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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

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* (*Lb. 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 ROS 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].

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

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An important part of a "tool kit" for these studies is the methods to assess the antioxidative properties of probiotics. This paper provides an overview of such methods. These methods can be divided into two distinct groups: those assessing the effects on the oxidative status systems signaling and those testing the biochemical antioxidant properties of probiotics.

Oxidative Status and Inflammation Systems Signaling-Based Techniques

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

Generally, the basic interactomic approach [24] is used in this type of studies: probiotics are analyzed with respect to their ability to induce a set of genes (or protein products) regulated by a single transcription factor or being a part of a signaling pathway—e.g. NFE2L2, AP-1, NF-κB, etc. For example, Chauhan et al. tested antioxidant properties of *Lb. fermentum* Lf1 through assessing the NFE2L2/AP-1 and PPARGC1A pathways activation in the HT-29 cells [15]. Endo et al. tested the effects of MIYAIRI 588 probiotic on rats using similar approach; however, they only assessed NFE2L2/AP-1 targets (*NQO1*, *HMPOX1*, *TXN*) on the protein level [25]. Gao et al. used the NFE2L2 protein expression assessment-based variation of the method, together with numerous biochemical tests, to study the signaling antioxidant activity of the *Lactobacillus plantarum* FC225 strain [26]. The effects of probiotic *Lactobacillus reuteri* ATCC PTA 6475 on pro-inflammatory cytokines regulated by the AP-1 component JUN were revealed by Lin et al. [27].

The same approach is sometimes employed using prooxidant/pro-inflammatory signaling systems as the reporters. For example, the effects of the combined *Lactobacillus delbruekii* and *Lb. fermentum* probiotic on the NF-κB signaling pathway at the protein level were studied by Hegazy and El-Bedewy [28]. A similar investigation focused at probiotic *Lb. rhamnosus* GR-1 was performed by Karlsson et al. [29]. Being involved in cytokine signaling, NF-κB is often in the focus of the studies dedicated to testing of probiotics effects on the human gut microbiota interactions [30].

A study involving a combined analysis of activation of NF-κB and AP-1 was undertaken by Wehkamp and co-authors; these investigators tested the signaling effects of *Escherichia coli* Nissle 1917 probiotic on the human intestinalepithelial cells [31, 32]. Schlee et al. investigated several oxidative status related and non-related pathways to assess the signaling effects of several probiotics and of a probiotic cocktail [33].

Biochemical Approaches to Probiotics Antioxidant Properties Testing

There are several group of methods, and several variances within these methods, which are routinely used for the antioxidant properties of probiotics. These methods range from those analyzing total pro- or antioxidant capacities of the reporter system, to those quantifying separate indices of oxidative status of the reporter system. Several methods rely on detection of changes in free radical production modulated by probiotics introduced into the radical-generating systems. Other methodological options are also available (varying in reporter substance type, e.g., fluorescent probes and primary exogenous ROS) that are not routinely used in probiotics screening and research but are of potential interest for the field.

Reviewing of the biochemical methods starts with the total prooxidant/antioxidant assays. Please note, brief ready-to-use protocols are given in the papers cited.

Total Prooxidant/Antioxidant Assays

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]. Biochemical principles and mechanisms underlying these assays are described in detail elsewhere in numerous experimental reports and reviews.

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, peroxyl 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 peroxyl radical generators are a group of azo-compounds, e.g., 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH) (hydrophilic) and 2,2′-azobis(2,4-dimethylnaleronitrile (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

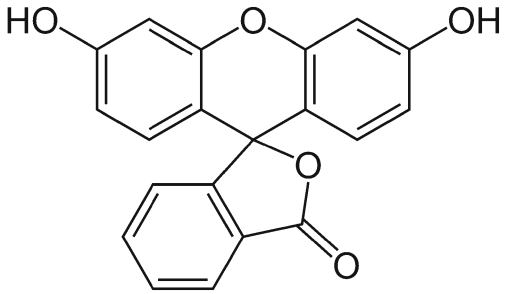

Fig. 1 Structural formula of fluorescein—a common fluorescent probe used in the ORAC methods

fluorescein (Fig. 1) [38]. The ORAC method was used to test the antioxidant properties of the *Lb. 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 (Fig. 2). Its absorbance at this wavelength decreases in the presence of antioxidants [36]. Another example of TEAC chromophores is 2,2-diphenyl-1-picrylhydrazyl with maximum absorption at 520 nm(Fig. 3) [36, 39]. In a large-scale study by Amaretti et al., TEAC assay was used to test several probiotics, including 7 *Bifidobacterium*, 11 *Lactobacillus*, 6 *Lactococcus*, and 10 *Streptococcus thermophilus* strains [40].

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

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

The TAS method is based on the generation of hydroxyl radical via Fenton reaction, and the rate of the reaction is monitored via the analysis of absorbance of colored dianisidyl radicals (absorbance is registered at ~440 nm)

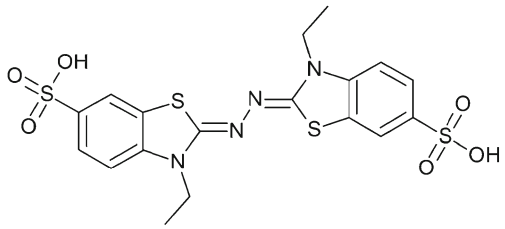

Fig. 2 Structural formula of ABTS—a common chromophor used in the SET methods

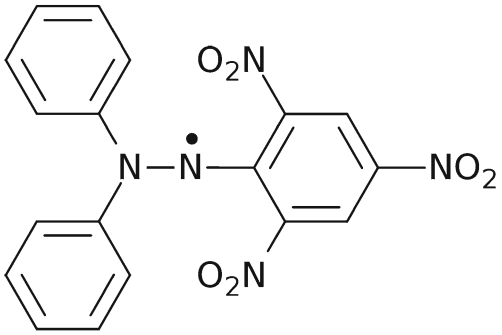

Fig. 3 Structural formula of 2,2-diphenyl-1-picrylhydrazyl—a common chromophor used in the SET methods

[41]. The mixture of ortho-dianisidine, ferrous ammonium sulfate, and hydrogen peroxide solution produces oxidized o-dianisidine molecules into dianisidyl radicals, leading to a bright yellow-brown color development (Fig. 4). Antioxidants suppress the color formation [36]. The TAS method has been used to study several probiotics, including Protexin [43].

The TOS/TOC assay is calibrated with hydrogen peroxide and results are expressed in terms of hydrogen peroxide micromolar equivalents per liter (μmol H2O2 Eq/L), whereas the TAS assay is calibrated with a stable antioxidant standard solution, which is traditionally the Trolox, and results are expressed as mmol Trolox Eq/L [36]. Total oxidant status assay is relatively rarely used for studying the probiotics properties. An example of such a study is the one performed by Anwar et al. on the Protexin probiotic [43].

Unfortunately, total oxidative/antioxidant indices reflect too complex events, and thus they are not readily reproducible. Different TOC/TAC assays sometimes do not correlate with each other, and even considering the same method or methods with similar mechanisms, the results are often conflicting [36].

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

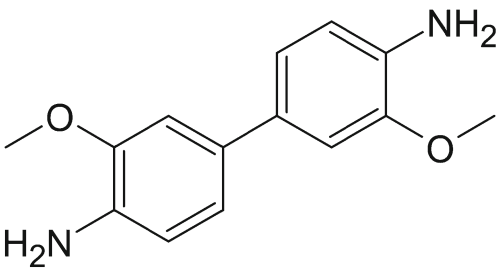

Fig. 4 Structural formula of ortho-dianisidine, a common chromophor-producing substance used in the SET methods

Lipid Peroxidation Detection Using TBA

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

Endpoint Products of Redox Processes

Over the years, MDA and TBARS were the most often analyzed markers of oxidative stress. In line with these markers, oxidized LDLs, antibodies to oxidized LDLs, 4-hydroxynonenal (4-HNE), acrolein, advanced lipid oxidation products, advanced protein oxidation products, advanced glycation end products, disulfides, carbonyls, 3-nitrotyrosine, reactive aldehydes, reduced sugars, 8-oxy-2-deoxyguanosine were also used [25, 36, 44, 46]. These factors are easily detected using respective specific techniques, from ELISA to HPLC with UV detection (HPLC-UV), ultra-performance liquid chromatography with tandem mass spectrometry (HPLC/UPLC-MS/MS), and gas chromatography–mass spectrometry (GC-MS) [46]. These markers are also supplemented with more accurate ones, such as isoprostanes and their metabolites, and allantoin [46].

Isoprostanes

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

Allantoin

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

Radical-Generating Systems Used for the Antioxidant Assays

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

Pyrogallol Autoxidation

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

DPPH Radical-Generating/Reporting System

The DPPH (1,1-diphenyl-2-picrylhydrazyl) solution is a stable radical-generating system [50]. Usually, the 0.1 mM DPPH solution in methanol [26] or ethanol [50] is mixed with the test compound or a probiotic. The decrease in absorbance at 517 nm is measured at 0 and 5 min and then every 15 min until the reaction reaches its plateau. The percentage of DPPH remaining at the steady-state is calculated as a function of the

molar ratio of antioxidant to DPPH [26]. *Lb. plantarum* FC225 strain antioxidant effect was studied using this method [26].

1,10-Phenanthroline/Ferrous Sulfate Radical-Generating System

In this assay, the hydroxyl radical scavenging activity of the test compound is analyzed using the mixture of 1,10-phenanthroline (0.75 mM), $FeSO_4$ (0.75 mM), and H_2O_2 (0.01%) producing a colored product registered at 536 nm [26]. This method was used to study *Lb. plantarum* FC225 strain's antioxidant properties [26].

Anti-lipid Peroxidation Activity Test—the Egg Yolk/Ferrous Sulfate System

According to this approach, anti-lipid peroxidation activity is determined following a simple procedure. Equal volumes of PBS and fresh egg yolk are mixed and stirred for 10 min, and then the mix is diluted 1:25 with PBS. One milliliter of the resulting solution, 0.5 mL of the sample, 1 mL PBS, and 1 mL $FeSO_4$ (0.01 mM) are mixed; the mixture is shaken at 37 °C for 15 min, and then 1 mL of 2.5% trichloroacetic acid is added. The solution is thoroughly mixed, centrifuged at 4000 g for 20 min, then 3 mL of the supernatant is mixed with 2 mL 0.8% 2-thiobarbituric acid and heated to 100 °C for 10 min. The absorbance of the mixture is measured at 532 nm [26].

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

Superoxide Anion Detection Methods

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

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

Biosensors in Assessment of Antioxidative and Related Properties of Probiotics

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 et al. [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-κB 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*, *Bif. breve*, *Bif. longum*, *B if. pseudocatenulatum*, *Lactobacillus acidophilus*, *Lb. buchneri*, *Lb. farciminis*, *Lb. helveticus*, *Lb. plantarum*, *Lb. rhamnosus*, *Lb. lactis*, *Pediococcus acidilactici*, and *Streptococcus thermophilus*. As a result, anti-inflammatory properties of 11 strains were tested. It was also found that *B if. breve* and *Lactococcus lactis (Lc. lactis)*, in a composition of symbiotic preparations, significantly decreased proliferation of cancer cells.

It is worth considering the approach of in vitro screening of probiotic properties proposed in [38]. The antioxidant performance of *Lb. fermentum* LF31 with prebiotic supplement was shown in human colon cultured cells using oxygen radical absorbance capacity (ORAC) method and the potency of the strain was compared with that of the positive control, Trolox.

Authors observed a statistically significant free radical scavenging capacity of *Lb. fermentum LF31*.

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

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

A biosensor strain with the PsoxS promoter detects the presence of oxidants forming a cell superoxide anion radical in the medium. A characteristic feature of oxidative stress in *E. coli* is the induction of genes of the antioxidant system and increasing the activity of antioxidant enzymes encoded by these genes [65]. Therefore, in the genetic constructs that constitute the basis of biosensors responsive to oxidative stress, the promoters of these genes were used. The PsoxS promoter specifically reacts to the superoxide anion radicals. Biosensors with pRecA and pColD plasmids report on the presence of factors that cause DNA damage in the cell. The sensitivity of these biosensors is about 10^{-8} M of the inductor [64]. To activate SoxS promoter, paraquat (1,1′-dimethyl-4,4′-bipyridylium dichloride) was used. This compound triggers oxidative stress, switching the cell bioenergetics to generation of superoxide anion, instead of ATP synthesis [66, 67].

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

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

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

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

A Brief Summary of Methods Used to Assess the Antioxidative Properties of Probiotics

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

A Comparative Analysis of Methods under Description

As seen from current review, the methods utilized for measuring of antioxidant activity of probiotics can be subdivided into biochemical and signaling-based techniques. The most straightforward methods employ chemical systems for generation of ROS, and one can even choose a system producing specific radicals. In these methods, reporting substances are external just as the radical-generating systems, and no eukaryotic cells are required to run the test.

The second group of methods relies on external or cellular eukaryotic sources of ROS, while detected are cell-derived substances only. Remarkably, there are sub-groups of such techniques, and these reflect an important biological fact: ROS are produced all over the eukaryotic cell, in all of its compartments. Although ROS are generally universal, the consequences of their generation are dramatically different: most impact may fall on lipids, proteins, small molecules, DNA, and RNA. Consequently, this initial impact affects the secondary events. To list a few examples, lipid oxidation may lead to chain reactions of lipid peroxidation: oxidation of calcium channels of endoplasmic reticulum leads to cytoplasmic calcium flux further leading to endoplasmic reticulum stress

Table 1 A brief summary of assays used to study the oxidative status-modulating capabilities of 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	*B. coagulans* 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	*Enterococcus faecium* (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	*Lc. lactis* ssp. *lactis* CLFP 100, *Leuconostoc mesenteroides* CLFP 196, and *Lactobacillus sakei* 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	*Lb. fermentum* 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	*Clostridium butyricum* 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	*Lb. plantarum* 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	*Lb. reuteri* ATCC PTA 6475	The probiotic suppressed TNF transcription by inhibiting activation of MAP kinase-regulated c-Jun and the transcription factor, AP-1.	[27]
The NF-κB pathway activation assay	NF-κB and IL6 protein expression, and TNF RNA expression in colonic tissue of patients with chronic diarrhea	*Lb. delbruekii*/*Lb. fermentum*	The use of probiotic for 8 weeks significantly ameliorated the inflammation by decreasing the colonic concentration of IL-6, expression of TNF-α and NF-κB p65	[28]
The NF-κB pathway activation assay	NF-jB activity in *E. coli*-stimulated T24 bladder cells	*Lactobacillus rhamnosus* 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-κB pathway activation assay—luciferase gene reporter analysis approach	NF-κB and AP-1 binding to the target DNA in probiotic-treated Caco-2 cells	*E.coli* Nissle 1917	*E. coli* Nissle 1917 induced the luciferase gene expression via NF-κB 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	*Lb. fermentum* LF31	The probiotic/prebiotic mix confers remarkable antioxidant capacity	[38]
The Trolox equivalent antioxidant capacity assay	Trolox equivalent antioxidant capacity of the probiotics	7 *Bifidobacterium,* 11 *Lactobacillus*, six *Lactococcus*, and 10 *Str. thermophilus* strains	Strains *Bif. animalis* subsp. *lactis* DSMZ 23032, *Lactobacillus acidophilus* DSMZ 23033, and *Lb. brevis* DSMZ 23034 exhibited the highest TAA(AA), TAA(LA), TEAC, and TGSH values within the lactobacilli and bifidobacteria.	[40]
The lipid peroxidation detection assay with TBA	Lipid peroxidation in the human sperm samples	Combined probiotic: *Lb. brevis* CD2, *Lb. salivarius* FV2, and *Lb. plantarum* 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	*Lb. fermentum* 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]

Table 1 (continued)

Assay or biosensor type	Analyzed parameters	Probiotics studied	Brief results	References
Individual indices of oxidative status	Serum allantoin in infant rhesus monkeys fed with probiotic-containing diet	*Bif. animalis* subsp. *lactis* HN019	Probiotic diet caused increased allantoin	[48]
Bif. longum biosensor	Bioluminescence (insect luciferase)	*Bif. longum* with additional compounds	It was shown that bifidogenic prebiotics can sensitively improve the activity of synbiotic compounds	[74]
E. coli biosensor	Bioluminescence (bacterial luciferase)	*B. amyloliquefaciens* B-1895, *B. subtilis* 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)	*Bif. breve*, *Lc. lactis*, prebiotic glucooligosaccharide and synbiotic preparations	An anti-inflammatory effect and anti-proliferative activity were shown for symbiotic preparations	[62]
Human colon cultured cells	Fluorescence	*Lb. fermentum* LF31	Free radical scavenging activity of *Lactobacillus fermentum* LF31 confirmed	[38]

and oxidation of cytoplasmic signaling proteins leads to induction of redox-activated transcription factors controlling ROS-generating enzymes. Thus, different analytical methods are required and employed to study specific roles of probiotics in development of the primary and secondary events and their consequences. In addition, just as it is true for the first group of methods, specific methods of the second group are used to test particular properties in a given probiotic. The rationale is standard for biomedicine: therapeutical intervention should be as targeted as possible. This is especially true for modulation of redox process, because there are many sources of ROS inside the cell, and these ROS have numerous physiological functions that are spatiotemporally specific.

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

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

In summary, antioxidant activity testing methods used in probiotics research are extremely versatile. They range from cell-free radical generation testing (for assessing the direct inherent antioxidant activity) and to RNA/protein expression analysis in eukaryotic cells co-culture and animal models. Firstly, this vast variety allows one to choose the most appropriate method for assessing a specific property of the probiotic strain being under consideration or development. Secondly, in probiotics development, these methods are conveniently combined into a panel of tests of increasing complexity thus making positive and negative selection of strains both fast and cheap. Moreover, each type (or step in the multi-step approach) of testing methods is represented by generally interchangeable techniques varying in details, such as radical generation system used, mode of detection, positive control or reference substance, pathway targets analyzed, etc. Consequently, these groups of tests are flexible enough to meet the needs and capabilities of every given lab. At the same time, utilization of several techniques within the same group of methods allows to account non-intended interactions of the agent (probiotic) being tested and the assay system components. Thus, the abovementioned wide variety of methods, which can be used to assess antioxidant properties of probiotics, lays a solid basis for reliable data interpretation.

Moreover, probiotics, as a group of antioxidants, are inherently much more "customizable" than any other group of antioxidants, and their potential redox roles are far more complex and wide-ranged that these of the latter. On the other hand, it is evident that cellular antioxidants such as probiotics, have much more complex effects when used as treatment, when compared to molecular antioxidants. Thus, much more complex testing is required for probiotics than for molecular antioxidants. As a consequence, the antioxidant properties testing "toolbox" used in probiotics research should be more diverse than that used in molecular antioxidants research, and concomitant use of tests from different groups is required to comprehensively characterize the intrinsic complexity of effects of probiotics in modulation of redox processes and oxidative status of the host cells.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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