

# Probiotics and Antimicrobial Proteins



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To Whom It May Concern:

This is to confirm that the manuscript PAAP-D-17-00262 entitled “Methods to assess the antioxidative properties of probiotics” by P.V. Zolotukhin, E.V. Prazdnova, and V.A. Chistyakov was submitted to Probiotics and Antimicrobial Proteins on November 14, 2017. The manuscript was reviewed by three specialists in the field of the study and was suggested for publication after minor revision (November 28, 2017).

Michael Leonidas Chikindas, Ph.D.

A handwritten signature in blue ink, consisting of several overlapping, fluid strokes that form a cursive representation of the name.

Editor-in-Chief,  
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# Probiotics and Antimicrobial Proteins

## Methods to assess the antioxidative properties of probiotics

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<b>Abstract:</b>	<p>Probiotics prove useful in correcting and preventing numerous health conditions, including those having severe impact on society, e.g. obesity and cancer. Notably, these capabilities of probiotics appear to be associated with their antioxidant properties. The mechanisms of antioxidant action of probiotics range from immediate biochemical scavenging of reactive substances to induction of signaling events leading to increased capacity of the host's cytoprotective systems. Since the antioxidant effects of probiotics significantly vary in types and details, a broad selection of methods of assessment of these properties is required in order to identify, characterize and develop novel probiotics for medical purposes, as well as to explain the mechanisms of action of probiotics already in use in healthcare. This review revises the versatile toolbox, which can be used to assess the antioxidant properties of probiotics.</p>	
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1

**Methods to assess the antioxidative properties of probiotics**

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**Abstract**

Probiotics prove useful in correcting and preventing numerous health conditions, including those having severe impact on society, e.g. obesity and cancer. Notably, these capabilities of probiotics appear to be associated with their antioxidant properties. The mechanisms of antioxidant action of probiotics range from immediate biochemical scavenging of reactive substances to induction of signaling events leading to increased capacity of the host’s cytoprotective systems. Since the antioxidant effects of probiotics significantly vary in types and details, a broad selection of methods of assessment of these properties is required in order to identify, characterize and develop novel probiotics for medical purposes, as well as to explain the mechanisms of action of probiotics already in use in healthcare. This review revises the versatile toolbox, which can be used to assess the antioxidant properties of probiotics.

**Keywords:** antioxidant assays; probiotics; preventive healthcare; biochemical scavenging; signaling

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## Introduction

There are numerous reports on the ability of probiotic bacteria to correct negative effects of various non-infectious pathologies such as allergies, toxicoses of different etiology, obesity, etc.; in addition, some probiotics appear to be capable of preventing cancer [1-7]. The broad spectrum of health-promoting activity of probiotics can be attributed to their metabolic products protecting eukaryotic host's cells from negative influence of various factors, including oxidative stress [6].

Besides displaying a plethora of health promoting functions which are often strain specific [8, 9], some probiotic bacteria demonstrate strong antioxidative potentials [10]. Specifically, *Lactobacillus fermentum* strains were shown to have resistance to several reactive oxygen species (ROS) such as hydrogen peroxide, superoxide and hydroxyl radicals [11]. In addition, some metabolites, such as exopolysaccharides, synthesized and excreted by probiotic bacteria, were shown to have antioxidant activity [12]. Extracts of *Bifidobacterium animalis* 01 were found to scavenge ROS in vitro and in vivo [13]. Also, oxidative stress associated with type 2 diabetes was shown to be decreased by multispecies probiotic preparations, and *Lactobacillus rhamnosus* demonstrated strong antioxidant activity in situations of elevated physical stress in humans [14].

One of the widely investigated topics in dietary-based biomedicine is probiotics for amelioration of oxidative stress-related diseases by direct sequestration of reactive oxygen species and augmentation of antioxidant defense systems operating in the human body [15-17]. The production of free radicals at high levels in the gut can exert cytotoxic effects on the membrane phospholipids of the intestinal epithelial cells, resulting in the formation of toxic products such as malondialdehyde (MDA). Similarly, the occurrence of severe peroxidative changes in the gut due to lipids and free radicals reaction resulting in enhanced lipid peroxidation has been found to be commonly associated with the onset of numerous diseases.

Thus, probiotics are an important factor affecting oxidative status of the gut by exhibiting direct antioxidant properties and by inducing the intrinsic human signaling antioxidant defense [15, 18].

48 Studying the mechanisms underlying health-promoting functions of probiotic bacteria will  
49 enhance our knowledge of symbiotic microbe-host interactions [19-23]. As a result, we expect to  
50 find new approaches in using nature-derived biologically active substances in gastro-intestinal  
51 health care, immunomodulation, prophylaxis of cancer, stress (UV and radiation)-protection, and  
52 growth/regeneration promotion.

53 An important part of a "tool kit" for these studies is the methods to assess the antioxidative  
54 properties of probiotics. This paper provides an overview of such methods.

55 These methods can be divided into two distinct groups: those assessing the effects on the  
56 oxidative status systems signaling, and those testing the biochemical antioxidant properties of  
57 probiotics.

### 58 **Oxidative status and inflammation systems signaling-based techniques**

59 As far as humans and animals have evolved genetic programs through intervention of  
60 antioxidative enzymes for protection against oxidative stresses, the level of expression of some  
61 eukaryotic genes could be used to indirectly assess the antioxidant capacity of probiotics  
62 administered to the objects. Therefore, nuclear factor erythroid 2-related factor 2 (NFE2L2, also  
63 known as NRF2) has been recognized as one of the key transcriptional factors that can play a  
64 significant protective role by controlling the antioxidant response element-dependent gene  
65 regulation in response to oxidative stress [15].

66 Generally, the basic interactomic approach [24] is used in this type of studies: probiotics are  
67 analyzed with respect to their ability to induce a set of genes (or protein products) regulated by a  
68 single transcription factor or being a part of a signaling pathway - e.g. NFE2L2, AP-1, NF-κB, etc.  
69 For example, Chauhan et al. tested antioxidant properties of *L. fermentum* Lf1 through assessing the  
70 NFE2L2/AP-1 and PPARGC1A pathways activation in the HT-29 cells [15]. Endo and colleagues  
71 tested the effects of MIYAIRI 588 probiotic on rats using similar approach; however, they only  
72 assessed NFE2L2/AP-1 targets (*NQO1*, *HMPOX1*, *TXN*) on the protein level [25]. Gao and co-

74 authors used the NFE2L2 protein expression assessment-based variation of the method, together  
175 with numerous biochemical tests, to study the signaling antioxidant activity of the *Lactobacillus*  
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376 *plantarum* FC225 strain [26]. The effects of probiotic *Lactobacillus reuteri* ATCC PTA 6475 on  
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677 proinflammatory cytokines regulated by the AP-1 component JUN were revealed by Lin and  
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878 colleagues [27].  
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1179 The same approach is sometimes employed using pro-oxidant/pro-inflammatory signaling  
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1380 systems as the reporters. For example, the effects of the combined *Lactobacillus delbruekii* and  
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1681 *Lactobacillus fermentum* probiotic on the NF- $\kappa$ B signaling pathway at the protein level were  
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1882 studied by Hegazy and El-Bedewy [28]. A similar investigation focused at probiotic *Lactobacillus*  
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2183 *rhamnosus* GR-1 was performed by Karlsson and colleagues [29]. Being involved in cytokine  
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2384 signaling, NF- $\kappa$ B is often in the focus of the studies dedicated to testing of probiotics effects on the  
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2585 human gut-microbiota interactions [30].  
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2886 A study involving a combined analysis of activation of NF- $\kappa$ B and AP-1 was undertaken by  
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3087 Wehkamp and co-authors; these investigators tested the signaling effects of *Escherichia coli* Nissle  
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3388 1917 probiotic on the human intestinal epithelial cells [31, 32]. Schlee and colleagues investigated  
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3589 several oxidative status related and non-related pathways to assess the signaling effects of several  
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3890 probiotics and of a probiotic cocktail [33].  
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## 40 41 4292 **Biochemical approaches to probiotics antioxidant properties testing** 43

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4593 There are several group of methods, and several variances within these methods, which are  
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4794 routinely used for the antioxidant properties of probiotics. These methods range from those  
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5095 analyzing total pro- or antioxidant capacities of the reporter system, to those quantifying separate  
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5296 indices of oxidative status of the reporter system. Several methods rely on detection of changes in  
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5597 free radical production modulated by probiotics introduced into the radical-generating systems.  
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5798 Reviewing of the biochemical methods starts with the total prooxidant/antioxidant assays.  
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5999 Please note, brief ready-to-use protocols are given in the papers cited.  
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101 *Total prooxidant/antioxidant assays*

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Several generalized indices of oxidative status of biological systems are used to date. These are, for example, oxygen radical antioxidant capacity (ORAC), total oxidant capacity (TOC), also known as total oxidant status (TOS); total antioxidant capacity (TAC), also known as total antioxidant status (TAS), total antioxidant response (TAR), antioxidant potential (AOP), or non-enzymatic antioxidant capacity (NEAC) [34-37].

Antioxidant activity can be monitored by a variety of methods based on different mechanisms such as hydrogen atom transfer (HAT), single electron transfer (SET), reducing power, etc.

Oxygen radical antioxidant capacity (ORAC) is the most used HAT method. Other HAT-based methods share the same principle, with the examples being total radical trapping antioxidant parameter (TRAP) and crocin bleaching assays. In these methods, peroxy radicals produced by a generating system react with a probe resulting in the loss of fluorescence or absorbance that is registered as decay curves. Commonly used peroxy radical generators are a group of azo-compounds, e.g. 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH) (hydrophilic) and 2,2'-azobis(2,4-dimethylvaleronitrile (AMVN) (hydrophobic). A model antioxidant, Trolox (a vitamin E analog) is usually used as reference, and ORAC values of the tested antioxidants/probiotics are reported as Trolox equivalents [36]. A commonly used reporter fluorescent probe is fluorescein (Figure 1) [38]. The ORAC method was used to test the antioxidant properties of the *Lactobacillus fermentum* LF31 [38].

SET-methods typically use Trolox as standard antioxidant. Among SET-based methods, the Trolox equivalent antioxidant capacity (TEAC) assay is one of the most frequently used to date. The assay measures the ability of antioxidants to scavenge the stable radical cation 2,2'-azinobis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), a chromophore with maximum absorption at 734 nm (Figure 2). Its absorbance at this wavelength decreases in the presence of antioxidants [36].

126 Another example of TEAC chromophores is 2,2-Diphenyl-1-picrylhydrazyl with maximum  
127 absorption at 520 nm (Figure 3) [36, 39]. In a large-scale study by Amaretti and colleagues, TEAC  
128 assay was used to test several probiotics, including 7 *Bifidobacterium*, 11 *Lactobacillus*, 6  
129 *Lactococcus*, and 10 *Streptococcus thermophilus* strains [40].

130 The reducing power of antioxidants can be measured through their redox reactions with  
131 transition metal ions - iron (ferric reducing antioxidant potential, FRAP) and copper (Cupric  
132 reducing antioxidant capacity, CUPRAC). The TAS and TOS/TOC methods by Erel et al. [41]  
133 employ oxido-reduction of iron ions [36].

134 The TOS/TOC assay is based on the oxidation of ferrous ion to ferric ion in the presence of  
135 oxidants in acidic medium [36]. The resulting complex ferric ion-xylene orange is colored [36],  
136 and can be measured at 560 nm [42].

137 The TAS method is based on the generation of hydroxyl radical via Fenton reaction, and the  
138 rate of the reaction is monitored via the analysis of absorbance of coloured dianisidyl radicals  
139 (absorbance is registered at ~440 nm) [41]. The mixture of ortho-dianisidine, ferrous ammonium  
140 sulfate and hydrogen peroxide solution produces oxidized o-dianisidine molecules into dianisidyl  
141 radicals, leading to a bright yellow-brown colour development (Figure 4). Antioxidants suppress the  
142 color formation (36). The TAS method has been used to study several probiotics, including Protexin  
143 [43].

144 The TOS/TOC assay is calibrated with hydrogen peroxide and results are expressed in terms  
145 of hydrogen peroxide micromolar equivalents per litre ( $\mu\text{mol H}_2\text{O}_2 \text{ Eq/L}$ ), whereas the TAS assay  
146 is calibrated with a stable antioxidant standard solution, which is traditionally the Trolox, and  
147 results are expressed as mmol Trolox Eq/L [36]. Total oxidant status assay is relatively rarely used  
148 for studying the probiotics properties. An example of such a study is the one performed by Anwar  
149 and colleagues on the Protexin probiotic [43].

150 Unfortunately, total oxidative/antioxidant indices reflect too complex events, and thus they  
151 are not readily reproducible. Different TOC/TAC assays sometimes do not correlate with each

152 other, and even considering the same method or methods with similar mechanisms, the results are  
153 often conflicting [36].

154 Thus, more specific methods are often used together or apart from the total  
155 prooxidant/antioxidant assays. These methods are subdivided into two categories: those assessing  
156 dynamics of isolated redox processes, and those analyzing end-point products of such processes.  
157 One of the most frequently used assays of the first category is the lipid peroxidation detection.

#### 159 *Lipid peroxidation detection using TBA*

160 Several variations of the basic principle of the assay [44] are used today. An acidified (with  
161 1% phosphoric acid) homogenate is treated with TBA (0.6%), and the mixture is then heated on a  
162 boiling water bath for 45 min. At this stage, the reaction occurs. 2-thiobarbituric acid reacts with  
163 MDA or other chemically similar molecules (TBA-reactive substances) at 25°C. One molecule of  
164 MDA or other chemically related substance reacts with 2 molecules of 2-thiobarbituric acid via a  
165 Knoevenagel-type condensation to yield chromophores with absorbance maximum at 532 nm.  
166 These chromophores require extraction, thus subsequently, an equal volume of n-butanol is added  
167 to the sample, and the solution is thoroughly mixed to allow for extraction of the products of the  
168 reaction. The butanol phase is then separated by centrifugation, and absorbance is measured at 520  
169 and 535 nm [15]. For preparation of the standard curve, overnight digestion of various  
170 concentrations of 1,1,3,3-tetraethoxypropane (0.1 mM) in presence of 0.2 N HCl can be used [15].  
171 The method was used, for example, to study the antioxidant properties of *Lactobacillus brevis* CD2,  
172 *L. salivarius* FV2, and *L. plantarum* FV9 [45].

#### 174 *End-point products of redox processes*

175 Over the years, MDA and TBARS were the most often analysed markers of oxidative stress.  
176 In line with these markers, oxidized LDLs, antibodies to oxidized LDLs, 4-hydroxynonenal (4-  
177 HNE), acrolein, advanced lipid oxidation products, advanced protein oxidation products, advanced

178 glycation end-products, disulfides, carbonyls, 3-nitrotyrosine, reactive aldehydes, reduced sugars, 8-  
179 oxy-2-deoxyguanosine were also used [25, 36, 44, 46]. These factors are easily detected using  
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180 respective specific techniques, from ELISA to HPLC with UV detection (HPLC-UV), ultra-  
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181 performance liquid chromatography with tandem mass spectrometry (HPLC/UPLC-MS/MS), and  
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182 gas chromatography–mass spectrometry (GC-MS) [46]. These markers are also supplemented with  
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183 more accurate ones, such as isoprostanes and their metabolites, and allantoin [46].  
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### 134 14 15 *Isoprostanes* 16

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186 Isoprostanes are prostaglandin (PG) isomers that are generated from polyunsaturated fatty  
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187 acids, mainly from arachidonic acid (and additionally from docosahexaenoic and eicosapentaenoic  
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238 acids) by a non-enzymatic process that involves in situ peroxidation of membrane phospholipids by  
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189 free radicals and reactive oxygen species [46]. Isoprostanes are reliable markers of oxidative  
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190 damage in vivo and in vitro [46]. Isoprostanes are suitable oxidative stress markers: they are stable  
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301 specific products of ROS-induced lipid peroxidation, and they have been found in detectable  
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33 quantities as a free form in all biological fluids and as esterified form in normal tissues and they are  
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393 unaffected by lipid content in diet. Current methods for determination of isoprostanes are ELISA,  
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394 LC-MS and GC-MS [46]. Isoprostanes served as a marker of oxidative status in human subjects that  
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405 were assigned with a diet containing the *Lactobacillus fermentum* ME-3 probiotic [47].  
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### 42 43 44 457 *Allantoin* 46

478 In humans, allantoin is the end product of non-enzymatic oxidation of uric acid. Allantoin is  
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199 a promising biomarker of systemic oxidative status in humans because concentration of allantoin  
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520 does not depend on variations of uric acid level, it is stable regardless of the storage or sample  
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501 preparation, and additionally it is easily detected in biological material of human samples [46].  
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56  
502 Allantoin is an extremely polar compound, therefore quantitative determination in plasma, serum or  
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203 urine is difficult. It requires the use of sensitive and specific analytical techniques: capillary zone  
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204 electrophoresis (CZE), enzymatic assay and enzyme cycling method, capillary electrophoresis with  
205 UV detection (CE-UV), HPLC-UV, HPLC/UPLC-MS/MS, and GC-MS [46]. Allantoin was among  
206 compounds tested in infant rhesus monkeys fed with diet containing the *Bifidobacterium animalis*  
207 *subsp. lactis* HN019 probiotic [48].

## 209 **Radical-generating systems used for the antioxidant assays**

210 Several radical-generating systems are routinely used in antioxidants and probiotics testing.

### 211 *Pyrogallol autoxidation*

212 The method utilizes the iron ions or luminol-enhanced autoxidation of pyrogallol  
213 accompanied by release of superoxide anion [26, 49]. In this method, the test compound or a  
214 probiotic affect the rate of release of chromophoric products of the reaction (detection at 320 nm or  
215 420 nm) [26, 49]. This method was used, for example, in a study by Gao and co-authors where the  
216 antioxidant activity of the *Lactobacillus plantarum* FC225 strain was elucidated [26].

### 218 *DPPH radical-generating/reporting system*

219 The DPPH (1,1-diphenyl-2-picrylhydrazyl) solution is a stable radical-generating system  
220 [50]. Usually, the 0.1 mM DPPH solution in methanol [26] or ethanol [50] is mixed with the test  
221 compound or a probiotic. The decrease in absorbance at 517 nm is measured at 0 and 5 min and  
222 then every 15 min until the reaction reaches its plateau. The percentage of DPPH remaining at the  
223 steady-state is calculated as a function of the molar ratio of antioxidant to DPPH [26]. *Lactobacillus*  
224 *plantarum* FC225 strain antioxidant effect was studied using this method [26].

### 226 *1,10-phenanthroline/ferrous sulfate radical-generating system*

227 In this assay, the hydroxyl radical scavenging activity of the test compound is analyzed  
228 using the mixture of 1,10-phenanthroline (0.75 mM), FeSO<sub>4</sub> (0.75 mM) and H<sub>2</sub>O<sub>2</sub> (0.01%)

229 producing a colored product registered at 536 nm [26]. This method was used to study

230 *Lactobacillus plantarum* FC225 strain's antioxidant properties [26].

231  
232 *Anti-lipid peroxidation activity test - the Egg yolk/ferrous sulfate system*

233 According to this approach, anti-lipid peroxidation activity is determined following a simple  
234 procedure. Equal volumes of PBS and fresh egg yolk are mixed and stirred for 10 min, and then the  
235 mix is diluted 1:25 with PBS. One mL of the resulting solution, 0.5 mL of the sample, 1 mL PBS  
236 and 1 mL FeSO<sub>4</sub> (0.01 mM) are mixed, the mixture is shaken at 37°C for 15 min, and then 1 mL of  
237 2.5% trichloroacetic acid is added. The solution is thoroughly mixed, centrifuged at 4000 g for 20  
238 min, then 3 mL of the supernatant is mixed with 2 mL 0.8% 2-thiobarbituric acid and heated to  
239 100°C for 10 min. The absorbance of the mixture is measured at 532 nm [26].

240 Although effects of probiotics are often analyzed with respect to chemical content of egg  
241 yolk [51, 52], the method is rarely used to test for anti-lipid peroxidation activity.

### 243 **Superoxide anion detection methods**

244 Numerous substances react with superoxide and allow for its detection via calorimetric or  
245 fluorescent methods [25]. The most frequently used ones are: redox-sensitive fluorescent dye  
246 dihydroethidium (compatible with tissues samples) [25]; ferricytochrome C (when reduced, it can  
247 be measured spectrophotometrically at 550 nm) [53]; nitroblue tetrazolium (the reaction product  
248 absorbance is measured at 550 nm) [54, 55]; 4-chloro-7-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl)  
249 (upon reacting with superoxide anion, it produces a product with absorbance measured at 470 nm;  
250 the same product is also fluorescent, with ex./em. maxima of 470/550 nm) [55]; 2,3-bis(2-methoxy-  
251 4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide (XTT) (the reaction  
252 product absorbance is measured at 470 nm) [55].

253 These and other fluorescent/chromophor probes can and are readily used for biochemical  
254 assessment of antioxidant properties of probiotics in cell-free and cellular assays [56-58].

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256 **Biosensors in assessment of antioxidative and related properties of probiotics**

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*In vivo* studies on animal objects are usually rather laborious and time-consuming. To identify potential protectors among probiotics, much simpler model systems are required [59].

The considerable universality of the antioxidant defense mechanisms, a consequence of evolutionary antiquity of its mechanisms, allows using simple unicellular system, including prokaryotes, as model objects to test antioxidant properties of bioactive factors. An approach implying using of biosensors is an example of such solution [60].

A biosensor is defined as an analytical device, which integrates a biological recognition element with a physical transducer to generate a measurable signal proportional to the concentration of the analyzed compound [61].

The biosensor approach is not too common; however, there were some attempts to utilize it in probiotics studies. A typical approach for using cellular biosensors was proposed by Grimoud and colleagues [62]. Briefly, for the screening of potential protective (anti-inflammatory and anti-proliferative) properties, the authors used a two-stage screening system based on a modified eukaryotic cell line. The first step of screening was based on the HT-29 cells with modified expression pattern. The pattern of inflammation was characterized by analyzing the secreted interleukins. Secreted substances were quantified using classical chemiluminescent ELISA test. Then, further testing was carried out using the inflammatory cell culture model consisting of inflammatory-activated transgenic Caco-2 cells transfected with a reporter gene under the control of the NF- $\kappa$ B inducible promoter. This method is attributed to biosensor type because the detecting system consists of the biological part (cells) and the technical component (a luminometer). In this study, the following microorganisms were screened: *Bifidobacterium bifidum*, *B. breve*, *B. longum*, *B. pseudocatenulatum*, *Lactobacillus acidophilus*, *L. buchneri*, *L. farciminis*, *L. helveticus*, *L. plantarum*, *L. rhamnosus*, *L. lactis*, *Pediococcus acidilactici*, and *Streptococcus thermophilus*. As a result, anti-inflammatory properties of 11 strains were tested. It was also found that *B. breve* and

281 *Lactococcus lactis*, in a composition of symbiotic preparations, significantly decreased proliferation  
282 of cancer cells.

283 It is worth considering the approach of in vitro screening of probiotic properties proposed in  
284 [38]. The antioxidant performance of *Lactobacillus fermentum* LF31 with prebiotic supplement was  
285 shown in human colon cultured cells using oxygen radical absorbance capacity (ORAC) method  
286 and the potency of the strain was compared with that of the positive control, trolox. Authors  
287 observed a statistically significant free radical-scavenging capacity of *L. fermentum* LF31.

288 Speaking of single-cell systems, it should be noted that bacteria grow faster and are easier to  
289 operate with when compared to eukaryotic cell culture. Signal system based on luminescence is a  
290 tool of choice in bacterial biosensor studies, since luminescent signal is one of the most easily  
291 detected. If methods under review are to be applied in large-scale pharmacological research, the  
292 speed of screening will be a crucial factor.

293 In one of our own studies [63] bacterial biosensors based on *E. coli* MG 1655 (pSoxS-lux),  
294 *E. coli* MG1655 (pRecA-lux), and *E. coli* MG1655 pCold-lux were used as a single-cell model  
295 systems. These biosensors are the genetically modified strains of *E. coli*, containing the plasmid  
296 carrying luxCDABE operon from *Photobacterium luminescens* under the control of appropriate  
297 promoters, SoxS, RecA, etc. This operon is responsible for bioluminescence and provides luciferase  
298 used in this test as a reporter [64].

299 A biosensor strain with the PsoxS promoter detects the presence of oxidants forming a cell  
300 superoxide anion radical in the medium. A characteristic feature of oxidative stress in *E. coli* is the  
301 induction of genes of the antioxidant system and increasing the activity of antioxidant enzymes  
302 encoded by these genes [65]. Therefore, in the genetic constructs that constitute the basis of  
303 biosensors responsive to oxidative stress, the promoters of these genes were used. The PsoxS  
304 promoter specifically reacts to the superoxide anion radicals. Biosensors with pRecA and pCold  
305 plasmids report on the presence of factors that cause DNA damage in the cell. The sensitivity of  
306 these biosensors is about  $10^{-8}$  M of the inductor [64]. To activate SoxS promoter, paraquat (1,1'-



307 dimethyl-4,4'-bipyridylium dichloride) was used. This compound triggers oxidative stress,  
308 switching the cell bioenergetics to generation of superoxide anion, instead of ATP synthesis [66,  
309 67].

310 In addition, an activity of *Bacillus amyloliquefaciens* B-1895 (soil isolate) and *Bacillus*  
311 *subtilis* KATMIRA1933 (isolated from the fermented dairy product YoguFarm™) was studied.  
312 Probiotic properties of *B. amyloliquefaciens* B-1895 manifest in stimulation of growth and tolerance  
313 to pathogens of fish and birds [68, 69]. The subtilisin preparation obtained from *B. subtilis*  
314 KATMIRA1933 was confirmed as being safe for human tissues, having spermicidal activity [70],  
315 and active against foodborne [71] and vaginal [72, 73] pathogens. Preparations of both fermentates  
316 demonstrated anti-oxidant activity [68-73].

317 In another work [74], a similar approach for use of biosensors was proposed with some  
318 modifications: genetically engineered constructs were made in *Bifidobacterium longum*. Authors  
319 constructed a bifidobacteria-based biosensor that could be used to analyze the metabolic state of the  
320 cells. In this case, the probiotic strain itself was a biosensor. An insect (*Pyrophorus*  
321 *plagiophthalmus*) luciferase gene was introduced into the genome of the bacteria to construct a  
322 bifidobacterial luminescent biosensor that could be used for a quick screening. Light emission is the  
323 signal of the metabolic state changes of cells. Experiments with luminescent *B. longum* indicate  
324 that, under acidic stress condition, bifidogenic prebiotics such as FOS or lactulose can considerably  
325 improve the cell physiology.

326 Applying this approach makes it possible to study the metabolic activity of the probiotic  
327 preparation under different conditions, which allows choosing the optimum combination of  
328 additional compounds in synbiotic preparations, for example, in order to help bifidobacteria to  
329 survive gastric transit, or to increase its beneficial properties. This approach seems promising and,  
330 with minor modification (e.g., introduction of stress-inducible promoters to the construct) can be  
331 applied to problems discussed above.

332 In general, we can conclude that there is some trend in applying biosensors in probiotic  
333 screening. Most of authors use microplate tests, with luminescent or fluorescent signal as detection  
334 tool.

### 336 **A brief summary of methods used to assess the antioxidative properties of probiotics**

337 Table 1 summarizes the brief results of the studies that employed the reviewed methods of  
338 assessment of the oxidative status-modulating properties of probiotics.

### 340 **A comparative analysis of methods under description**

341 As seen from current review, the methods utilized for measuring of antioxidant activity of  
342 probiotics can be subdivided into biochemical and signaling-based techniques.

343 The most straightforward methods employ chemical systems for generation of reactive  
344 oxygen species, and one can even choose a system producing specific radicals. In these methods,  
345 reporting substances are external just as the radical-generating systems, and no eukaryotic cells are  
346 required to run the test.

347 The second group of methods relies on external or cellular eukaryotic sources of reactive  
348 oxygen species, while detected are cell-derived substances only. Remarkably, there are sub-groups  
349 of such techniques, and these reflect an important biological fact: reactive oxygen species are  
350 produced all over the eukaryotic cell, in all of its compartments. Although ROS are generally  
351 universal, the consequences of their generation are dramatically different: most impact may fall on  
352 lipids, proteins, small molecules, DNA and RNA. Consequently, this initial impact affects the  
353 secondary events. To list a few examples, lipid oxidation may lead to chain reactions of lipid  
354 peroxidation; oxidation of calcium channels of endoplasmic reticulum leads to cytoplasmic calcium  
355 flux further leading to endoplasmic reticulum stress; oxidation of cytoplasmic signaling proteins  
356 leads to induction of redox-activated transcription factors controlling ROS-generating enzymes.

357 Thus, different analytical methods are required and employed to study specific roles of probiotics in

358 development of the primary and secondary events and their consequences. In addition, just as it is  
359 true for the first group of methods, specific methods of the second group are used to test particular  
360 properties in a given probiotic. The rationale is standard for biomedicine: therapeutic intervention  
361 should be as targeted as possible. This is especially true for modulation of redox process, because  
362 there are many sources of ROS inside the cell, and these ROS have numerous physiological  
363 functions that are spatiotemporally specific.

364 The third groups of methods resembles the second one, with one essential difference: the  
365 detection is based on cellular signals deriving from cellular sensing of and reactions towards redox  
366 processes. As redox regulation is vast and diverse, specific signaling systems and levels of these  
367 systems (pre-mRNAs, mRNA, proteins and their modifications) to be analyzed are chosen based on  
368 research needs. For example, pre-mRNAs, mRNAs and signaling proteins modifications are used to  
369 address changes in cellular signaling pattern, while proteins quantities and enzymatic activities are  
370 analyzed to assess the cellular response.

371 The fourth group of methods is somewhat similar to the third group in being based on  
372 assessment of cellular reactions towards ROS, rather than on assessment of direct chemical  
373 consequences of generation of ROS, but it is distinct, as it utilizes prokaryotic and eukaryotic  
374 biosensors.

375 In summary, antioxidant activity testing methods used in probiotics research are extremely  
376 versatile. They range from cell-free radical generation testing (for assessing the direct inherent  
377 antioxidant activity) and to RNA/protein expression analysis in eukaryotic cells co-culture and  
378 animal models. Firstly, this vast variety allows one to choose the most appropriate method for  
379 assessing a specific property of the probiotic strain being under consideration or development.  
380 Secondly, in probiotics development, these methods are conveniently combined into a panel of tests  
381 of increasing complexity thus making positive and negative selection of strains both fast and cheap.  
382 Moreover, each type (or step in the multi-step approach) of testing methods is represented by  
383 generally interchangeable techniques varying in details, such as radical generation system used,

384 mode of detection, positive control or reference substance, pathway targets analyzed, etc.

385 Consequently, these groups of tests are flexible enough to meet the needs and capabilities of every  
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386 given lab. At the same time, utilization of several techniques within the same group of methods  
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387 allows to account non-intended interactions of the agent (probiotic) being tested and the assay  
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388 system components. Thus, the above-mentioned wide variety of methods, which can be used to  
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389 assess antioxidant properties of probiotics, lays a solid basis for reliable data interpretation.  
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14 Moreover, probiotics, as a group of antioxidants, are inherently much more “customizable”  
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16 than any other group of antioxidants, and their potential redox roles are far more complex and wide-  
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18 ranged than those of the latter. On the other hand, it is evident that cellular antioxidants such as  
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21 probiotics, have much more complex effects when used as treatment, when compared to molecular  
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23 antioxidants. Thus, much more complex testing is required for probiotics than for molecular  
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25 antioxidants. As a consequence, the antioxidant properties testing ‘toolbox’ used in probiotics  
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27 research should be more diverse than that used in molecular antioxidants research, and concomitant  
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29 use of tests from different groups is required to comprehensively characterize the intrinsic  
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31 complexity of effects of probiotics in modulation of redox processes and oxidative status of the host  
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400 cells.

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#### 406 **Conflict of interest**

407 The authors declare that they have no conflict of interest.  
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614 Table 1. A brief summary of assays used to study the oxidative status-modulating capabilities of  
 615 probiotics.

Assay or biosensor type	Analyzed parameters	Probiotics studied	Brief results	References
Nitroblue tetrazolium superoxide probe assay	Superoxide anion scavenging detection in a cell-free system	<i>B. coagulans</i> RK-02	The probiotic had significant antioxidant and free radical scavenging activities	58
DPPH radical-generating system	Superoxide anion scavenging detection in a cell-free system	<i>Enterococcus faecium</i> (BDU7)	The probiotic had significant superoxide radical scavenging activities	75
Nitroblue tetrazolium superoxide probe assay	Superoxide anion production by the head kidney leucocytes of rainbow trout	<i>Lactococcus lactis</i> ssp. <i>lactis</i> CLFP 100, <i>Leuconostoc mesenteroides</i> CLFP 196, and <i>Lactobacillus sakei</i> CLFP 202	A significant increase in the superoxide anion production was observed in the groups fed with CLFP 100 and CLFP 196	76
The NFE2L2/AP-1 pathway activation assay - RNA level	RNA expression of the NFE2L2/AP-1 and PPARGC1A target genes in the HT29 cells	<i>Lactobacillus fermentum</i> Lf1	A significant increase in the expression of genes of antioxidant enzymes was found	15
The NFE2L2/AP-1 pathway activation assay - protein level	Nfe2l2 and its targets protein expression assessed in livers of rats treated with the probiotic	<i>Clostridium butyricum</i> MIYAIRI 588	The probiotic-treated rats showed remarkable induction of liver NFE2L2 and its target enzymes	25
The NFE2L2/AP-1 pathway activation assay - protein level	Murine liver Nfe2l2 protein expression after the probiotic-containing diet	<i>Lactobacillus plantarum</i> FC225	C225 markedly elevated the gene expression of Nfe2l2	26
The NFE2L2/AP-1 pathway activation assay - RNA level	TNF RNA expression in human LPS-activated monocytes and primary monocyte-derived macrophages treated with the probiotic	<i>Lactobacillus reuteri</i> ATCC PTA 6475	The probiotic suppressed TNF transcription by inhibiting activation of MAP kinase-regulated c-Jun and the transcription factor, AP-1.	27

Assay or biosensor type	Analyzed parameters	Probiotics studied	Brief results	References
The NF- $\kappa$ B pathway activation assay	NF- $\kappa$ B and IL6 protein expression, and TNF RNA expression in colonic tissue of patients with chronic diarrhea	<i>Lactobacillus delbruekii</i> / <i>Lactobacillus fermentum</i>	The use of probiotic for 8 weeks significantly ameliorated the inflammation by decreasing the colonic concentration of IL-6, expression of TNF- $\alpha$ and NF- $\kappa$ B p65	28
The NF- $\kappa$ B pathway activation assay	NF-jB activity in E. coli-stimulated T24 bladder cells	<i>Lactobacillus rhamnosus</i> GR-1	Viable GR-1 cells were found to potentiate NF-jB activity, while heat-killed lactobacilli demonstrated a marginal increase in NF-jB activity.	29
The NF- $\kappa$ B pathway activation assay - luciferase gene reporter analysis approach	NF- $\kappa$ B and AP-1 binding to the target DNA in probiotic-treated Caco-2 cells	<i>Escherichia coli</i> Nissle 1917	<i>E. coli</i> Nissle 1917 induced the luciferase gene expression via NF- $\kappa$ B and AP-1 binding	31
Total antioxidant status and total oxidant status assays	Total antioxidant activity and total oxidant capacity of the blood samples of the White Leghorn birds	Protexin	The overall total antioxidant capacity was increased, whereas total oxidant status was reduced	43
Oxygen radical absorbance capacity assay	Oxygen radical absorbance capacity of the probiotic plus prebiotic-treated HT-29 cells	<i>Lactobacillus fermentum</i> LF31	The probiotic/prebiotic mix confers remarkable antioxidant capacity	38
The trolox-equivalent antioxidant capacity assay	Trolox-equivalent antioxidant capacity of the probiotics	7 <i>Bifidobacterium</i> , 11 <i>Lactobacillus</i> , six <i>Lactococcus</i> , and 10 <i>Streptococcus thermophilus</i> strains	Strains <i>Bifidobacterium animalis</i> subsp. <i>lactis</i> DSMZ 23032, <i>Lactobacillus acidophilus</i> DSMZ 23033, and <i>Lactobacillus brevis</i> DSMZ 23034 exhibited the highest TAA(AA), TAA(LA), TEAC, and TGS values within the lactobacilli and bifidobacteria.	40

Assay or biosensor type	Analyzed parameters	Probiotics studied	Brief results	References
The lipid peroxidation detection assay with TBA	Lipid peroxidation in the human sperm samples	Combined probiotic: <i>Lactobacillus brevis</i> CD2, <i>Lactobacillus salivarius</i> FV2, and <i>Lactobacillus plantarum</i> FV9	The combined probiotic prevented sperm lipid peroxidation that was induced in vitro by a ferrous ion promoter, thus preserving sperm motility and viability.	45
Individual indices of oxidative status	Peroxidized lipoproteins, oxidized LDLs, 8-isoprostanes, glutathione redox ratio in humans consuming non-fermented and fermented goats' milk	<i>Lactobacillus fermentum</i> ME-3	Consumption of fermented goats' milk prolonged resistance of the lipoprotein fraction to oxidation, lowered levels of peroxidized lipoproteins, oxidized LDL, 8-isoprostanes and glutathione redox ratio, and enhanced total antioxidative activity.	47
Individual indices of oxidative status	Serum allantoin in infant rhesus monkeys fed with probiotic-containing diet	<i>Bifidobacterium animalis</i> subsp. <i>lactis</i> HN019	Probiotic diet caused increased allantoin	48
<i>Bifidobacterium longum</i> biosensor	Bioluminescence (insect luciferase)	<i>Bifidobacterium longum</i> with additional compounds	It was shown that bifidogenic prebiotics can sensitively improve the activity of synbiotic compounds	74
<i>Escherichia coli</i> biosensor	Bioluminescence (bacterial luciferase)	<i>Bacillus amyloliquefaciens</i> B-1895, <i>Bacillus subtilis</i> KATMIRA1933	A screening of antioxidant and DNA-protective activity of a number of probiotics was performed. Strains exhibiting protective activity have been found.	63
HT-29 biosensor; Caco-2 biosensor	Secreted inflammation biomarkers (IL-8, NF-κB) detected in ELISA test (chemiluminescent signal)	<i>Bifidobacterium breve</i> , <i>Lactococcus lactis</i> , prebiotic glucooligosaccharide and synbiotic preparations	An anti-inflammatory effect and anti-proliferative activity were shown for symbiotic preparations	62
Human colon cultured cells	Fluorescence	<i>Lactobacillus fermentum</i> LF31	Free radical-scavenging activity of <i>Lactobacillus fermentum</i> LF31 confirmed	38



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**Figure legends**

Figure 1. Structural formula of fluorescein - a common fluorescent probe used in the ORAC methods.

Figure 2. Structural formula of ABTS - a common chromophor used in the SET methods.

Figure 3. Structural formula of 2,2-Diphenyl-1-picrylhydrazyl - a common chromophor used in the SET methods.

Figure 4. Structural formula of ortho-dianisidine, a common chromophor-producing substance used in the SET methods.

## Figures

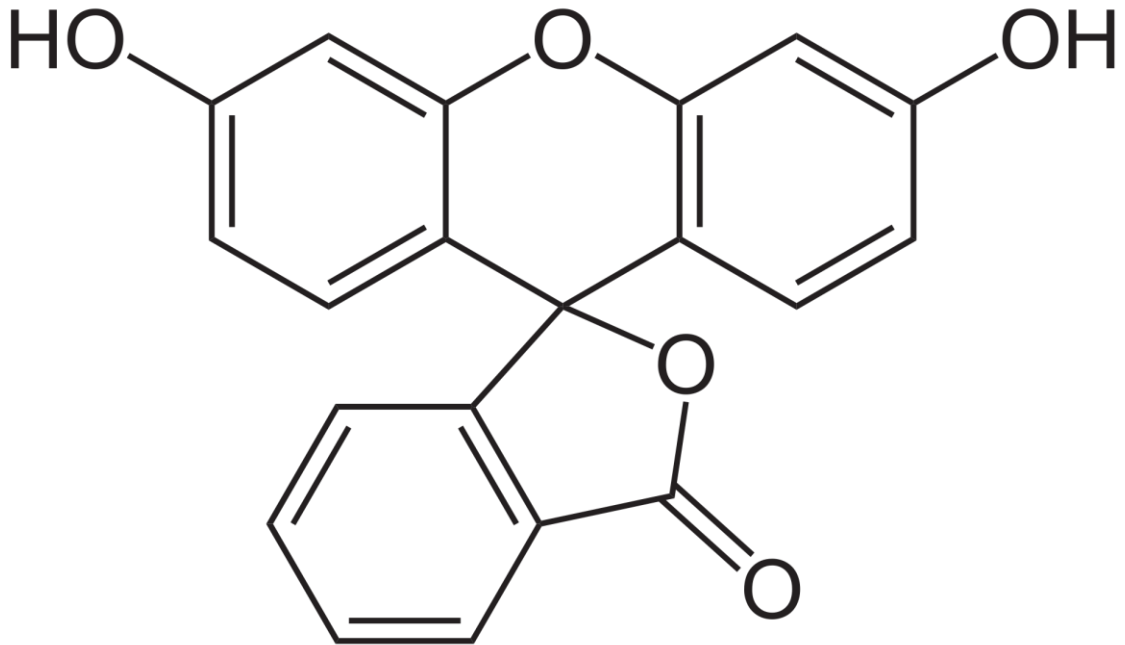


Fig. 1

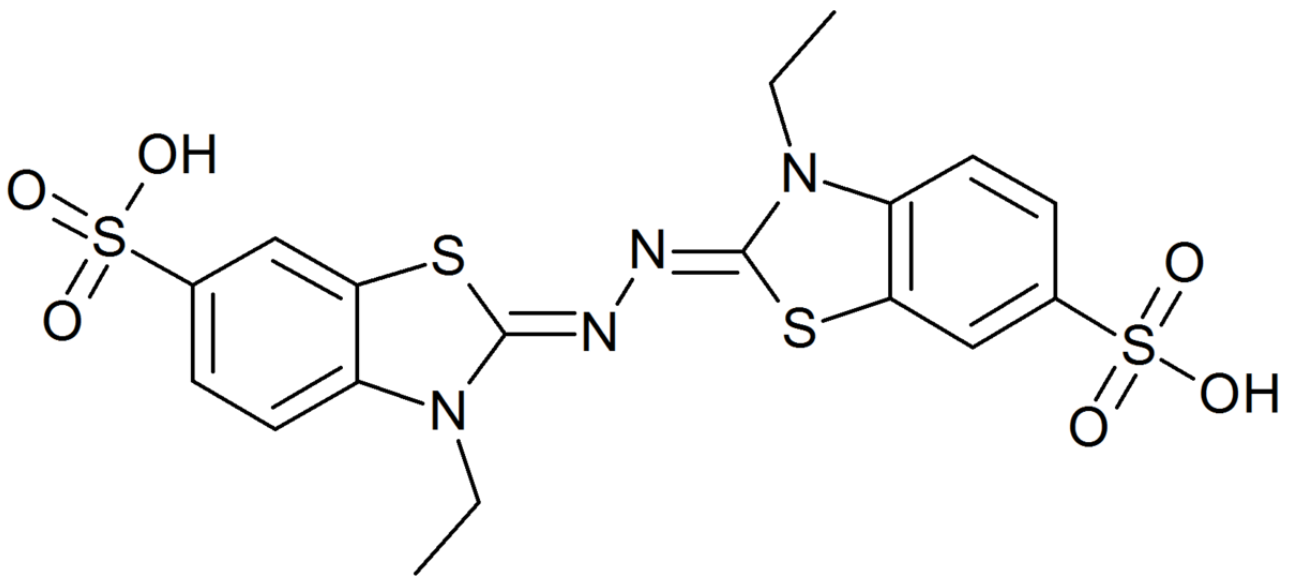


Fig. 2

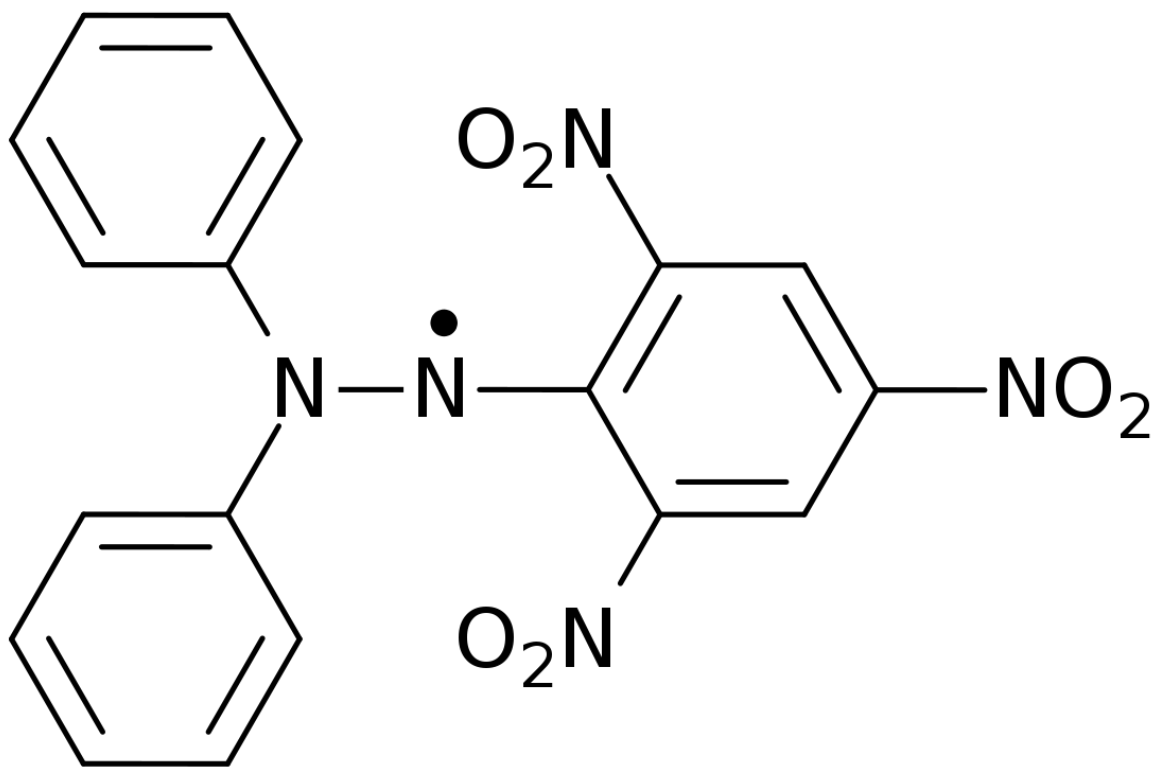


Fig. 3

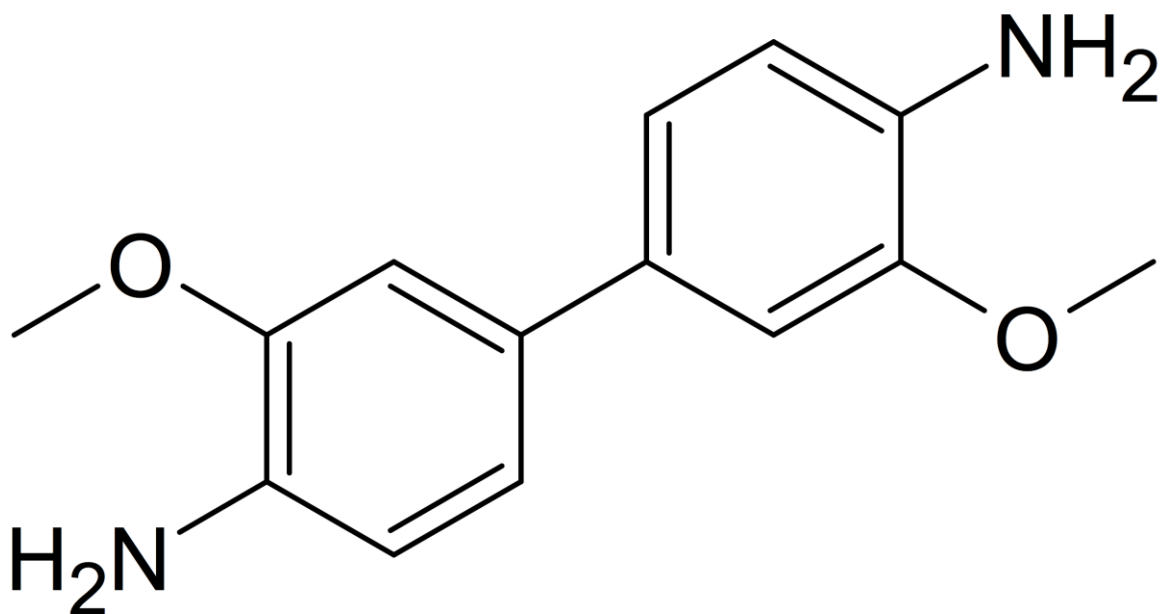


Fig. 4

