Toxicological significance of azo dye metabolism by human intestinal microbiota

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1. ABSTRACT

Approximately 0.7 million tons of azo dyes are synthesized each year. Azo dyes are composed of one or more R_1-N=N-R_2 linkages. Studies have shown that both mammalian and microbial azoreductases cleave the azo bonds of the dyes to form compounds that are potentially genotoxic. The human gastrointestinal tract harbors a diverse microbiota comprised of at least several thousand species. Both water-soluble and water-insoluble azo dyes can be reduced by intestinal bacteria. Some of the metabolites produced by intestinal microbiota have been shown to be carcinogenic to humans although the parent azo dyes may not be classified as being carcinogenic. Azoreductase activity is commonly found in intestinal bacteria. Three types of azoreductases have been characterized in bacteria. They are flavin dependent NADH preferred azoreductase, flavin dependent NADPH preferred azoreductase, and flavin free NADPH preferred azoreductase. This review highlights how azo dyes are metabolized by intestinal bacteria, mechanisms of azo reduction, and the potential contribution in the carcinogenesis/mutagenesis of the reduction of the azo dyes by intestinal microbiota.

2. INTRODUCTION

One of the remarkable abilities of the human eye is to recognize colors; therefore compounds like dyes are used to enhance the attractiveness of objects. Today, there are thousands of various types of dyes used widely in foods, pharmaceuticals, cosmetics, textiles and plastics (36, 90, 128), some of which may pose a risk to human and ecological health due to their toxic properties. Dyes have been used for thousands of years and approximately 0.7 million tons of dyes are synthesized annually worldwide (41, 110). Azo dyes are a group of compounds containing one or more R_1-N=N-R_2 bonds, some of the reduced products of the dyes are carcinogenic (59). Azo dyes represent the majority of all the dyes used in today's society, because the azo bond facilitates π-electron delocalization, which gives rise to the absorption at visible spectrum wavelengths (78).

Microorganisms have evolved in a long-lasting mutual interactions with human beings to influence host physiology (67). Although the study of intestinal bacteria was hindered by the fact that most of the bacteria were unculturable *ex vivo* (67), recent molecular and genomic sequencing approaches have revealed a vast and dynamic

$$R1-N{=}N-R2 \xrightarrow[\text{Azoreductase}]{\text{NAD(P)H} \quad \text{NAD(P)}^+} R1-NH_2 \; + \; H_2N-R2$$
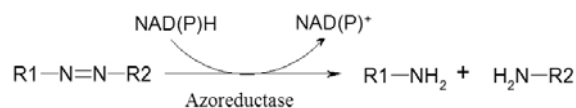
Figure 1. The reduction of azo dyes by NAD(P)H catalyzed by azoreductase.

consortium of bacteria, with an estimated 100 trillion bacteria inhabiting in the human gut (68). The function of intestinal bacteria includes enhancing host digestive efficiency, providing instructive signals for several aspects of intestinal development, and protecting their hosts against pathogenic infections (68). As a result of enhancing host digestive efficiency, germ-free rodents must consume about 30% more calories to sustain their body weight than that of conventionally raised animals (6). The relationship of host immune system and the intestinal microbiota has been reviewed recently (68). Colonization barrier disruption of the intestinal bacteria has been linked to several types of diseases to the host. For example, inflammatory bowel disease (IBD) was associated with activating inappropriately the host mucosal immune system by losing of tolerance to gut commensals (51, 82).

Human intestinal bacteria also play a role in degrading various xenobiotics to metabolites, some of which are involved in genotoxicity and carcinogenesis. Human exposure to xenobiotics including azo dyes occurs via ingestion, skin contact, or inhalation. Azo dyes can be biotransformed into colorless aromatic amines, some of these may become less toxic than the original dye, but others, such as arylamines and free radicals, are potentially carcinogenic (40). Sudan azo dyes such as Sudan I, II, III, IV and Para Red are often illegally used to enhance and/or maintain the appearance of food products and they have been found as food contaminants (28, 138). The initial step in the anaerobic metabolism of azo dyes by intestinal microbiota is reduction of the azo bond by the NAD(P)H dependent azoreductases. Three distinct groups of azoreductases have been found, flavin dependent NADH preferred azoreductase, flavin dependent NADPH preferred azoreductase, and flavin free NADPH preferred azoreductase (31, 87). The flavin dependent NADH preferred azoreductase has been purified from *Escherichia coli* (95), *Enterococcus faecalis* (33), and *Pseudomonas aeruginosa* (134). The flavin dependent NADPH preferred azoreductase has been purified from *Bacillus cereus* OY1-2 (129), and similar genes were found from *Rhodobacter sphaaeroides* (16), *Staphylococcus aureus* (32), *and Saccharomyces cerevisiae* (80). The flavin free NADPH preferred azoreductase has been purified from *Xenophilus azovorans* KF46F (18) and *Pigmentiphaga kullae* K24 (19, 31). There are also some studies on the characterization and crystal structure of azoreductases in *B. subtilis* (93), *E. coli* (69, 70), *Ent. faecalis* (34, 81, 83), *P. aeruginosa* (113, 134, 135), and *S. cerevisiae* (80). Azo dyes can be reduced by NAD(P)H catalyzed by azoreductases as shown in Figure 1

The aim of this review is to summarize the mechanism of azo dye degradation by human intestinal bacteria and the potential toxicity of azo dyes and their metabolites. We also provide a brief discussion on azoreductases from intestinal bacteria and diversity of intestinal bacteria and their capability to reduce azo dyes.

4. HUMAN INTESTINAL MICROBIOTA

The human gastrointestinal tract contains about 10^{14} bacterial cells, 10 times the number of human cells present in our bodies. The largest diversity of the human associated microbes is found in the intestinal tract with about 10^{11} per gram in stool (121). It is reported that most of the intestinal bacteria are members of the *Firmicutes* and *Bacteroidetes* phyla (52). The Gram-positive *Firmicutes* phyla include *Clostridia* class and the families of *Enterococcaceae* and *Lactobacillacea*. The Gram-negative *Bacteroidetes* are comprised of several *Bacteroides* species. The other relatively few (less than 10%) intestinal bacteria belong to *Proteobacteria*, *Actinobacteria*, *Fusobacteria*, and *Verrucomicrobia* phyla (52).

With recent advances in genome sequencing, our understanding of role of commensal microbiota in causing disease and mutualistic relationship to host has been advancing (49). The human intestinal microbiota are essential to individual health. They play important roles in the digestion, vitamin synthesis, epithelial development, pathogen resistance, and immune responses (15). A classic example in nutrition is polysaccharide degradation. Most of the polymers are not degraded by the host, while, they are energy sources of the intestinal microbiota. Herbivores can get up to 70% of their energy intake from the digestion of polymers by intestinal bacteria (57). Germ-free mice need additional vitamin K and certain B vitamins (137), because the intestinal bacteria produce these vitamins in conventional animals (66). Moreover, the intestinal microbiota are able to change the metabolic potential of liver by influencing hepatic gene expression through altering its xennobiotic response to drugs (17).

An initial barrier in studying the microbial ecology of the human gastrointestinal tract was that most of the intestinal bacteria were difficult to culture in the laboratory. Recently, modern molecular methods including small-subunit rRNA (16S rRNA) gene sequencing (52), quantitative PCR coupled to denaturing gradient gel electrophoresis (DGGE) (94) have been applied to study the uncultured intestinal microbial community. Metagenomics, a culture independent genomic analysis method, provides a valuable tool for the study of gut microbial diversity. By employing high resolution nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS), metabolomics can simultaneously analyze hundreds to thousands of small molecule metabolites in the samples, therefore, it has been also used in the study of intestinal bacteria, especially in the study of host-microbe interactions (64).

4. AZO DYES

All azo dyes contain one or more azo bonds. Although azo dyes can supply a complete rainbow of colors in theory, most of commercial azo dyes supply yellow,

orange, and red colors. Among all the dyes synthesized annually worldwide, 15% of the used dyes produced enter the environment as industrial effluents (110), which reduces sunlight penetration into the deeper layer of the water. Therefore, serious pollution occurs (119). Environmental release of azo dyes can also cause contamination of agricultural products. Some physical and chemical techniques including adsorption, coagulation and membrane processes have been developed to remove azo dyes from the wastewaters (127). However, the treatments are limited by the cost and the possibility of causing secondary pollution. Presently, bio-decolorization is being intensively investigated because of its low cost and environmental friendly nature (43, 60, 71, 74, 120, 122, 126).

4.1. Azo dyes containing amine group

Based on the structure, azo dyes can be divided into two groups, one group contains aromatic amine radical (R_1-N=N-R_2-NH_2), such as 4-aminoazobenzene, and the other group can produce amine components only after reduction (R_1-N=N-R_2), for example, Methyl Red. Some azo dyes have been used safely in foods, drugs and cosmetics, while others are carcinogenic.

Most of the azo dyes containing amine group are carcinogenic. As is the case for 4-aminoazobenzene, the compound has high hepatocarcinogenicity to male mice when given as a single intraperitoneal injection, and a linearly relationship has been found between the hepatoma multiplicity induced by the dye and the dose of 0.017 to 0.15 μmol/g body weight. However, female mice were resistant to tumor induction under these conditions due to the insensitivity of them to 4-aminoazobenzene and other hepatic carcinogens (48). The dye also produces liver tumors in rats by oral administration and epidermal tumors by application to the skin (2).

o-Aminoazotoluene can produce tumors of urinary bladder, gall bladder and liver in dogs (96). The carcinogenicity of the dye was also found in mice, rats and hamsters producing tumors of the urinary bladder, gall bladder, lung and liver (2). It is strongly mutagenic to *Salmonella* Typhimurium TA98 and TA100 with S9 activation (47). The structures of this type of azo dye are included in Table 1.

4.2. Azo dyes used in food

Azo dyes can provide a complete rainbow of colors for use in the food industry. Azo dyes are used as food additives to enhance color of the manufactured foods, which account for approximately 60-70% of all dyes used in the food process. Tartrazine (1H-pyrazole-3-carboxylic acid, 4,5-dihydro-5-oxo-1-(4-sulfophenyl)-4-((4-sulfophenyl)azo)-,tri-sodium salt, Table 1) is a yellow coloring agent, and commonly used in food and pharmaceutical products. Tartrazine has no mutagenic and carcinogenic potential with a daily intake of 7.5 mg/kg body weight (53). Allura Red (Table 1) is classified as nongenotoxic, and is permitted for use as a food coloring agent. Sunset Yellow (Table 1) is not mutagenic in *Salmonella* Typhimurium testing strain (26). The other

non-food azo dyes further being discussed in this paper are all mutagenic/carcinogenic or have the potential to be genotoxic in mammals (Table 1).

4.3. Sudan dyes as food contaminants

Sudan dyes are non-ionic fat-soluble azo dyes used in plastics, printing inks, waxes, leather, fabrics, and floor polish (8, 139). The chemical name of Sudan I, II, III, IV, and Para Red are 1-phenylazo-2-naphthol, 1-[(2,4-dimethylphenyl)azo]-2-nahpthalenol, 1-(4-phenylazophenylazo)-2-nahpthalenol, 1-[[2-methyl-4-[(2-methylphenyl)azo]phenyl]azo]-2-naphthalenol, and 1-(4-nitrophenylazo)-2-naphthol, respectively, and the structures of these dyes are shown in Table 1. These dyes are classified by the International Agency for Research on Cancer (IARC) as category 3 carcinogens to humans. Although Sudan dyes are banned for food usage in most countries, they are illegally used to maintain the color in food products because of their low cost, bright staining, and wide availability. In May 2003, some chili powder and a variety of foodstuffs were found contaminated by Sudan dyes throughout Europe and Asia (63). Sudan I was also found in China in chili sauce, meat and eggs (65). Therefore, accurate determination of the concentration of Sudan dyes in foodstuffs by regulatory authorities is very important. The analytical techniques for determination of Sudan dyes have been reviewed recently (108). Several methods based on liquid chromatography (LC), enzyme-linked immunosorbent assay (ELISA), and gas chromatography-mass spectrometry (GC-MS) have been developed to detect Sudan dyes. The LC methods are the main methods to analyze Sudan dyes. In most cases, C18 columns were used as the stationary phases, and the mixtures of acetonitrile with acetate or formic acid buffers used as mobile phases. The ultra-violet visible (UV-vis) absorbance and photodiode array (PDA) detectors are most widely used in the analysis of Sudan dyes. ELISA is a sensitive, selective, and rapid method for the determination of Sudan dyes. The ELISA method has been developed for Sudan dyes in chili powder, ketchup and egg yolk (8). GC-MS also has been used to determine Sudan dyes in chili powder (65).
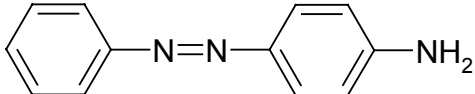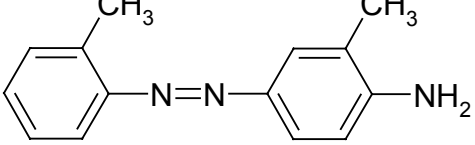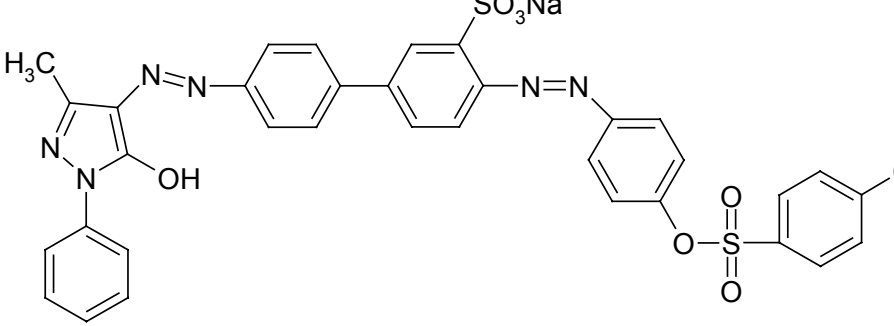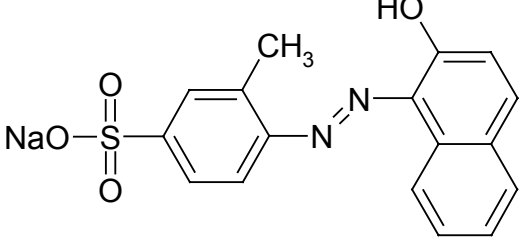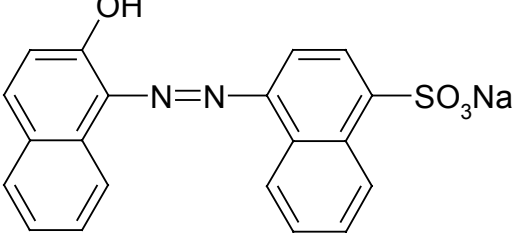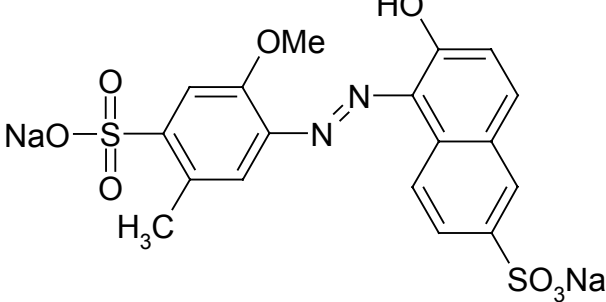
4.4. Mechanism of the reduction of azo dyes

Azo dyes are degraded mainly via reductive reactions. When the azo dyes enter body through ingestion, both the intestinal microorganisms and the enzymes in liver are responsible for the reduction of the dyes. Some studies showed that the intestinal bacterial reductases may be more important than the liver enzymes in metabolism of the azo dyes (38, 39, 53). The azo dyes are reduced to aromatic amines by a type of reductase named azoreductases, which are widely distributed in intestinal bacteria (Figure 1). In some cases, the azo dyes can be degraded via oxidative reactions catalyzed by oxidases and peroxidases (30). The oxidative metabolites of azo dyes were also found in the bile of rat (86, 117, 131, 136).
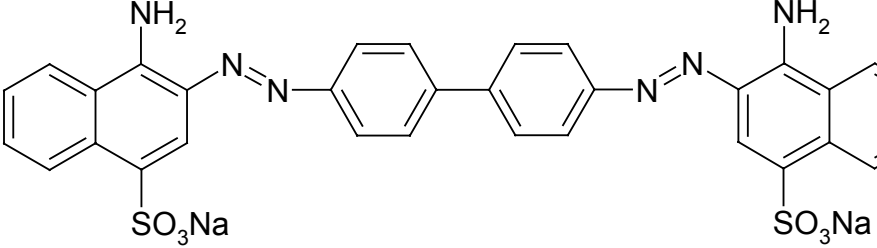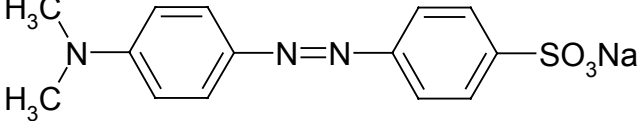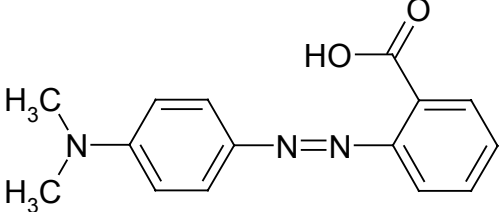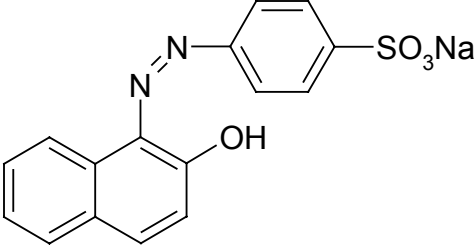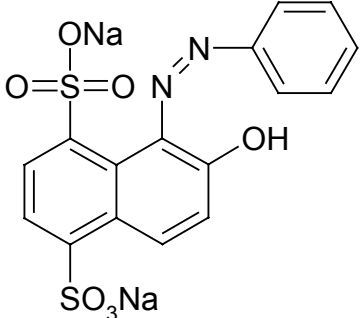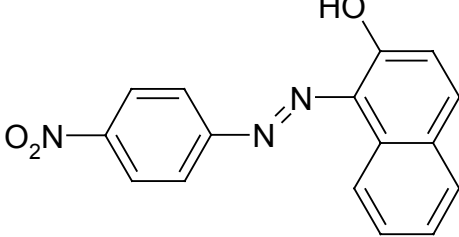
Three groups of azoreductases have been found in bacteria (31). Two groups are flavin dependent, which are composed of NADH-preferred and NADPH-preferred azoreductase. Another group is flavin free azoreductase.
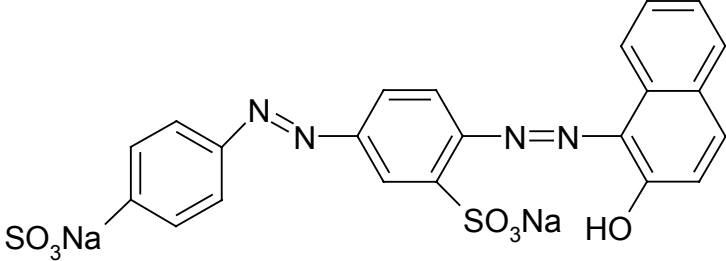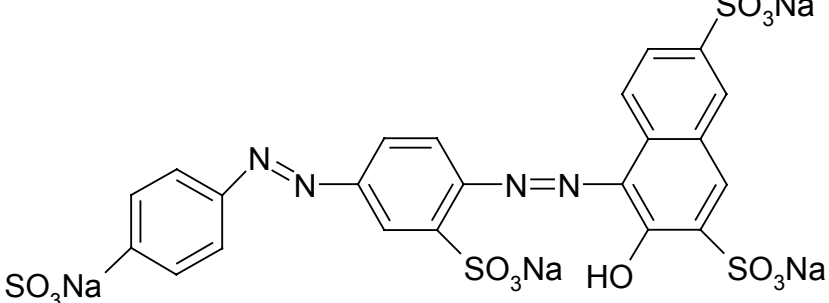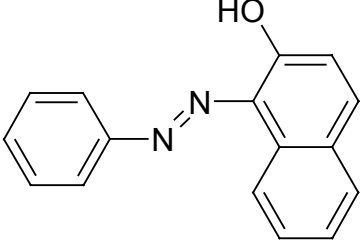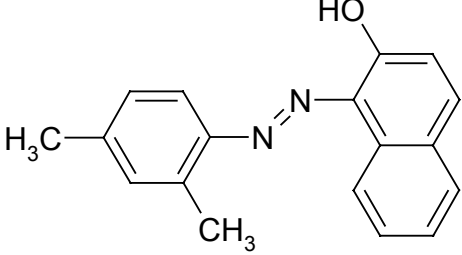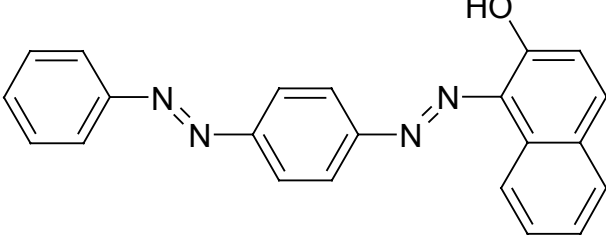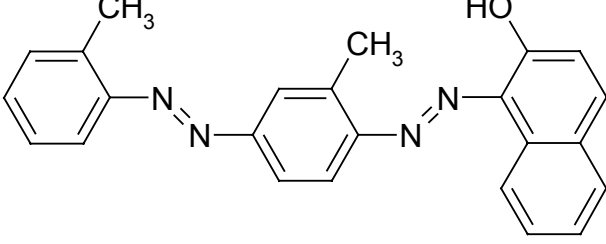
Table 1. Chemical structure of azo dyes

Azo dye	Structure	Group (a)
—		2B[b]
o-Aminoazotoluene		2B
Acid Orange 63		– [c]
Acid Orange 8		–
Acid Red 88		–
Allura Red		–
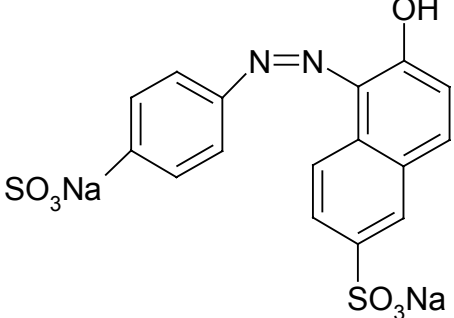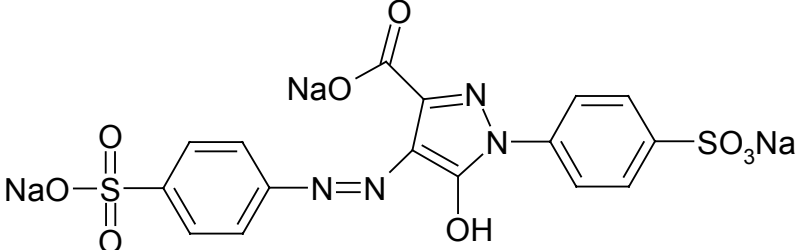
Congo Red		-
Methyl Orange		-
Methyl Red		3 [(d)]
Orange II		-
Orange G		3
Para Red		-

Ponceau BS		-
Ponceau S		-
1-Phenylazo-2-naphthol		3
1-[(2,4-Dimethylphenyl)azo]-2-nahpthalenol		3
1-(4-Phenylazophenylazo)-2-nahpthalenol		3
1-[[2-Methyl-4-[(2-methylphenyl)azo]phenyl]azo]-2-naphthalenol		3

Sunset Yellow		3
Tartrazine		-

(a), Classified by the IARC monographs, (b), Possibly carcinogenic to humans, (c), Not on the list of classification by International Agency for Research on Cancer, (d), Not classifiable as to its carcinogenicity to humans

The catalytic mode of flavin dependent enzymes, such as the azoreductases from *E. coli* (AzoR) and from *Ent. faecalis* (AzoA), is a ping-pong Bi-Bi mechanism, the electrons from NAD(P)H are transferred to substrate via FMN (33, 95). AzoA has two separate active sites, and FMN lies in one site, the other site provides room for both NAD(P)H and substrate binding (33, 34, 81). In the case of Methyl Red reduction by AzoA, NADH firstly transfers the electron to FMN and then releases from the enzyme as NAD^+, after that, Methyl Red bind with the enzyme and the electron is transferred from $FMNH_2$ to Methyl Red. As for reducing Methyl Red, two cycles of the ping-pong mechanism are required, as shown in Figure 2. In theory, a hydrazo intermediate should exist in the reduction process. However, only one research group has detected the intermediate from the reduction of Methyl Red using MS/MS technique. The authors also reported that the intermediate is not stable and can be reduced quickly to the amine (16). A different mechanism is found in the flavin free azoreductase. In the case of Orange I reduction by azoreductase from *P. kullae* K24 (AzoB), AzoB joins both substrates (NADPH and Orange I) together in the active site at the same time, and then a direct hydride transfer occurs in the reaction center of AzoB. This mechanism requires a sophisticated binding mode, therefore, this group of azoreductases shows narrow substrate specificity, only Orange I can be degraded by AzoB in the tested azo dyes (31). On the other hand, the flavin dependent azoreductase families have a broad substrate specificity. AzoA from *Ent. faecalis* can degrade Methyl Red, Orange II, Amaranth, Ponceau BS, Ponceau S, and Menadione (33).

The solubility of azo dyes and the distribution of azoreductase in the cell also affect the dye biodegradation. As for the water-soluble dyes, for example Methyl Red, the dye molecules were first dispersed in solution, then went through the bacterial cell membrane, and subsequently reduced in the cytoplasm by the azoreductase (55). As for the water-insoluble dyes, for example 1-[(2,4-dimethylphenyl)azo]-2-naphthalenol, the dye particles were quickly bound to the cells after being added to the culture, and then reduced on the cell membrane. After that, the cells bound other dye particles from the culture, therefore, the amount of dye bound to the cells kept constant until the dye disappeared in the supernatant of the culture (139). Because almost 100% of azoreductase activity of *E. coli* was found in the cytoplasm, *E. coli* lacks the ability to significantly reduce water-insoluble azo dyes. However, about 55% of the azoreductase activity of *Ent. faecalis* was bound in the membrane, therefore, some water-insoluble azo dyes including 1-phenylazo-2-naphthol, 1-[(2,4-dimethylphenyl)azo]-2-naphthalenol, 1-(4-phenylazophenylazo)-2-naphthalenol, 1-[[2-methyl-4-[(2-methylphenyl)azo]phenyl]azo]-2-naphthalenol, and 1-(4-nitrophenylazo)-2-naphthol can be reduced by the bacterium (139). The mutants of *Shewanella oneidensis* MR-1 generated by random transposon also confirmed that the outer membrane, cytoplasmic membrane, and the periplasm all participated in the reduction of azo dyes (24).

5. TOXICITY OF AZO DYE METABOLITES PRODUCED BY HUMAN INTESTINAL BACTERIA

Azo dyes are degraded mainly via reductive reactions, therefore, we will briefly discuss the toxicity of some of the reduction products of the dyes in this review. Both mutagenicity and carcinogenicity assays are taken into account for the azo dyes and their metabolites. In addition, some azo dyes or metabolites showed marked selectively toxic/carcinogenic, for example, 4-aminoazobenzene demonstrates hepatocarcinogenicity to male mice, but not to female mice. Therefore the clinical data and the literature should be retrospectively analyzed (25).
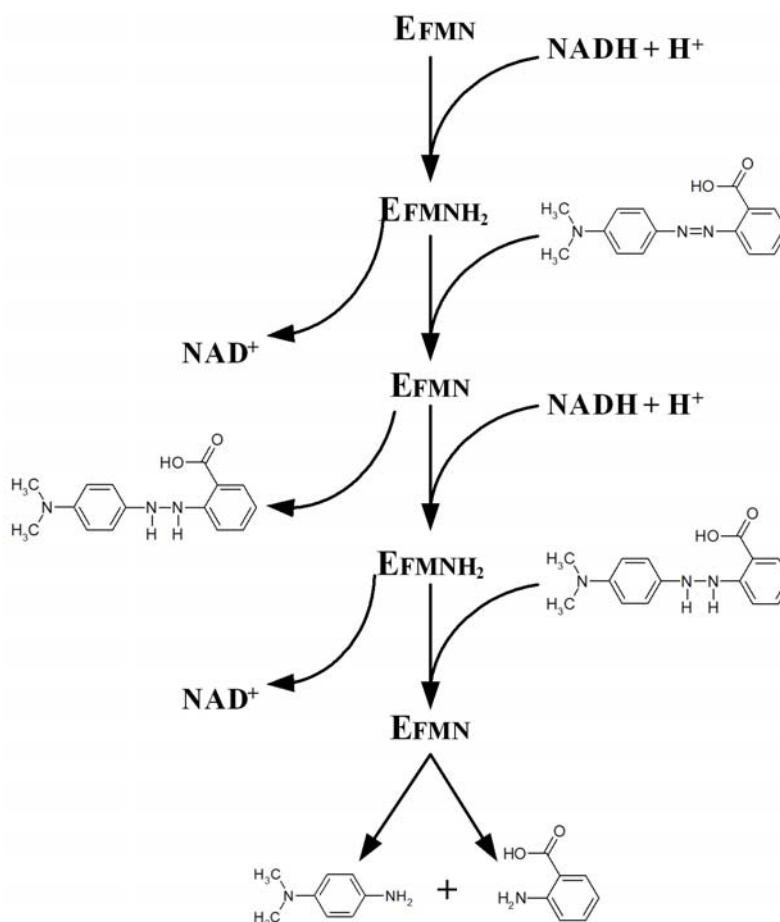
Figure 2. Proposed pathway of the reduction of Methyl Red by *Ent. faecalis* AzoA. EFMN, flavin dependent azoreductase; EFMNH$_2$, reduced flavin dependent azoreductase.

5.1. Genotoxicity of metabolites of azo dyes

The reduction of Orange G, 1-phenylazo-2-naphthol, and 1-(4-phenylazophenylazo)-2-nahpthalenol can produce aniline (Table 2). Aniline is one of the most important compounds of industrial chemistry. About 2.3 million tons aniline were produced in 1996 by more than 40 companies around the world. Aniline is mainly used in the synthesis of diphenylmethane-4,4'-diisocyanate (MDI), rubber chemicals, dyes, pesticides, pharmaceuticals, and fibers (132). The genotoxic activities of aniline has been reviewed by Bomhard and Herbold (21). When *Salmonella* Typhimurium and *E. coli* systems were employed in the mutagenicity test, aniline was proved to be non-mutagenic with or without metabolic activation (by S9 mix). Aniline did not induce direct DNA damage to *E. coli* and *Bacillus subtilis* in *rec* assays and in the Umu test with *S.* Typhimurium (84, 88, 115). But at the high concentrations (156-624 μg/plate), aniline has a weakly positive role in the DNA repair assay without metabolic activation system (S9) (45, 46). At the HPRT locus of V79 cells, aniline did not induce mutations even at concentrations over 1,860 μg/ml (54). At a relatively high concentration aniline can induce the chromosome aberrations, which means a clastogenicity by aniline. Aniline can induce tumors in the spleen of rats (21), but does not induce bladder cancer in humans or animals (5). The data from a contact allergen surveillance network between 1992 and 2004 showed that aniline is not an independent sensitizer, but may elicit allergic reactions in its substituted compounds (132).
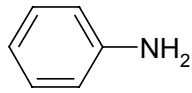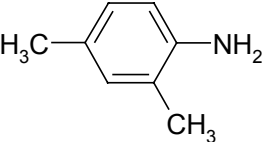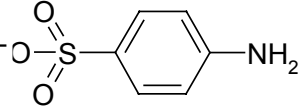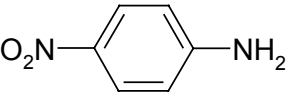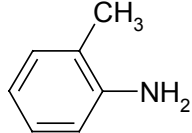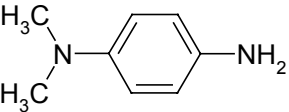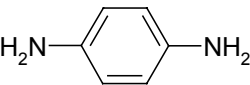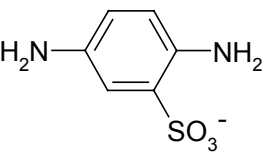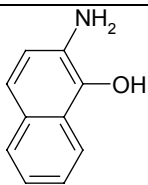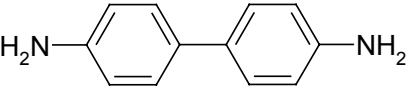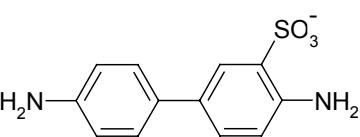
2,4-Dimethylaniline can elicit positive DNA repair responses and possess mutagenicity and carcinogenicity (141). In the *Vibrio* test system, 2,4-dimethylaniline showed direct genotoxicity (97). In the *Xenopus* frog test, 2,4-dimethylaniline showed no mortality even at a concentration of 100 mg/L, but possessed teratogenic properties (98). One of the metabolites of 1-[(2,4-dimethylphenyl)azo]-2-naphthalenol is 2,4-dimethylaniline (Table 2).

It was shown that 4-aminobenzenesulfonic acid (4-ABS) did not possess a mutagenic effect with or without S9 (13, 14, 85). It should be noted that 4-ABS is a metabolite of food dyes of Sunset Yellow and Tartrazine.

Table 2. Chemical structure of metabolites from azo dyes

Name	Structure	Substrate	Group[a]
Aniline		Orange G, 1-phenylazo-2-naphthol, 1-[(2,4-dimethylphenyl)azo]-2-naphthalenol	3[b]
2,4-Dimethylaniline		1-[(2,4-Dimethylphenyl)azo]-2-naphthalenol	3
4-Aminobenzenesulfonic acid		Methyl Orange, Orange II, Ponceau BS, Ponceau S	-[c]
p-Nitroaniline		Para Red	-
o-Toluidine		1-[[2-Methyl-4-[(2-methylphenyl)azo]phenyl]azo]-2-naphthalenol	1[d]
N,N-Dimethyl-p-phenylenediamine		Methyl Orange, Methyl Red	-
p-Phenylenediamine		1-(4-Phenylazophenylazo)-2-naphthalenol	3
2,5-Diminobenzene sulfonic acid		Ponceau BS, Ponceau S	-
1-Amino-2-naphthol		Acid Orange 8, Acid Red 88, Orange II, Para Red, Ponceau BS, 1-phenylazo-2-naphthol, 1-[(2,4-dimethylphenyl)azo]-2-naphthalenol, 1-(4-phenylazophenylazo)-2-nahpthalenol, 1-[[2-methyl-4-[(2-methylphenyl)azo]phenyl]azo]-2-naphthalenol	-
Benzidine		Congo Red	1
Benzidine-3-Sulfonic Acid		Acid Orange 63	-

(a), Classified by the IARC monographs, (b), Not classifiable as to its carcinogenicity to humans, (c), Not on the list of classification by International Agency for Research on Cancer, (d), Carcinogenic to human

This compound can also be produced in the reduction of Methyl Orange, Orange II, Ponceau BS, and Ponceau S (Table 2).

p-Nitroaniline was first reported as very weakly mutagenic or non-mutagenic in the presence of S9 mix in *S.* Typhimurium strain TA98 (124). The mutagenicity of the compound was later confirmed in the test system of *S.* Typhimurium strain TA98 with or without S9 activation, but the results were negative for all other strains. Equivocal evidence of carcinogenic activity was found in mice (3). *p-N*itroaniline is a metabolite of Para Red (Table 2).

o-Toluidine was first synthesized in 1844, and was mainly used as a chemical intermediate in dye, rubber, and pharmaceutical production (44, 123). *o*-Toluidine is a mutagen, and is carcinogenic to experimental animals (4). The aromatic amine *o*-toluidine has been recognized as carcinogenic (Group 1) to humans by IARC. The carcinogenicity of *o*-toluidine as a human bladder carcinogen has been reviewed (123). The recent research also supports the hypothesis that *o*-toluidine is a human bladder carcinogen (29). *o*-Toluidine also has genotoxic activity. *o*-Toluidine can produce DNA damage and induce aneuploidy in fungi and mammalian cultured cells. The genetic toxicology of *o*-toluidine has been reviewed (44). The *p*-toluidine-*m*-sulfonic acid did not show the mutagenicity, and the carcinogenicity of this compound is unknown (141). *o*-Toluidine is a metabolite of 1-[[2-methyl-4-[(2-methylphenyl)azo]phenyl]azo]-2-naphthalenol (Table 2).

N,N-Dimethyl-*p*-phenylenediamine (DMPD) possesses strong mutagenicity with or without metabolic activation (13, 14, 37). The mutagenicity of DMPD did not depend on the dose, because the highest mutagenic effect was obtained with the lowest dose (10 µg/plate) (13). Since the nonmethylated compound *p*-phenylenediamine is not a mutagen, the dimethyl group contributes to the mutagenicity of DMPD, when located in the para position to the amino group on the benzene ring (13). A similar result was also reported in the mutagenicity of other dyes showing methyl substituents were more genotoxic than that of the nonmethylated dyes (118). DMPD is a metabolite of Methyl Orange and Methyl Red, which are used widely in laboratory (Table 2).

p-Phenylenediamine is one of the primary intermediates in the azo dyes. Poisoning with *p*-phenylenediamine causes angioneurotic edema, intravascular hemolysis, rhabdomyolysis with acute renal failure (9). It was reported that there was no convincing evidence of carcinogenicity in rats and mice by dietary administration of *p*-phenylenediamine dihydrochloride (1). *In vitro*, a weak genotoxic potential was found. In the *S.* Typhimurium test systems, *p*-phenylenediamine possessed weak genotoxicity (58). The carcinogenicity of hair dyes which contained *p*-phenylenediamine has been reviewed (20). In the Umu test using *S.* Typhimurium, 2,5-diaminotoluene showed positive genotoxicity with S9 mix

(140). It is reported that 2,5-diaminobenzene sulfonic acid induced mutagenicity, while the carcinogenicity is unknown (141). The compounds of *p*-phenylenediamine and 2,5-diaminotoluene can be produced from the reduction of 1-(4-phenylazophenylazo)-2-naphthalenol and 1-[[2-methyl-4-[(2-methylphenyl)azo]phenyl]azo]-2-naphthalenol, respectively. The compound of 2,5-diaminobenzene sulfonic acid is a product of the reduction of Ponceau BS and Ponceau S (Table 2).

1-Amino-2-naphthol, which is one of the components of an important group of 1-amino-2-naphthol-based azo dyes, has been reported to be a carcinogen. Although there is a study reporting that it was not mutagenic when tested in the *Salmonella*/microsome test system (36, 37), the later studies showed that this compound is mutagenic (50, 61). In the standard protocols with S9, 1-amino-2-naphthol was mutagenic to *S.* Typhimurium strain TA100 but not to strain TA98 (50). When Orange II was reduced, the toxicity increased almost 100-fold. While, when the structurally similar compound Sunset Yellow was reduced, the toxicity slightly decreased. Therefore, the product of 1-amino-2-naphthol should contribute to the increased toxicity (61). This study also proved that the metabolite of Sunset Yellow 1-amino-2-naphthol-6-sulphonate was not mutagenic, which could be due to the decrease in membrane permeability of this compound (61). A study about the mutagenicity of 1-amino-2-naphthol-based azo dyes also indicated that sulphonated 1-amino-2-naphthol at strategic sites inhibits the mutagenicity (111). Acid Orange 8, Acid Red 88, Orange II, Para Red, Ponceau BS, 1-Phenylazo-2-naphthol, 1-[(2,4-dimethylphenyl)azo]-2-naphthalenol, 1-(4-phenylazophenylazo)-2-naphthalenol, and 1-[[2-methyl-4-[(2-methylphenyl)azo]phenyl]azo]-2-naphthalenol can be reduced to produce 1-amino-2-naphthol; while, the food dyes of Allura Red and Sunset Yellow can produce 1-amino-2-naphthol-6-sulphonate (Table 2).

Benzidine is known as carcinogen for the human urinary bladder (62). In Japan, a recent study found that the concentration of benzidine in placemats made of cotton exceeds the EU regulation limits (72). In the testing systems with mouse liver enzyme preparations, benzidine was mutagenic to *S.* Typhimurium strain TA98 and TA100 (75). In the testing systems with FMN and hamster liver S9, benzidine was also mutagenic (102). The nitro derivative of benzidine was more mutagenic than that of parent compound in TA98 without S9 (142). While, the addition of a sulfonic acid group to benzidine reduced the mutagenicity (10). In addition, when both sides of the azo linkage of the azo dyes were sulfonated, the compounds were not carcinogenic in any animal species (27). The similar situation was also found with the mutagenicities of aniline and 4-aminobenzenesulfonic acid, 1-amino-2-naphthol, and 1-amino-2-naphthol-6-sulphonate as discussed before. In United States today, the production and use of benzidine has been banned (11). Benzidine is a product of the reduction of Congo Red, and the sulfonated benzidine is a product of the reduction of Acid Orange 6 (Table 2).

5.2. Capability of human intestinal microbiota to reduce azo dyes

The main intestinal bacteria and their roles in the reduction of azo dyes are shown in Table 3. *Bacteroides vulgatus* is one of the most abundant bacterial species in the human colon (92), and may play a role in the pathogenesis of Crohn's disease (112). *B. vulgatus* can reduce 1-(4-phenylazophenylazo)-2-naphthalenol and 1-[[2-methyl-4-[(2-methylphenyl)azo]phenyl]azo]-2-naphthalenol completely, and can partially reduce 1-phenylazo-2-naphthol and Para Red (139).

Bacteroides ovatus is a Gram-negative intestinal bacterium and can causes serum antibody responses in IBD patients (114). Azoreductase activity was already found in *B. ovatus* (87). The strain *B. ovatus* ATCC 8483 can reduce 1-phenylazo-2-naphthol, 1-[(2,4-dimethylphenyl)azo]-2-naphthalenol, 1-(4-phenylazophenylazo)-2-naphthalenol, 1-[[2-methyl-4-[(2-methylphenyl)azo]phenyl]azo]-2-naphthalenol and Para Red (139).

Bacteroides distasonis is one of the most common species from human feces, and was reclassified as *Parabacteroides distasonis* (116). *P. distasonis* has the activity to reduce 1-phenylazo-2-naphthol, 1-[(2,4-dimethylphenyl)azo]-2-naphthalenol, 1-(4-phenylazophenylazo)-2-naphthalenol, 1-[[2-methyl-4-[(2-methylphenyl)azo]phenyl]azo]-2-naphthalenol, and Para Red (139).

Baceroides fragilis is one of the most common species from the normal human colonic microbiota, however the bacterium can cause abscesses, soft-tissue infections and bacteremias to the host (91). The crude extract of *B. fragilis* has a strong fibrinogenolytic activity suggesting an important biological factor in *Bacteroides* infection (35). *B. fragilis* can reduce 1-phenylazo-2-naphthol, 1-(4-phenylazophenylazo)-2-naphthalenol, 1-[[2-methyl-4-[(2-methylphenyl)azo]phenyl]azo]-2-naphthalenol and can partially degrade Para Red (139).

Bacteroides thetaiotaomicron is an anaerobe and predominate in the intestinal microbiota, accounting for 6% of all bacteria in the human intestine (143). *B. thetaiotaomicron* has a central role in nutrient metabolism, especially in the starch utilization (42, 109) and has the activity to reduce Amaranth, Sunset Yellow (38, 130), Tartrazine, Ponceau SX, Methyl Orange, Orange II, Allura Red (38), 1-phenylazo-2-naphthol, 1-[(2,4-dimethylphenyl)azo]-2-naphthalenol, 1-(4-phenylazophenylazo)-2-naphthalenol, 1-[[2-methyl-4-[(2-methylphenyl)azo]phenyl]azo]-2-naphthalenol, and Para Red (139).

Bifidobacterium longum is used as a probiotic in functional foods (12). The bacterium is acquired from the mother during delivery and habits in the newborn intestine (7). 1-(4-Phenylazophenylazo)-2-naphthalenol, 1-[[2-methyl-4-[(2-methylphenyl)azo]phenyl]azo]-2-naphthalenol, and Para Red can be reduced by *B. longum* (139).

Bifidobacterium adolescentis is also a type of probiotic bacteria, and a butanol extract of the bacterium can increase the activity of host immune system (76). *B. adolescentis* was also studied as a gene delivery system to solid tumors (79). 1-(4-Phenylazophenylazo)-2-naphthalenol, and 1-[[2-methyl-4-[(2-methylphenyl)azo]phenyl]azo]-2-naphthalenol can be reduced by *B. adolescentis* (139).

Bifidobacterium infantis as probiotic bacterium has the ability to reduce intestinal inflammation (22, 23). It is reported that *B. infantis* can decolorize 1-phenylazo-2-naphthol, 1-[(2,4-dimethylphenyl)azo]-2-naphthalenol, 1-(4-phenylazophenylazo)-2-nahpthalenol, 1-[[2-methyl-4-[(2-methylphenyl)azo]phenyl]azo]-2-naphthalenol, and Para Red within 2 days (139).

It is reported that *Clostridium* species from human gastrointestinal tract have the azoreductase activity and these azoreductases share a similar enzyme structure (106, 107). The enzyme from *Clostridium perfringens* had the highest azoreductase activity in the test bacteria (105). The azoreductase was purified and addition of FAD enhanced the enzyme activity. The enzyme also reduced the nitro substituent of nitroaromatic compounds (103, 104). McBain and Macfarlane reported a similar finding that culture samples of *C. perfringens* can reduce amaranth and nitrobenzoic acid (87). *C. perfringens* also reduced 1-phenylazo-2-naphthol, 1-(4-phenylazophenylazo)-2-naphthalenol, 1-[[2-methyl-4-[(2-methylphenyl)azo]phenyl]azo]-2-naphthalenol, and Para Red (139).

Clostridium botulinum known for the ability to form botulinum neurotoxin has the similar azoreductase activity to *C. perfringens* (99). *C. botulinum* can reduce 1-phenylazo-2-naphthol, 1-(4-phenylazophenylazo)-2-naphthalenol, 1-[[2-methyl-4-[(2-methylphenyl)azo]phenyl]azo]-2-naphthalenol, and Para Red (139).

Clostridium difficile infection associated with some gastrointestinal diseases (125). *C. difficile* toxin A caused acute colitis in rats and can be inhibited by a compound contained azo bond, APAZA, which is a molecule of 5-aminosalicylic acid linked to another molecule of 4-aminophenylacetic acid via the azo bond. The reduced product of APAZA was responsible for protection against colitis (89). *C. difficile* can metabolize azo dyes similar to *C. butyricum* (139).

Ent. faecalis is commonly isolated from mammalian gastrointestinal tract (56). The azoreductase from *Ent. faecalis* has a higher specific activity compared to the enzyme from several other intestinal bacteria (133). The azoreductase from *Ent. faecalis* has been purified and characterized (33). The enzyme is FMN-depended and NADH is a preferred electron donor for its activity. The structure of the enzyme has been studied and the FMN binding site has been characterized (34, 81). The azoreductase from *Ent. faecalis* (AzoA) was overexpressed in *E. coli* and the results showed that cytoplasmic azoreductase is involved in the decolorization of dyes *in*

Table 3. Some prevalent intestinal bacteria and their activities for the reduction of azo dyes

Organism	Azo dyes	Azoreduction products	Reference
Bacteroides vulgatus	1-Phenylazo-2-naphthol, 1-(4-phenylazophenylazo)-2-nahpthalenol, 1-[[2-methyl-4-[(2-methylphenyl)azo]phenyl]azo]-2-naphthalenol, Para Red	Aniline, 1-amino-2-naphthol, *p*-phenylenediamine, *o*-toluidine, 2-methyl-1,4-benzenediamine, *p*-nitroaniline	(139)
Bacteroides ovatus	1-Phenylazo-2-naphthol, 1-[(2,4-dimethylphenyl)azo]-2-naphthalenol, 1-(4-phenylazophenylazo)-2-nahpthalenol, 1-[[2-methyl-4-[(2-methylphenyl)azo]phenyl]azo]-2-naphthalenol, Para Red	Aniline, 1-amino-2-naphthol, 2,4-dimethylaniline, *p*-phenylenediamine, *o*-toluidine, 2-methyl-1,4-benzenediamine, *p*-nitroaniline	(139)
Bacteroides distasonis	1-Phenylazo-2-naphthol, 1-[(2,4-dimethylphenyl)azo]-2-naphthalenol, 1-(4-phenylazophenylazo)-2-nahpthalenol, 1-[[2-methyl-4-[(2-methylphenyl)azo]phenyl]azo]-2-naphthalenol, Para Red	Aniline, 1-amino-2-naphthol, 2,4-dimethylaniline, *p*-phenylenediamine, *o*-toluidine, 2-methyl-1,4-benzenediamine, *p*-nitroaniline	(139)
Baceroides fragilis	1-Phenylazo-2-naphthol, 1-(4-phenylazophenylazo)-2-nahpthalenol, 1-[[2-methyl-4-[(2-methylphenyl)azo]phenyl]azo]-2-naphthalenol, Para Red	Aniline, 1-amino-2-naphthol, *p*-phenylenediamine, *o*-toluidine, 2-methyl-1,4-benzenediamine, *p*-nitroaniline	(139)
Bacteroides thetaiotaomicron	Amaranth, Sunset Yellow, Tartrazine, Ponceau SX, Methyl Orange, Orange II, Allura Red, 1-phenylazo-2-naphthol, 1-(4-phenylazophenylazo)-2-nahpthalenol, 1-[[2-methyl-4-[(2-methylphenyl)azo]phenyl]azo]-2-naphthalenol, Para Red	4-Aminobenzenesulfonic acid, 4-amino-3-hydroxy-2,7-naphthalenedisulfonic acid, 4-aminobenzenesulfonic acid, 4-amino-3-hydroxy-7-naphthalenesulfonic acid, 5-hydroxy-1-(4-sulforatophenyl)-4-amino-1H-pyrazole-3-carboxylate, 1-amino-2,4-dimethyl-5-benzenesulfonic acid, 3-amino-4-hydroxy-1-naphthalenesulfonic acid, *N,N*-dimethyl-*p*-phenylenediamine, 1-amino-2naphthol, 1-amino-2-methoxy-5-methyl-4-benzenesulfonic acid, aniline, *p*-phenylenediamine, *o*-toluidine, 2-methyl-1,4-benzenediamine, *p*-nitroaniline	(38, 130, 139)
Bifidobacterium longum	1-(4-Phenylazophenylazo)-2-nahpthalenol, 1-[[2-methyl-4-[(2-methylphenyl)azo]phenyl]azo]-2-naphthalenol, Para Red	Aniline, 1-amino-2-naphthol, *p*-phenylenediamine, *o*-toluidine, 2-methyl-1,4-benzenediamine, *p*-nitroaniline	(139)
Bifidobacterium adolescentis	1-(4-Phenylazophenylazo)-2-nahpthalenol, 1-[[2-methyl-4-[(2-methylphenyl)azo]phenyl]azo]-2-naphthalenol	Aniline, 1-amino-2-naphthol, *p*-phenylenediamine, *o*-toluidine, 2-methyl-1,4-benzenediamine	(139)
Bifidobacterium infantis	1-Phenylazo-2-naphthol, 1-[(2,4-dimethylphenyl)azo]-2-nahpthalenol, 1-(4-phenylazophenylazo)-2-nahpthalenol, 1-[[2-methyl-4-[(2-methylphenyl)azo]phenyl]azo]-2-naphthalenol, Para Red	Aniline, 1-amino-2-naphthol, 2,4-dimethylaniline, *p*-phenylenediamine, *o*-toluidine, 2-methyl-1,4-benzenediamine, *p*-nitroaniline	(139)
Clostridium perfringens	Amaranth, 1-phenylazo-2-naphthol, 1-[(2,4-dimethylphenyl)azo]-2-naphthalenol, 1-(4-phenylazophenylazo)-2-nahpthalenol, 1-[[2-methyl-4-[(2-methylphenyl)azo]phenyl]azo]-2-naphthalenol, Para Red	4-Animobenzenesulfonic acid, 4-amino-3-hydroxy-2,7-naphthalenedisulfonic acid, aniline, 1-amino-2-naphthol, 2,4-dimethylaniline, *p*-phenylenediamine, *o*-toluidine, 2-methyl-1,4-benzenediamine, *p*-nitroaniline	(87, 139)
Clostridium botulinum	1-Phenylazo-2-naphthol, 1-[(2,4-dimethylphenyl)azo]-2-nahpthalenol, 1-(4-phenylazophenylazo)-2-nahpthalenol, 1-[[2-methyl-4-[(2-methylphenyl)azo]phenyl]azo]-2-naphthalenol, Para Red	Aniline, 1-amino-2-naphthol, 2,4-dimethylaniline, *p*-phenylenediamine, *o*-toluidine, 2-methyl-1,4-benzenediamine, *p*-nitroaniline	(139)
Clostridium difficile	1-Phenylazo-2-naphthol, 1-[(2,4-dimethylphenyl)azo]-2-nahpthalenol, 1-(4-phenylazophenylazo)-2-nahpthalenol, 1-[[2-methyl-4-[(2-methylphenyl)azo]phenyl]azo]-2-naphthalenol, Para Red	Aniline, 1-amino-2-naphthol, 2,4-dimethylaniline, *p*-phenylenediamine, *o*-toluidine, 2-methyl-1,4-benzenediamine, *p*-nitroaniline	(139)
Enterococcus faecalis	1-Phenylazo-2-naphthol, 1-[(2,4-dimethylphenyl)azo]-2-nahpthalenol, 1-(4-phenylazophenylazo)-2-nahpthalenol, 1-[[2-methyl-4-[(2-methylphenyl)azo]phenyl]azo]-2-naphthalenol, Para Red, Methyl Red, Orange II, Orange G, Amaranth, Ponceau BS, Ponceau S	Aniline, 1-amino-2-naphthol, 2,4-dimethylaniline, *p*-phenylenediamine, *o*-toluidine, 2-methyl-1,4-benzenediamine, *p*-nitroaniline, *N,N*-dimethyl-*p*-phenylenediamine, 4-animobenzenesulfonic acid, 4-amino-3-hydroxy-5,8-naphthalenedisulfonic acid, 4-amino-3-hydroxy-2,7-naphthalenedisulfonic acid, 2,5-diminobenzenesulfonic acid	(33, 139)
Enterococcus faecium	1-Phenylazo-2-naphthol, 1-[(2,4-dimethylphenyl)azo]-2-nahpthalenol, 1-(4-phenylazophenylazo)-2-nahpthalenol, 1-[[2-methyl-4-[(2-methylphenyl)azo]phenyl]azo]-2-naphthalenol,	Aniline, 1-amino-2-naphthol, 2,4-dimethylaniline, *p*-phenylenediamine, *o*-toluidine, 2-methyl-1,4-benzenediamine, *p*-nitroaniline	(139)

	Para Red		
Escherichia coli	Methyl Red, Ponceau BS, Orange II	Aniline, *N,N*-dimethyl-*p*-phenylenediamine, 4-animobenzenesulfonic acid, 2,5-diminobenzenesulfonic acid, 1-amino-2-naphthol	(55, 95)
Lactobacillus paracasei	Methyl Orange, Acid Orange 8, Tartrazine, Acid Orange 63, 1-phenylazo-2-naphthol, 1-[(2,4-dimethylphenyl)azo]-2-nahpthalenol, 1-(4-phenylazophenylazo)-2-nahpthalenol, 1-[[2-methyl-4-[(2-methylphenyl)azo]phenyl]azo]-2-naphthalenol, Para Red	*N,N*-Dimethyl-*p*-phenylenediamine, 4-animobenzenesulfonic acid, 4-amino-3-methylbenzenesulfonic acid, 1-amino-2-naphthol, 4-aminobenzenesulfonic acid, 5-hydroxy-1-(4-sulforatophenyl)-4-amino-1H-pyrazole-3-carboxylate, 3-methyl-4-amino-1-phenyl-1H-pyrzaol-5ol, benzidine-3-sulfonic acid, 4-methylphenyl-4-aminobenzenesulfonate, aniline, 2,4-dimethylaniline, *p*-phenylenediamine, *o*-toluidine, 2-methyl-1,4-benzenediamine, *p*-nitroaniline	(101, 139)

vivo (55). *Ent. faecalis* has been shown to possess higher azoreductase activity than those of many other intestinal bacteria (133). The bacterium can degrade a broad spectrum of azo dyes, including 1-phenylazo-2-naphthol, 1-[(2,4-dimethylphenyl)azo]-2-naphthalenol, 1-(4-phenylazophenylazo)-2-naphthalenol, 1-[[2-methyl-4-[(2-methylphenyl)azo]phenyl]azo]-2-naphthalenol, Para Red, Methyl Red, Orange II, Orange G, Amaranth, Ponceau BS, and Ponceau S (33, 138, 139).

Enterococcus faecium is an intestinal bacterium that also has been isolated from patients with severe infection in hospital and possesses a broad spectrum of antibiotic resistance (73). Interestingly, some strains of *Ent. faecium* are gaining biotechnological interest since they may produce bacteriocins (77). The azoreductase from *Ent. faecium* was purified, and shared 67% identity with the enzyme (AzoA) from *Ent. faecalis* (83). Like AzoA, the enzyme also possesses a broad substrate spectrum of azoreductase activity (83). *Ent. faecium* can metabolize 1-phenylazo-2-naphthol, 1-[(2,4-dimethylphenyl)azo]-2-naphthalenol, 1-(4-phenylazophenylazo)-2-naphthalenol, 1-[[2-methyl-4-[(2-methylphenyl)azo]phenyl]azo]-2-naphthalenol, and Para Red (139).

The azoreductase gene of *E. coli* was annotated first as an acyl carrier protein phosphodiesterase (*acpD*) gene. The gene product was purified, and it was found that the enzyme can not convert holo-acyl carrier protein into the apo form, and instead the azoreductase activity was identified, therefore, the *acpD* gene was redesigned as *azoR* (95). AzoR is a FMN-dependent and NADH-preferred enzyme, and catalyzes the reduction of azo dyes by ping-pong mechanism. The crystal structure of AzoR with FMN has been reported (69). The *E. coli* strain contained the overexpressed AzoR has a similar decolorization activity at aerobic and anaerobic conditions (Unpublished data), which means the azoreductase from *E. coli* is oxygen tolerant. Methyl Red, Ponceau BS, and Orange II can be reduced by *E. coli* (55, 95).

Lactobacillus paracasei is a facultative anaerobic bacteria found in intestinal tracts. It is reported that this bacterium can metabolize azo dyes under anaerobic conditions (101). The bacterium can cause red-color spoilage in cucumber pickle products when the azo dye Tartrazine was used as yellow coloring (100). 1-Phenylazo-2-naphthol, 1-[(2,4-dimethylphenyl)azo]-2-naphthalenol, 1-(4-phenylazophenylazo)-2-naphthalenol, 1-[[2-methyl-4-[(2-methylphenyl)azo]phenyl]azo]-2-naphthalenol, Para Red, Methyl Orange, Acid Orange 8, Tartrazine, and Acid Orange 63 can be reduced by the bacterium (101, 139).

6. SUMMARY

Azo dyes are used widely in consumer products. Human intestinal microbiota play a key role in the metabolism of azo dyes and azoreductase from intestinal bacteria can reduce the azo bond of the dyes via NAD(P)H. There are three groups of azoreductases: flavin dependent NADH preferred azoreductase, such as the enzymes from *Ent. faecalis*, *Ent. faecium*, and *E. coli*; flavin dependent NADPH preferred azoreductase, such as the enzymes from *S. aureus*, *B. cereus*, and *B. subtilis*; and flavin free NADPH preferred azoreductase, such as the enzymes from *P. kullae* and *X. azovorans*. A portion of the intestinal microbiota community can metabolize both water-soluble and water-insoluble azo dyes. Some of metabolites of the dyes produced by these microorganisms are carcinogenic, for example, *o*-toluidine and benzene are two of the metabolites of 1-[[2-methyl-4-[(2-methylphenyl)azo]phenyl]azo]-2-naphthalenol and Congo Red, respectively; and they are carcinogenic to humans and classed as group 1 by IARC. Recently, we demonstrated that human microbiota can reduce Sudan dyes to form toxic aromatic amines, which indicates the potential risk to consume food products contaminated with these dyes. Further studies on the enzymatic reduction mechanisms of both water-soluble and water-insoluble azo dyes by human intestinal microbiota along with the potential to produce genotoxic aromatic compounds are needed.

7. ACKNOWLEDGEMENTS

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