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ORIGINAL PAPER

Decolorization of water and oil-soluble azo dyes by *Lactobacillus acidophilus* and *Lactobacillus fermentum*

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Abstract The capability of *Lactobacillus acidophilus* and *Lactobacillus fermentum* to degrade azo dyes was investigated. The bacteria were incubated under anaerobic conditions in the presence of 6 μg/ml Methyl Red, Ponceau BS, Orange G, Amaranth, Orange II, and Direct Blue 15; 5 μg/ml Sudan I and II; or 1.5 μg/ml Sudan III and IV in deMann–Rogosa–Sharpe broth at 37°C for 36 h, and reduction of the dyes was monitored. Both bacteria were capable of degrading all of the water-soluble azo dyes to some extent. They were also able to completely reduce the oil-soluble diazo dyes Sudan III and IV but were unable to reduce the oil-soluble monoazo dyes Sudan I and II to any significant degree in the concentrations studied. Growth of the bacteria was not significantly affected by the presence of the Sudan azo dyes. Metabolites of the bacterial degradation of Sudan III and IV were isolated and identified by liquid chromatography electrospray ionization tandem mass spectrometry analyses and compared with authentic standards. Aniline and *o*-toluidine (2-methylaniline), both potentially carcinogenic aromatic amines, were metabolites of Sudan III and IV, respectively.

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Abbreviations

DMSO	Dimethyl sulfoxide
MRS	deMann–Rogosa–Sharpe
HPLC	High-performance liquid chromatography
LC/ESI-MS/MS	Liquid chromatography electrospray ionization tandem mass spectrometry

Introduction

Azo dyes, which are characterized by one or more azo bonds, are a major group of colorants used in textiles, plastics, pharmaceuticals, cosmetics, and food products [11]. Many synthetic azo dyes can be converted to colorless aromatic amines, some of which are carcinogenic, by azoreductases that catalyze a nicotinamide adenine dinucleotide phosphate NAD(P)H-dependent reduction [8]. Many food, drug, and textile dyes are sulfonated azo dyes, which are the most prevalent types of chemical dyes in use [7]. Sudan I, II, III, and IV are a family of oil-soluble azo dyes widely used in industrial processes for making plastics, printing inks, waxes, leather, fabrics, and floor polishes [2, 4]. These Sudan dyes are classified as category three carcinogens by the International Agency for Research on Cancer (IARC) [1]. Although Sudan dyes are not legal for use in foods, they have been found as contaminants in the food supply [4, 24, 26]. The level of Sudan dyes in foods is monitored by international food safety agencies. The recent detection of Sudan dyes in various food commodities, including hot chili products, emphasizes the need for toxicological evaluation by regulatory agencies to determine the impact of these dyes on human health [4, 24, 31].

Human exposure to azo dyes mainly occurs through ingestion of food contaminated with the dyes. The human gastrointestinal tract harbors a complex and diverse microbial community composed of several thousand species [16]. The human intestinal microbiota provide nourishment, regulate epithelial development, instruct innate immunity, act as a barrier to colonization of the intestinal tract by pathogenic bacteria, and metabolize both dietary and in-situ-produced compounds [6, 7]. These microorganisms also play a key role in the degradation of azo dyes, with azo reduction being the most crucial reaction related to toxicity and mutagenicity [8, 11, 12, 19, 30].

Lactic acid bacteria are normal inhabitants of the digestive tracts of humans and animals and play an important role in degrading xenobiotics [20]. Lactobacilli are facultative anaerobic or microaerophilic lactic acid bacteria, strictly fermentative and present throughout the gastrointestinal tract [13]. The *Lactobacillus* group represents 1–6% of the intestinal microbiota and is essential to the maintenance and restoration of human health, providing metabolic, nutritional, and protective functions [15]. *Lactobacillus acidophilus* and *L. fermentum* are predominant lactobacilli in the human intestine and display probiotic properties [32]. They are the most industrially important species used in making yogurt and dietary supplements [27]. *L. acidophilus* is among the most versatile and practically applied lactobacillus species, commonly used as starter culture. The bacterium is believed to have a beneficial effect on human health and is found in many probiotic products [23]. *L. fermentum* is commonly present in fermented milk products and has been recognized as one of the dominant microorganisms in sourdough fermentations and in the preparation of many traditional fermented foods [17, 22]. Azo dyes are found in many *Lactobacillus*-fermented food products, some of which are potentially at risk of being contaminated with oil-soluble Sudan dyes.

Although a few reports describe the ability of some lactic acid bacteria to degrade water-soluble azo dyes [28], little is known about the degradation of oil-soluble azo dyes by lactic acid bacteria. Furthermore, no report regarding the degradation of azo dyes by *L. acidophilus* or *L. fermentum* has been published.

The focus of our research is to investigate the biodegradation and bioconversion of azo dyes in food products, drugs, and cosmetics by the commensal microbiota. Recently, we demonstrated that human intestinal microbiota are able to reduce azo dyes [33, 35]. Furthermore, we found that Sudan dyes are degraded to form potentially carcinogenic aromatic amines [35] by human fecal suspensions. In this study we examined the ability of *L. acidophilus* and *L. fermentum* to degrade azo dyes and analyzed the metabolites of the bacterial degradation of Sudan III and IV using liquid chromatography electrospray ionization tandem mass spectrometry (LC/ESI-MS/MS).

Materials and methods

Chemicals

Methyl Red, Ponceau BS, Orange G, Amaranth, Orange II, Direct Blue 15, Sudan I (1-[(2,4-dimethylphenyl)azo]-2-naphthol), Sudan II (1-(phenylazo)-2-naphthol), Sudan III (1-[4-(phenylazo)phenylazo]-2-naphthol), Sudan IV (*o*-tolyazo-*o*-tolyazo-*β*-naphthol), aniline, and *o*-toluidine (2-methylaniline) were purchased from Sigma Chemical Co. Acetonitrile was purchased from J.T. Baker. Dimethyl sulfoxide (DMSO) and ethyl acetate were purchased from Fisher Scientific Co. Stock solutions of Methyl Red, Sudan I, II, III, and IV were made by dissolving each dye in DMSO (1 mg/ml). Chemical structures of the dyes are shown in Table 1.

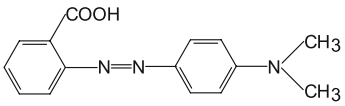
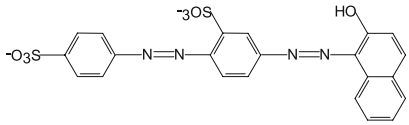
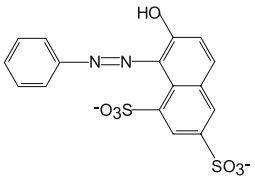
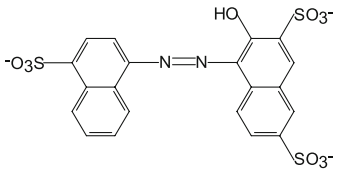
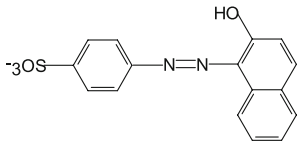
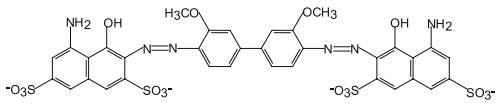
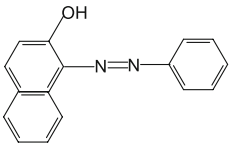
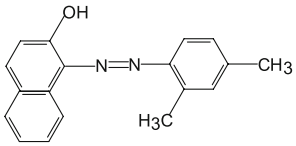
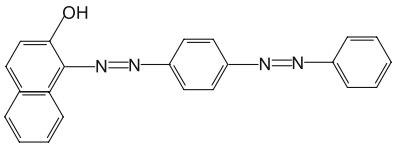
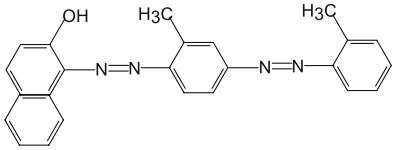
Bacteria and culture conditions

L. acidophilus ATCC 4356 and *L. fermentum* ATCC 23271 were obtained from the American Type Culture Collection (ATCC). The strains were preserved for long-term storage at −80°C in 10–15% glycerol stocks and revived as needed. They were routinely cultured in deMann–Rogosa–Sharpe (MRS) broth or on MRS agar medium (Becton Dickinson & Company) at 37°C under an anaerobic atmosphere of 91% nitrogen, 4% hydrogen, and 5% carbon dioxide in an anaerobic chamber (Coy Laboratory Products, Inc.) [18]. *L. acidophilus* and *L. fermentum* were grown anaerobically at 37°C in MRS broth supplemented with various azo dyes. A loopful of each strain was cultured at 37°C for 24 h in an Erlenmeyer flask containing 100 ml medium for use as starter culture. Flasks containing 100 ml MRS broth were inoculated with 1.5 ml of the bacterial inoculum culture. Azo dye stock solutions were added to the medium at final concentrations of 6 μg/ml (Methyl Red and water-soluble azo dyes), 5 μg/ml (Sudan I and II), and 1.5 μg/ml (Sudan III and IV) due to the insolubility of the dyes, especially the Sudan dyes, and the cultures were incubated at 37°C in an anaerobic chamber for 36 h without agitation. Three control incubations that consisted of sterile liquid medium, sterile liquid medium with bacteria, and sterile liquid medium with dyes were used for LC/ESI-MS/MS analyses. For the effect of Sudan dye on growth of bacterium, only one control (without dye) was included in the experiment.

Assay of the decolorization of azo dyes by *L. acidophilus* and *L. fermentum*

For measurements of decolorization of Methyl Red and water-soluble dyes (Ponceau BS, Orange G, Amaranth, Orange II, and Direct Blue 15), the cells in the cultures were

Table 1 Chemical structures of water-soluble and oil-soluble azo dyes used in this study

Chemical structure of azo dye	Chemical structure of azo dye
Methyl Red 	Ponceau BS 
Orange G 	Amaranth 
Orange II 	Direct Blue 15 
Sudan I 	Sudan II 
Sudan III 	Sudan IV 

removed by centrifugation at 6,000×*g* at 4°C for 10 min. The decolorization of the dyes was determined by measuring the absorption at the relevant wavelength for each dye (Table 2). For measurements of decolorization for oil-soluble Sudan dyes, 3 × 20 ml samples were collected from the cultures at the end of the incubation time and extracted twice with equal volumes of ethyl acetate. The extracts were evaporated in a rotary evaporator at 40°C, and the trace of solvent remaining was removed by evaporation at room temperature overnight. Each residue was dissolved in 4 ml acetonitrile and filtered through a 0.2-μm syringe filter. Samples of 40 μl were analyzed with a Hewlett-Packard 1050 high-performance liquid chromatograph (HPLC) equipped with a module variable wavelength detector (detection wavelengths were 250 and 500 nm), an autosampler, and a reversed-phase Luna C18 [2] column (150 × 3.0 mm, particle size 5 μm, Phenomenex) with a guard column (40 × 3.0 mm, Phenomenex). The mobile phase was composed of a linear gradient of acetonitrile from 30% to 95% in H_2O with 0.1% formic acid for 40 min. The peak area was used to calculate the concentration of Sudan dyes. Reduction of Sudan dyes in the cultures was directly determined by monitoring the disappearance of the absorption peak for each dye at 500 nm after the extraction.

The time-course of Sudan dye degradation by *L. acidophilus* and *L. fermentum* was monitored using HPLC and

Table 2 Decolorization of azo dyes by *Lactobacillus acidophilus* and *L. fermentum*

Azo dye	Absorption max. (nm)	*L. acidophilus* reduction (%)*	*L. fermentum* reduction (%)
Methyl Red	430	100	86
Ponceau BS	502	43	30
Orange G	477	100	73
Amaranth	520	78	61
Orange II	483	65	71
Direct Blue 15	601	87	80
Sudan I	500	–**	–
Sudan II	500	–	–
Sudan III	500	100	100
Sudan IV	500	100	100

* The cultures were incubated with each of azo dyes at 37°C in an anaerobic chamber for 36 h without agitation, and data are presented in percent by the averages from triplicate incubations with standard deviations of <5%

** No reduction detected

LC/ESI-MS/MS, as described below. Samples (5–10 ml) were aseptically collected every 4 h and analyzed for growth and reduction of the dyes. Then, extractions with ethyl acetate were performed as described above. To determine whether Sudan dyes inhibited bacterial growth, bacterial density in the cultures was determined by measuring the optical density (OD) of the cultures at OD_{600}.

Assay of metabolites formed from the biodegradation of Sudan dyes by *L. acidophilus* and *L. fermentum*

Identification of the metabolites of Sudan dyes was performed using a similar procedure to that described previously [35]. LC/ESI-MS/MS analyses were performed on the ThermoFinnigan Quantum Ultra mass spectrometer equipped with an Agilent 1100 Series HPLC and diode array detector (DAD). HPLC was performed with a Prodigy ODS [3] 2.0 × 250 mm 5 µm 100 A HPLC column (Phenomenex). The DAD was scanned from 200 to 550 nm at 2.5 Hz. The mass spectrometer was operated in the positive-ion electrospray ionization (ESI) mode with an in-source collision-induced dissociation (CID) offset of 0 V. Other ESI conditions were spray voltage 3.0 kV, capillary temperature 350°C, sheath gas 50 psi, ion sweep gas 1, and auxiliary gas 5. For MS/MS, argon collision gas was set at 1.5 mT; Q1 parent ions were alternated between *m/z* 94, 108, and 160, with a collision energies at 30, 20, and 20 eV, respectively; and Q3 was scanned *m/z* 30−170/0.5 s. Aromatic amine standards were recovered by the extraction procedure with ethyl acetate, dried, and then dissolved in a buffer with acetonitrile and 0.1% formic acid (5:95, v/v), respectively. Other samples were treated in a similar fashion. Much of the dried sample was insoluble, so samples were taken above the precipitate. For analyses of Sudan azo dye metabolites, the starting buffer was held 20 min, ramped to acetonitrile and 0.1% formic acid (95:5, v/v), respectively, at 40 min, and held to 70 min. Ethyl acetate extracts of incubation broths in which *L. acidophilus* and *L. fermentum* were incubated with Sudan III and IV and were dried. The residues were extracted with the starting buffer, and soluble metabolites were analyzed by LC/ESI-MS/MS. Product ion spectra, retention times, and ultraviolet (UV) data for metabolites were compared with those for authentic compounds for identification.

Results

Reduction of azo dyes by *L. acidophilus* and *L. fermentum*

Several water- and oil-soluble mono and diazo dyes (Table 1) were included in the degradation experiments. After 36 h cultivation, all water-soluble azo dyes were reduced to some extent in MRS medium by *L. acidophilus* and *L. fermentum*, which indicated that the bacteria were capable of degrading both monoazo and diazo water-soluble dyes (Table 2). *L. acidophilus* reduced Methyl Red and Orange G much quicker than did *L. fermentum*. However, both species reduced Ponceau BS, Amaranth, Orange II, and Direct Blue 15 at similar rates. Table 2 shows that *L. acidophilus* and *L. fermentum* were capable of completely degrading the oil-soluble diazo dyes Sudan III and IV at the concentration of 1.5 µg/ml. However, neither strain was able to degrade the oil-soluble monoazo dyes Sudan I and II at the concentration of 5 µg/ml. Furthermore, when the concentration of Sudan I and II in the medium was decreased to 1.5 µg/ml, no reduction of the dyes by the bacteria was observed.

Time-course study of the degradation of Sudan III and IV by *L. acidophilus* and *L. fermentum* and effects of the dyes on the growth of the bacteria

The degradation of the food contaminants Sudan III and IV by *L. acidophilus* and *L. fermentum* was investigated with a time-course experiment. *L. acidophilus* and *L. fermentum* were inoculated into MRS medium under anaerobic conditions to observe the effect of these two bacterial species on decolorization of individual Sudan dyes (Fig. 1). After a lag of 4 h, Sudan III and IV began to be reduced by *L. acidophilus*. Sudan IV was reduced more rapidly than Sudan III. Approximately 65% of Sudan IV was degraded in 16 h, and the dye had completely disappeared after 24 h (Fig. 1b). However, only about 49% of Sudan III was degraded in 16 h, and it took 28–32 h for the bacterium to completely

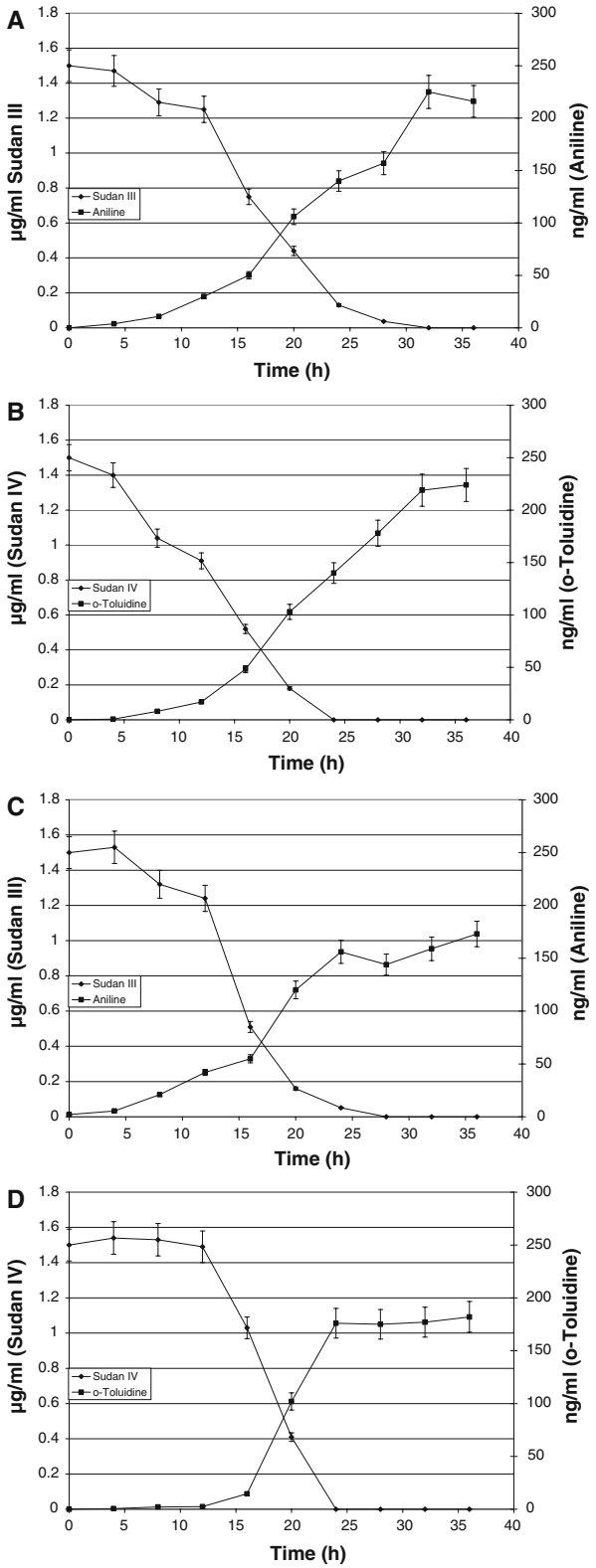


Fig. 1 High-performance liquid chromatography (HPLC) analysis of Sudan azo degradation and iquid chromatography electrospray ionization tandem mass spectrometry (LC/ESI-MS) analysis of metabolites by *Lactobacillus acidophilus* and *L. fermentum*. **a** Sudan III by *L. acidophilus*, **b** Sudan IV by *L. acidophilus*, **c** Sudan III by *L. fermentum*, **d** Sudan IV by *L. fermentum*. Concentration of Sudan azo dyes was 1.5 µg/ml

reduce this dye (Fig. 1a). The difference in degradation rates of Sudan III and IV by *L. fermentum* was less pronounced, with both dyes being completely metabolized in 24–28 h (Fig. 1c, d).

To understand the effects of Sudan III and IV on the growth of *L. acidophilus* and *L. fermentum*, the bacterial density in the media was determined at 600 nm at various time intervals from 0 to 36 h (Fig. 2). In the medium without Sudan dye, the maximal cell density of *L. acidophilus* was obtained after 28 h. In the medium with Sudan III or IV (1.5 µg/ml), no lag in growth was observed, and a final cell density similar to that without the dyes was reached (Fig. 2a). In *L. fermentum* cultures without dye, maximal cell density was obtained after 24 h. A small lag in growth of 1–2 h was observed in cultures with Sudan III or IV, and final cell densities reached 95% of that without dye (Fig. 2b). The negligible inhibitions on the growth of *L. acidophilus* and *L. fermentum* by the Sudan dyes indicated that the dyes were not toxic to the bacteria (Fig. 2) at low concentration (1.5 µg/ml). Furthermore,

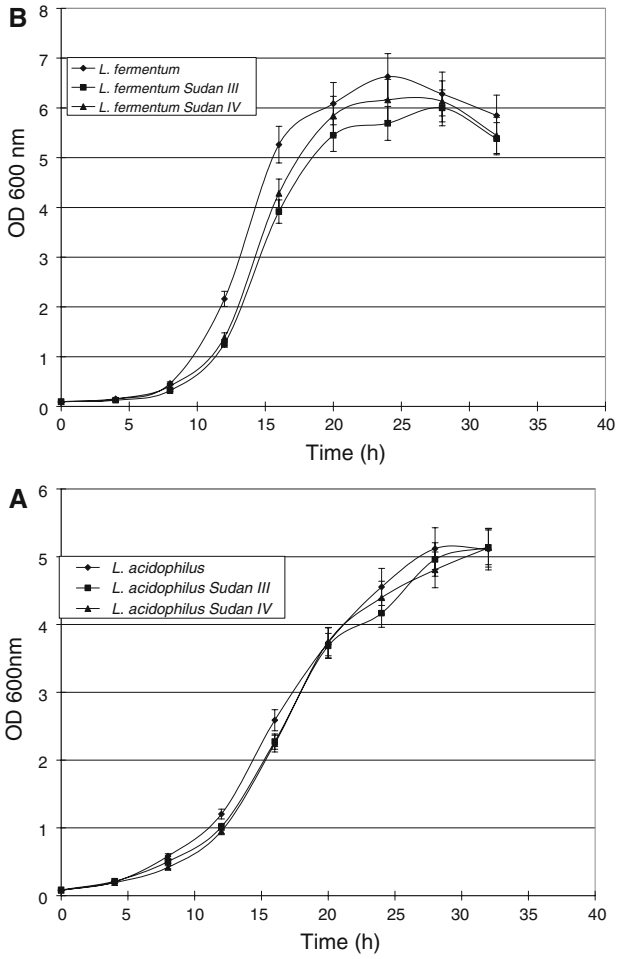


Fig. 2 Effects of Sudan azo dyes on growth of **a** *Lactobacillus acidophilus* and **b** *L. fermentum* . Concentration of Sudan azo dyes was 1.5 µg/ml

diazo Sudan dyes at higher concentration (5 μg/ml) did not significantly inhibit the growth of strains. In a similar experiment, the speed of bacterial cell growth of *L. acidophilus* and *L. fermentum* was not affected by the presence of Sudan I or II in the medium (not shown).

Identification of metabolites of Sudan III and IV by *L. acidophilus* and *L. fermentum* using LC/ESI-MS/MS

The metabolite from Sudan III was identified as aniline, based on a retention time of 4.1 min and a product ion mass spectrum identical to that of the standard (Fig. 3a–c). The protonated molecule at *m/z* 94 was fragmented to give ions at *m/z* 77 [MH^+-17] and 51. The metabolite from Sudan IV was identified as *o*-toluidine based on a retention time of 7.5 min and a product ion mass spectrum identical to the standard (Fig. 3d–f). The protonated molecule at *m/z* 108 was fragmented to give ions at *m/z* 93 [MH^+-15], 91 [MH^+-17], and 65. 1-Amino-2-naphthol from all tested Sudan dyes, 1,4-phenylenediamine from Sudan III, and 2,5-diaminotoluene from Sudan IV (Table 1) could not be detected in the extracted samples as demonstrated in samples inoculated with human fecal suspension [35]. Areas of the protonated molecules from LC/ESI-MS/MS data were used to determine the amounts of aniline and *o*-toluidine in cultures over time. This confirmed that the amounts of aniline and *o*-toluidine increased as Sudan III and IV, respectively, disappeared (Fig. 1).

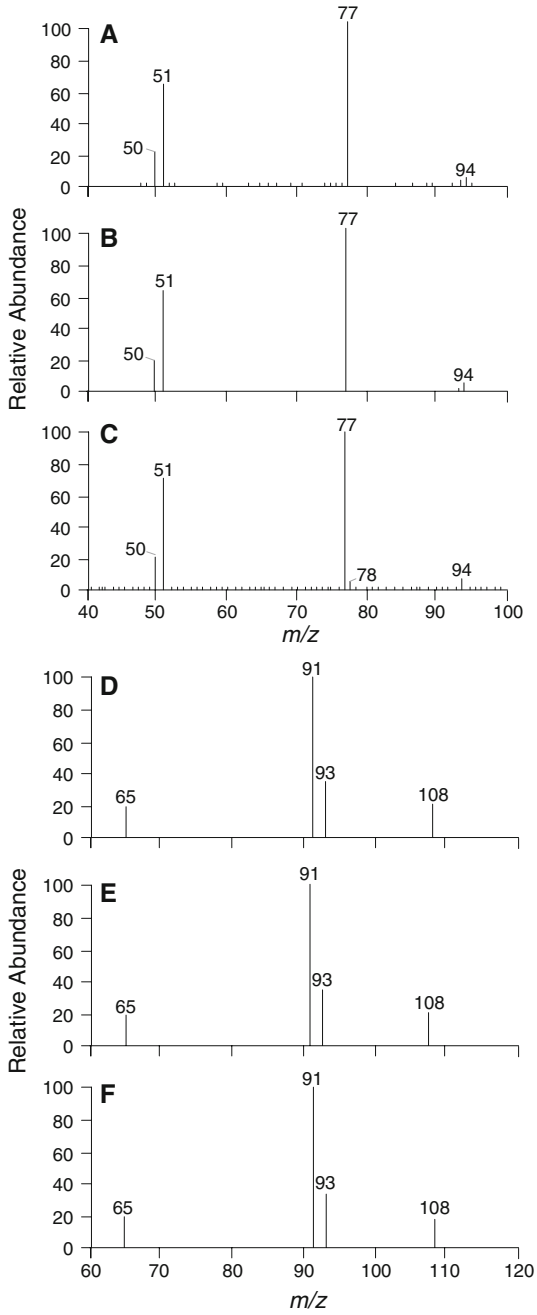


Fig. 3 Product ion spectra (*m/z* 94 at 30 eV) of metabolites from Sudan III with **a** *Lactobacillus acidophilus* or **b** *L. fermentum*, and **c** aniline standard, all eluting at 4.12 min. Product ion spectra (*m/z* 108 at 20 eV) of metabolites from Sudan IV with **d** *L. acidophilus* or **e** *L. fermentum*, and **f** *o*-toluidine standard, all eluting at 7.52 min

Discussion

L. acidophilus and *L. fermentum* were able to degrade both monoazo and diazo water-soluble dyes in a manner similar to that of *Lactobacillus casei* TISTR 1500 [28]. Previously, it was demonstrated that water-soluble azo dyes can freely move in medium, pass through cell walls more easily than water-insoluble azo dyes, and are degraded by azoreductases in several other bacteria [8]. *L. acidophilus* and *L. fermentum* were only able to degrade diazo Sudan III and IV dyes but not monoazo Sudan I and II dyes. This implies that these strains may have a preference for reducing oil-soluble diazo Sudan dyes over oil-soluble monoazo Sudan dyes, which is different from the results seen with fecal suspension inocula. As a bacterial consortium, intestinal microbiota were not only able to slowly reduce diazo Sudan III and IV dyes but also monoazo Sudan I and II dyes [35].

The Sudan dyes were not toxic to the growth of *L. acidophilus* and *L. fermentum* at the concentrations tested, which is similar to results obtained with two human commensal bacteria, *Staphylococcus aureus* [9] and *Enterococcus faecalis* [10] on water-soluble azo dyes and to results obtained with a human fecal consortium on Sudan dyes [35]. Sudan III was reduced by *L. acidophilus* and *L. fermentum*, resulting in the release of aniline (Figs. 1a, c, 3a, b). Aniline is considered hazardous because of its toxicity and carcinogenicity [3, 5]. Experimental animals that were dosed with aniline in feed developed cancer of the spleen [34]. Aniline exposure induces lipid peroxidation and

protein oxidation in the spleen, and an association between release of free iron and oxidative DNA damage, which could lead to mutagenic and/or carcinogenic responses in the spleen [34]. *o*-Toluidine (2-methylaniline), a metabolite of Sudan IV produced by *L. acidophilus* and *L. fermentum* (Figs. 1b, d, 3d, e), is metabolized in vivo into a number of compounds, some of which are active genotoxins [1]. It induces aneuploidy in both fungi and mammalian cultured cells and also produces DNA damage and causes cell transformation [21, 25]. *o*-Toluidine has been demonstrated to be a carcinogen in mice and rats and is a suspected human carcinogen [29]. It has a wide range of genetic effects and is a clastogen on prolonged exposure [14]. *o*-Toluidine has been classified by the US Environmental Protection Agency (EPA) as a probable human carcinogen.

In conclusion, our results demonstrated that *L. acidophilus* and *L. fermentum* are capable of degrading the diazo oil-soluble Sudan III and IV, producing potentially toxic aromatic amines. In addition, these bacteria are able to degrade both monoazo and diazo water-soluble dyes. Further investigations are needed on the metabolic mechanism of azo dyes in lactobacilli and the food contaminant issues of Sudan dyes, along with the potential for producing toxic metabolites.

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