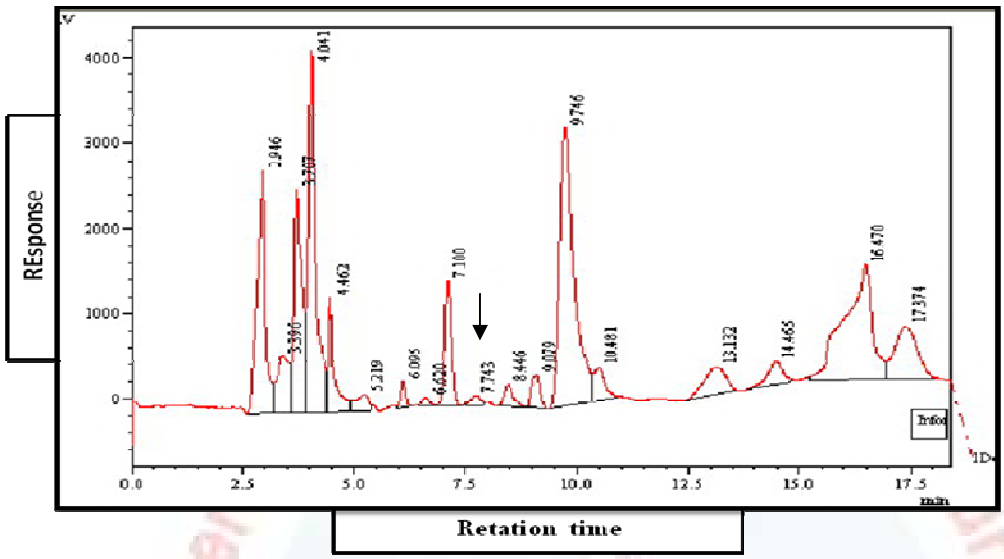


Figure (3) HPLC Separation of the Hydroxylation of D-Phenylalanine with 50 µM Ferrous Sulphate (The details are as mentioned in fig. (2)

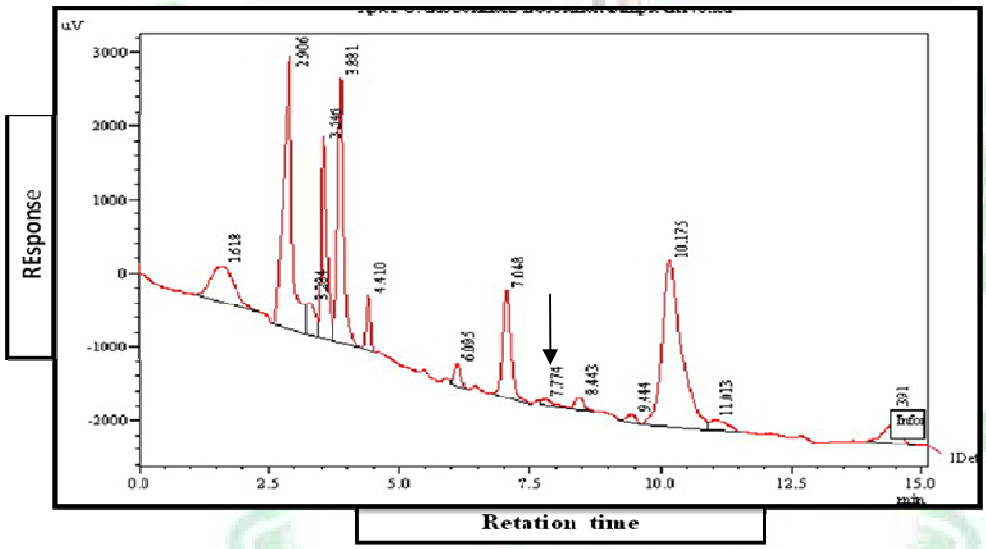


Figure (4) HPLC Separation of Hydroxylation of D-Phenylalanine by *E. faecalis* (Control) .

The details are as mentioned in figure (2)

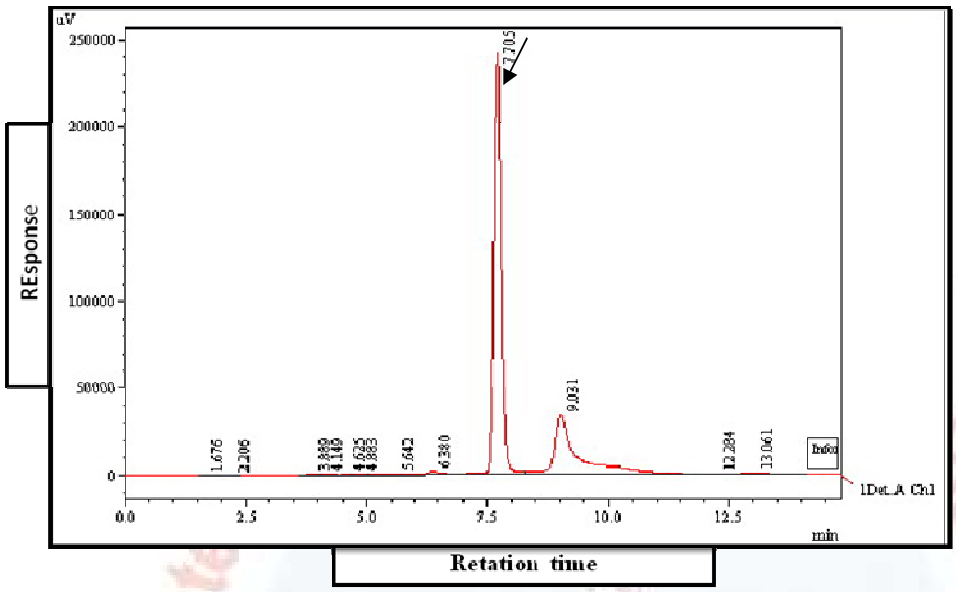
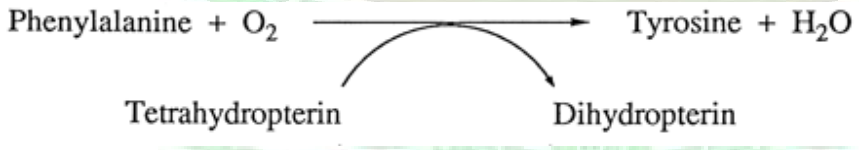


Figure (5) HPLC Separation of Hydroxylation of Para- Tyrosine (Standard)

The principle of this method depends on the attack Hydroxyl radical on D-phenylalanine , Phenylalanine hydroxylases are tetrahydropterin-dependent monooxygenases that catalyzed the hydroxylation of phenylalanine by dioxygen to produce tyrosine with the concomitant two-electron oxidation of a tetrahydropterin cofactor.

The overall transformations of the co-factor are illustrated in this reaction:



The results of this attack generate one of three isomers of tyrosine (ortho-, meta-, or Para-) under aerobic condition due to abstracting hydroxyl from the aromatic ring (Nukuna et al.,2001).

The requirement for iron in substrate hydroxylation and the uncoupling of hydroxylation from tetrahydropterin oxidation in the absence of iron indicate that iron participates in the hydroxylation process but is not required for the oxidation of tetrahydropterins. In the presence of iron, it reacts with 4a-hydroperoxy-$DMPH_4$ which could lead to 4a-hydroxy-$DMPH_4$ and a high valent iron-oxy species. The iron-oxy species might react with phenylalanine in the hydroxylation process (Dawei and Perry ,1998).

These isomers are regarded as induction to the production of hydroxyl radicals in cells, tissues, and animals (Biondi *et al* ., 2001; McLarty ,1997).

Quantitative Determination of $^{\cdot}$OH Produced by *E.faecalis* (*in vivo*)

All previous results were supported by the third test that investigated the generation of ·OH in the biological system .The method as shown below:

Dimethyl sulfoxide +·OH ⟶ methane sulfinic acid + CH_3

DMSO was oxidized by · OH to form a stable, non radical compound methanesulfinic acid. This was not normally found in living systems and which could be easily extracted from tissue and measured spectrophotometrically. The present method provided a simple, inexpensive assay for methane sulfinic acid in the biological materials. (Babbs and Steiner 1990; Turnipseed *et al* ., 1996).

The results of these experiments were estimated after supplying the rats with 100 mg of ferrous sulphate /kg diet for 4 months. There was a significant increase (P≤ 0.01) in the free radicals in the stool samples of rats fed dietary iron as compared with rats that fed standard diet. (Table 3).

Table 3: The Absorbance for ·OH Production from Stool Samples

	Type of treatment	Mean of Absorbance	Probability ≤
A	**Rats fed standard diet (Control)**	**0.5216±0.0009[a]**	**Non Significant**
B	**Rats fed basal diet with 9× $1o^8$*C.F.U. E.faecalis***	**0.5416±0.0015[b]**	**0.001**
C	**Rats fed basal diet with 12×10^8*C.F.U. E.faecalis***	**0.5456±0.0007[c]**	**0.001**
A	**Rat fed dietary iron (Control)**	**0.5233±0.0007[a]**	**Non Significant**
B	**Rats fed iron diet with 9× $1o^8$*C.F.U. E.faecalis***	**0.5596±0.009[d]**	**0.001**
C	**Rats fed iron diet with 12×10^8*C.F.U. E.faecalis***	**0.5646±0.0009[e]**	**0.001**

Different letters :Significant (P≤ 0.01) difference between means of the same column.

The study by Lund *et al* .,(1999) reported similar results using fecal samples from human volunteers taken iron supplements (19 mg iron /d).

The results of this study showed that a high absorption was in stool sample that taken from rats fed by ferrous sulpfate and colonized with both 9× $1o^8$ and 12×10^8 C.F.U./ml of *E.faecalis* (0.585 and 0. 602) respectively. *These results* were compared with the other groups of rats that fed standard diet and colonized with 9× $1o^8$ C.F.U./ml of *E.faecalis* and 12×10^8 *C.F.U./*ml of *E.faecalis* respectively.

From these results could be concluded:

The present results clearly indicated that oral iron supplementation increases generation of free radicals according to the equation above and supported the hypothesis that the presence of iron in the fecal stream might lead to generation of free radicals. These effects might be procarcinogenic for colon cancer.(Erhardt *et al* ,.1997; Lund *et al* .,1999). The results suggested that the iron supplement enhanced the production of ·OH by *E.faecalis* , via the Fenton reaction, and explained how the dietary factors (whether positively or negatively) were associated with these tumors. Hence, diets rich with meat are considered as a marker for the increase of iron concentration and might promote tumorgenesis through the bacterial metabolism in the colon(Nelson , 2001).

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