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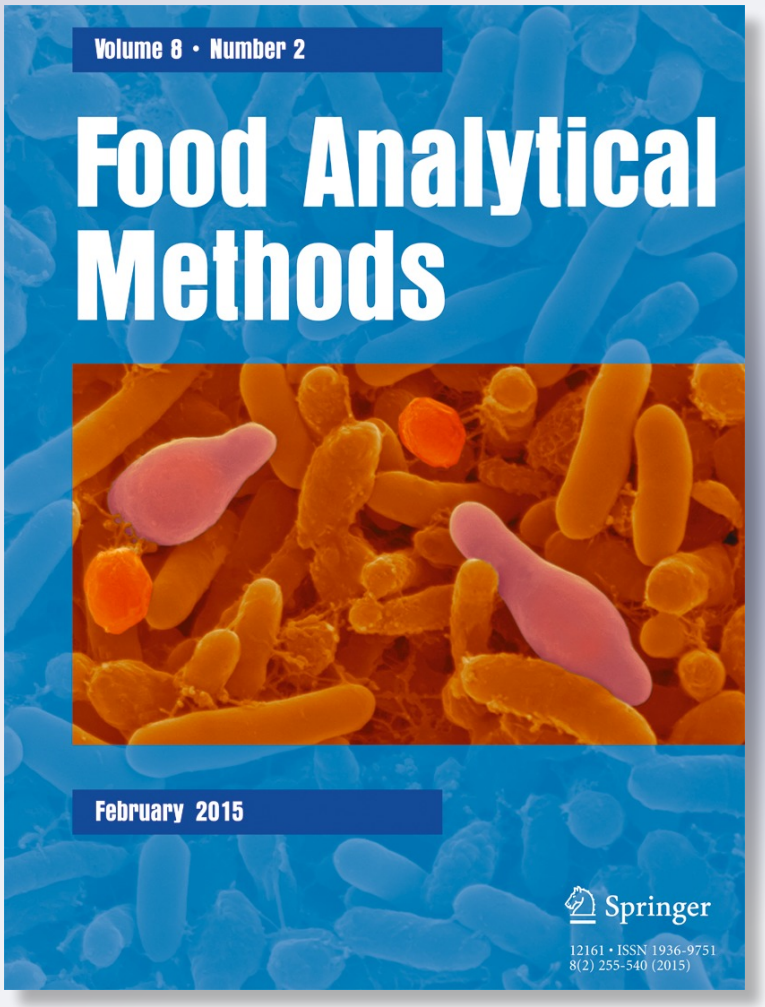

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Optimisation of the Measurement of the Antioxidant Activity of Probiotics and Pathogens: a Crucial Step Towards Evidence-Based Assessment of Health Claims and Production of Effective Functional Foods

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Abstract The consumer's attention is increasingly attracted by a multitude of health claims floated from the biggest food industries about probiotics. To date, the European Food Safety Authority (EFSA) has rejected all the applications for their health claims because they were not evidence based. It follows that more studies to characterise these microorganisms are needed, for instance regarding their antioxidant activity against reactive oxygen species involved in a number of illnesses and ageing. Questions as to whether the results have any bearing on effectiveness in the human body demand new methods able to estimate the antioxidant activity in a way that mimics the physiological defence against free radicals. In the following, (i) we report on the optimisation of a method complying with these requests and compare it to the classical Folin-Ciocalteu assay, (ii) we assert and explain its eligibility as regards probiotics on the basis of the mechanism of their antioxidant activity and (iii) we apply this method to highlight for the first time that probiotic bacteria and potential pathogens share the antioxidant capacity even if to a different extent. We provided food scientists with a tool to let them make educated guesses concerning the selection of effective ingredients for the production of functional foods.

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Keywords Briggs-Rauscher reaction · Health claims · Functional foods · Lactic acid bacteria vs. pathogens · Reactive oxygen species · Free radicals

Introduction

According to market statistics, the global functional food market is growing at a rate that is outpacing the traditional processed food market. In particular, new foods made by adding probiotic bacteria are appreciated by many consumers. Certainly, the consumer's attention was attracted by a multitude of health claims floated from the biggest food industries about probiotics. But, nowadays, the food safety authorities, in particular the European one, are reviewing all health claims made for probiotics to verify that they were scientifically well founded. To date, the European Food Safety Authority (EFSA) has rejected all the applications for health claims because they did not have sufficient evidence to evaluate them. So, more studies to characterise these microorganisms are needed. For instance, regarding the antioxidant power, a variety of in vitro chemical methods are being used to determine the antioxidant activity of food additives in general, but questions as to whether the results have any bearing on effectiveness in the human body are leading to the development of additional methods that may be more appropriate for screening potential antioxidant ingredients (Mermelstein 2008).

Reactive oxygen species (ROS) are unavoidable by-products of cellular respiration because some electrons bypass the electron transport chain and directly reduce oxygen molecules; four one-electron reductions of O_2 occur, and a superoxide anion radical ($O_2^{-}\cdot$), hydrogen peroxide, hydroxyl radical (OH·) and water are subsequently generated. One of the less reactive ROS is $O_2^{-}\cdot$. Since 23 is the estimated pKa for

this base in the membrane bilayer lipid environment, it is easily predicted to exist exclusively as its conjugated acid HOO· (perhydroxyl radical) that is much more operative than $O_2^{-}\cdot$ at initiating fatty acid peroxidation (Aikens and Dix 1991). The hydroxyl radical is often generated via the well-known Fenton chemistry

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + HO\cdot + HO^{-} \quad (1)$$

and it represents one of the most reactive and dangerous ROS (Brenneisen et al. 1998). The positive role of ROS, deliberately synthesised by dedicated enzymes for the protection of the biological system from bacteria or other foreign material and damaged tissue, cannot be overlooked; however, strongly reactive free radicals promote oxidation of nucleic acids, proteins and lipids (Sohal and Weindruch 1996; Camougrand and Rigoulet 2001) thereby causing a number of illnesses and ageing. Actually, our enzymes superoxide dismutase, catalase, glutathione peroxidase and reductase and non-enzymatic antioxidants such as vitamin C, vitamin E, co-enzyme Q10, glutathione and alpha lipoic acid limit the damage caused by ROS (Shindo et al. 1994).

ROS are deeply involved in inflammatory bowel diseases and colon cancer, and it was reported that lactic acid bacteria (LAB) that possess antioxidant properties may prevent these diseases (Koller et al. 2008). Oxidative stress results from an imbalance between the production of ROS in the intestine by various metabolic reactions and the antioxidant defences; hence, probiotic bacteria that exhibit antioxidant activities are the focus of interest of many research groups. It follows that the quantitative evaluation of the antioxidant ability of microorganisms is crucial for screening probiotic *Lactobacillus* strains since it is related to the therapeutic success seen with such strains in patients with inflammatory bowel diseases (which are characterised by increased endogenous formation of ROS) (Santosa et al. 2006).

There are many methods to assess free radical scavenging ability, and this implies that the measured antioxidant capacity of a sample depends on the analytical method used in the measurement (Cao et al. 1993; Cao and Cutler 1993; Halliwell and Gutteridge 1995). Antioxidant capacity assays can rely on hydrogen atom transfer (HAT) or on electron transfer (ET) reactions.

In HAT-based methods, the antioxidant and an oxidable fluorescent probe compete for peroxyl radicals (ROO·) thermally generated through the decomposition of an azo radical initiator, usually AAPH (2,2′-azobis-2-methyl-propanimidamide, dihydrochloride). The quantitation is obtained from the comparison of the fluorescence decay that follows the probe oxidation, in the presence or absence of the antioxidant. Assays with this feature include total radical trapping antioxidant parameter assay (TRAP) that makes use of R-phycoerythrin as a fluorescent probe, and oxygen radical absorbance capacity assay (ORAC) that uses fluoresceine as a fluorescent probe. ORAC assay is the most-used HAT method. Unfortunately, the concentration of the substrate is much less than that of the antioxidants, at variance with the real situation. Since these methods lack a chain propagation step and the nature of the damaging reaction is not characterised, the relevance of these approaches to the radical chain-breaking antioxidant capacity was considered low (Huang et al. 2005). The other HAT-based method, the crocin bleaching assays, measures the inhibition capacity of antioxidants in protecting the bleaching of crocin by AAPH, but it has found limited applications because the inhibited bleaching rates are not sensitive to the concentration changes of antioxidants. Moreover, interferences and the lot-to-lot variability of crocin limit its quantitative application (Huang et al. 2005). What is more, the radical initiator of the HAT-based method is different from those encountered in biological systems and only the antioxidant activity against particular (probably mainly peroxyl) radicals can be measured (Huang et al. 2005).

ET-based assays measure the ability of an antioxidant to reduce an oxidant, which changes colour when reduced. The degree of colour change is correlated with the sample's antioxidant concentrations.

In the ET-based Trolox equivalence antioxidant capacity assay (TEAC), the oxidant is $ABTS^{\cdot-}$ (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)). This method is not well founded on physical chemistry, since the TEAC values for pure antioxidant compounds are not correlated with the number of electrons an antioxidant can give away. Unfortunately, the reaction rate differences are not reflected in the TEAC values because the TEAC assay is an end-point assay and this lowers the reliability of TEAC results (Huang et al. 2005).

In the ferric ion reducing antioxidant power assay (FRAP), the FRAP values for a number of antioxidants are in conflict with their reductive properties, and this does not lend support to the test results; moreover, the test time (4 min) was demonstrated not to be long enough for polyphenols (Huang et al. 2005).

The ET-based DPPH (2,2-diphenyl-1-picrylhydrazyl) method uses a stable and commercially available organic nitrogen radical (2,2-diphenyl-1-picrylhydrazyl radical). The absorbance of the mixture fades as its reduction goes on, and it is monitored at 515 nm until it is stable. The percentage of the DPPH remaining is proportional to the antioxidant concentrations. Tested antioxidants never face those ROS responsible for the oxidative stress of the biological system, and this is easily predicted to impair the reliability of their estimates: in this respect, the stability of the commercially available DPPH is emblematic, since many antioxidants important from a biological point of view may be inert to it (Huang et al. 2005). Consequently, the antioxidant capacity is wrongly

rated. Furthermore, the reaction kinetics between DPPH and antioxidants does not linearly depend on DPPH concentrations and reversible reactions were also observed. Finally, the ratings are pH sensitive. All these issues lower the soundness of the DPPH assay that is the most widely used antioxidant capacity assay (Huang et al. 2005).

The most important ET-based method is the assay of total phenols by the Folin-Ciocalteu (FC) reagent. Obviously, the FC reagent is non-specific to phenolic compounds as it can be reduced by many non-phenolic compounds, such as vitamin C, Cu(I), etc. As other ET-based methods, it is not reliable since they are end-point methods, characterised by non-linear kinetics and reversible reaction; moreover, the tested reducing capacity of the antioxidants may not parallel their ability to prevent the oxidation of biological substrates by free radicals (Huang et al. 2005).

After this short review of the drawbacks of the existing antioxidant capacity assay, we further emphasise that, unfortunately, the tested antioxidant never faces those ROS that are the primary motive force of the oxidative damage in the human body. While superoxide anion and hydrogen peroxide are in vivo degraded to harmless species by SOD and catalase, no enzymatic action is known to scavenge HOO·, ROO· and HO·. The armoury against them relies on a variety of non-enzymatic antioxidants and free radical scavengers.

Taking into account the scarce reliability of the existing methods, and the fact that the correlation between in vitro antioxidant capacity estimates and health benefits was never demonstrated (Huang et al. 2005), the aim of this study was to apply the Briggs-Rauscher (BR) oscillating reaction method to estimate the antioxidant capacity of probiotics. Contrary to currently used antioxidant capacity assays, the sample antioxidants in the BR assay have to chemically face in the test mixture the same ROS responsible for the oxidative stress and particularly HOO· and H_2O_2. In this respect, the BR antioxidant capacity assay is the only in vitro test that simulates an in vivo assay. Moreover, it is able to detect the activity of those polyphenolic Fe^{2+} chelators able to exert a preventive antioxidant role avoiding the formation (via the Fenton's reaction) of the most disruptive and dangerous free radical, that is the hydroxyl radical (OH·) via Eq. (1): there is mounting evidence that this is actually the case of probiotics (Lee et al. 2006) thereby increasing the eligibility of the BR method to test their antioxidant activity. Notably, BR measurement data are interesting because the method works at pH≤2, i.e. in the pH range of human gastric fluids at which lipid peroxidation is amplified (Kanner and Lapidot 2001); hence, all results detailed in the following refer to bacteria transiting or residing in the stomach environment. Since the oscillation period is particularly sensitive to the temperature, it is important to thermostat the reaction mixture during the test. Another disadvantage is the fact that it works in a hydrophilic environment; however, it can be optimised also for lipophilic samples and it was actually used to estimate the antioxidant capacity of extra virgin olive oil (Cecchi et al. 2010a).

The BR assay has not only theoretical advantages, but also many practical benefits: it is easy, inexpensive and rapid, and that is why it was often used to test the antioxidant activity of a wide variety of samples (Cecchi and Alfei 2013; Cecchi et al. 2010a, b, 2011; Gajdos et al. 2005; Prenesti et al. 2005; Höner and Cervellati 2003; Cervellati et al. 2002a, b). The Folin-Ciocalteu method was also applied to compare the results of the BR method in order to highlight the main possible differences. Moreover, this study aims to compare the antioxidant activity of various species of bacteria able to survive in the intestinal tract in order to select those species characterised by the highest antioxidant capacity, not potentially pathogenic and eligible to be used as functional food ingredients. Furthermore, this method can scientifically support health claims for probiotics.

Materials and Methods

Bacterial Strains

Lactobacillus rhamnosus IMC 501® and *Lactobacillus paracasei* IMC 502® were obtained from Synbiotec S.r.l., Camerino, Italy. These strains are patented and experimentally documented (Cresci et al. 2005). These probiotic strains are able to overcome the gastric barrier, arriving alive and active in the bowel; they adhere and efficiently colonise the intestinal epithelium, with values greater than other strains commercially available; they have shown antipathogenic properties against the principal dangerous human microorganisms (Verdenelli et al. 2009, 2011). *Lactobacillus acidophilus-5*® *and Lactobacillus casei* 431® were obtained from Christian Hansen (Denmark). The other bacterial strains (*Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25093) were obtained from the American Type Culture Collection. All probiotics were cultured in MRS (de Mann, Rogosa, Sharpe) (Oxoid S.p.A., Milan, Italy) at 37 °C for 18 h anaerobically (BBL GasPak anaerobic system; Becton Dickinson and Company, Cockeysville, MD). *E. coli* ATCC 25922 and *S. aureus* ATCC 25093 were cultured in BHI (brain-heart infusion) (Oxoid) broth at 37 °C for 18 h aerobically. All strains were serially transferred at least three times prior to use in studies.

Preparation of Intact Cells, Intracellular Cell-Free Extract and Wall-Membrane Solution

The bacterial cells of each strain were harvested by centrifugation at 3,900*g* for 10 min after 18 h of incubation at 37 °C. For the intact cell preparation, cells were washed three times with 0.9 % NaCl solution and re-suspended in the same solution to reach a density of 5 McFarland. For the preparation

of intracellular cell-free extracts, cell pellets were then quickly washed twice with distilled water. Cells were re-suspended in distilled water to reach a density of 5 McFarland and then were subjected to ultrasonic disruption. Sonication was performed for 30 s with 1-min intervals in an ice bath for 30 cycles. Cell debris was removed by centrifugation at 10,000*g* for 10 min, and the resulting supernatant was the intracellular cell-free extract. The supernatant was collected, and the remaining pellet was diluted with distilled water to obtain wall-membrane solution.

Chemicals

Hydrogen peroxide (30 %, *w*/*w*, not stabilised), malonic acid (>99 %), $NaIO_3$, $MnSO_4 \cdot H_2O$, gallic acid (GA, >98 %), solvents, Folin-Ciocalteu reagent and acetic acid were from Sigma-Aldrich-Fluka (Milan, Italy).

Instruments

The inhibition time was measured by a potentiometer model 338 (Amel S.r.l., Milano, Italy) equipped with a combined platinum redox electrode (Ingold Pt 4805-57/120, Mettler-Toledo, Milan, Italy) and a temperature probe. The potentiometer was controlled, via the Hyperlink software, by a PC. Ultrapure water was produced via the Simplicity® Water Purification Systems (Millipore, Vimodrone, Milan, Italy). A PerkinElmer EZ 301 UV-VIS spectrophotometer equipped with quartz cuvettes was used for the spectrophotometric Folin-Ciocalteu assay.

Methods

BR mixtures were prepared by mixing the appropriate amounts of stock solutions from reagents using burettes in a 100-ml beaker to a total volume of 30 ml. Two millilitres of starch solution was then added to the mixture. Each reactant and sample were maintained at a controlled temperature (20.0 ±0.1 °C) by a thermostat (Haake Q model F-3) before and during the experiment, and the laboratory was thermostated at the same temperature. This temperature was chosen for the sake of simplicity because it is close to room temperature, but it is obviously possible to perform the assay at the gut environment temperature (37 °C): in this case, early scouts indicated that faster kinetics resulted in slightly decreased inhibition times. The order of addition was starch solution, H_2O_2, $NaIO_3$, $HClO_4$, malonic acid and $MnSO_4$. Oscillations started after the addition of the solution containing malonic acid and $MnSO_4$. The composition of the BR mixture was [malonic acid]=0.0500 M, $[Mn^{2+}]$=0.0067 M, $[HClO_4]$=0.03121 M, $[IO_3^-]$=0.0667 M and $[H_2O_2]$=0.8162 M. Intact cells and intracellular cell-free extract samples were prepared as described above. Gallic acid (GA) was prepared at a final concentration of 1,000 mg/l. A proper volume (100–2,000 μl) of each sample solution was added to the oscillating BR mixture after the third oscillation. To mark the beginning of the inhibition time, the last minimum potential before the flat region was considered; to mark the end of the inhibition time, the first minimum potential after the flat region was considered. Two hundred microlitres of 0.9 % NaCl was used as a blank to demonstrate that the BR oscillations were not perturbed.

The Folin-Ciocalteu method (Singleton and Rossi 1965) was used with some modifications. Briefly, 100 μl of each sample was mixed in a 5-ml screw cap test tube with 100 μl of Folin-Ciocalteu reagent diluted in 1,500 μl of ultrapure water for 2 min. Three hundred microlitres of sodium carbonate (200 g/l) was added; the mixture was mixed and then incubated for 1 h at room temperature. After the reaction period, the absorbance at 765 nm was measured. The calibration curve was constructed using gallic acid as a standard.

The whole experiment was run in duplicate. All measurements were performed in triplicate, and the mean value was considered.

Data Analysis, Calculations and Statistical Analysis

The measured inhibition times were regressed via least-squares monovariate fittings over the amount of (i) whole cells in 0.9 % NaCl solution (5 McFarland), (ii) cell membrane and wall in distilled water solution (5 McFarland) and (iii) cell extract in d.w. solution (5 McFarland). The linearity of the BR graphs of the samples was assessed via the software MacCurveFit 1.5.4 (Copyright © 1991–2000, Kevin Raner Software) that estimates the slope and intercept of the best straight line and their standard deviations.

From the ratio of the slope of the optimised straight lines and the slope of the GA (standard antioxidant molecule) optimised straight line, we calculated the BR antioxidant index (BRAI) (Cecchi et al. 2010b) of the probiotics that represents the mass of gallic acid (mg) equivalent per 10^9 cells.

The equation of the best straight line that describes the relationship between the inhibition time and the mass of GA (ranging from 0.02 to 0.04 mg) added to the BR mixture, optimised at 20.0 °C, is t_{in}=5,890·GA+13.9, R=0.9973; the standard deviations of the slope and intercept are, respectively, 252 and 7.7 (Cecchi et al. 2010b). The BRAI standard deviation is calculated from the standard deviations of the slopes of the optimised straight lines of the antioxidant sample and the standard molecule, as the usual standard deviation of a ratio.

Results and Discussion

The BR method is based on the kinetic response obtained by an oscillating reaction that generates ROS free radicals from

Table 1 Slopes, intercepts, SD and *R* of the linear relationship between the inhibition time and the amount of whole cell in NaCl 0.9 % solution (5 McFarland)

Strain	Slope ($s \times 10^9$/CFU)	Slope SD	Intercept (*s*)	Intercept SD	*R*	BRAI
L. acidophilus-5®	53.30	5.79	10.75	3.33	0.993	0.009
L. casei 431®	60.06	4.99	16.41	4.09	0.988	0.010
L. rhamnosus IMC 501®	78.25	4.64	46.71	7.01	0.997	0.013
L. paracasei IMC 502®	75.05	7.11	55.71	10.76	0.991	0.013
S. aureus ATCC 25093	57.90	3.39	15.57	5.12	0.997	0.010
E. coli ATCC 25922	74.67	10.78	11.67	12.57	0.990	0.013

SD standard deviation, *R* the correlation coefficient

hydrogen peroxide. The BR mixture also contains iodate, divalent manganese as catalyst in strongly acidic conditions and malonic acid ($CH_2(COOH)_2$), whose enolic hydrogen atom reduces free iodine to iodide.

The global reaction

$$IO_3^- + 2H_2O_2 + CH_2(COOH)_2 + H^+ \rightarrow ICH(COOH)_2 + 2O_2 + 3H_2O \quad (2)$$

involves many reactions working together (Furrow et al. 1985) and can follow both a radical and a non-radical path, according to the low or high iodide concentration. Each path produces products that favour the alternative path; hence, the concentration of iodide and the reaction electrochemical potential oscillate. The oscillation can be visually followed with the starch indicator, but the potentiometric monitoring of the potential is obviously more exact. If a ROS scavenger is added to the oscillating reaction, the oscillations are quenched: the higher the concentration and activity of the antioxidant, the longer the inhibition time followed by the regeneration of the oscillations. The inhibition time (t_{in}) was ascribed to the removal of hydroperoxy radical, HOO· (that is much more active than superoxide anion radical, O_2^-· at initiating fatty acid peroxidation (Aikens and Dix 1991)). To express the antioxidant activity, we did not compare the concentrations of a sample and a chosen standard that give the same inhibition time (Cervellati et al. 2002a, b) since this involves a single inhibition time specification; on the contrary, we measured the inhibition times at incremental antioxidant concentrations to increase the reproducibility of the estimates (Cecchi et al. 2010b). There is a linear relationship between the amount of added antioxidant and the measured t_{in}: the slope of the best straight line is proportional to the antioxidant capacity of the sample since it indicates the inhibition time increment given by the unitary antioxidant concentration increment.

To standardise the antioxidant capacity, the inhibition time was first measured on a standard molecule to construct a calibration curve and to obtain a reference scale. We selected gallic acid as reference compound because of three main reasons. Firstly, experimental evidence indicates that phenolics, particularly gallic acid, are likely to benefit the host by inhibiting pathogen growth and regulating commensal bacteria, including probiotics strains; gallic acid and polyphenols were therefore considered as prebiotics since they are metabolised by probiotics (Bialonska et al. 2010; Curiel et al. 2010; Hervert-Hernández et al. 2009; Rodríguez et al. 2008; Lee et al. 2006). Secondly, polyphenol active *Lactobacillus* strains gave antioxidant protection in mice subjected to intestinal oxidative stress (Jakesevic, et al. 2009). Thirdly, phenolic compounds were demonstrated to play a significant role against important pathogenic bacteria (Rodríguez Vaquero et al. 2010; Dolara et al. 2005).

Claims of linearity of the relationships between inhibition times and the amount of (i) whole cells in 0.9 % NaCl solution (5 McFarland), (ii) cell membrane and wall in distilled water solution (5 McFarland) and (iii) cell extract in distilled water solution (5 McFarland) are supported, respectively, by data in Tables 1, 2 and 3. They detail the slopes and their standard deviations, the intercepts and their standard deviations and the

Table 2 Slopes, intercepts, SD and *R* of the linear relationship between the inhibition time and the amount of cell membrane and wall in distilled water solution (5 McFarland)

Strain	Slope ($s \times 10^9$/CFU)	Slope SD	Intercept (*s*)	Intercept SD	*R*	BRAI
L. acidophilus-5®	43.94	3.57	15.93	3.99	0.989	0.007
L. casei 431®	37.18	0.98	14.81	2.07	0.998	0.006
L. rhamnosus IMC 501®	8.48	0.16	16.50	3.27	1.000	0.001
L. paracasei IMC 502®	7.24	0.66	15.00	1.31	0.996	0.001
S. aureus ATCC 25093	34.38	0.82	6.50	1.64	1.000	0.006
E. coli ATCC 25922	8.29	0.82	18.50	1.64	0.995	0.001

SD standard deviation, *R* correlation coefficient

Table 3 Slopes, intercepts, SD and *R* of the linear relationship between the inhibition time and the amount of cell extract in distilled water solution (5 McFarland)

Strain	Slope ($s \times 10^9$/CFU)	Slope SD	Intercept (s)	Intercept SD	*R*	BRAI
L. acidophilus-5®	39.78	6.94	14.94	4.01	0.996	0.007
L. casei 431®	387.66	21.78	3.75	0.78	0.987	0.066
L. rhamnosus IMC 501®	68.00	8.47	50.67	13.72	0.992	0.012
L. paracasei IMC 502®	59.33	3.47	20.00	5.61	0.998	0.010
S. aureus ATCC 25093	72.67	12.70	197.00	20.58	0.985	0.012
E. coli ATCC 25922	80.67	7.31	62.67	11.85	0.996	0.014

SD standard deviation, *R* the correlation coefficient

correlation coefficient of these linear functions. The slopes of these regression lines are a good estimate of the antioxidant capacity of the sample since the higher the slope, the longer the inhibition time given by a certain amount of cells. From the ratio of the slope of the optimised straight lines and the slope of the GA (standard antioxidant molecule; see "Data Analysis, Calculations and Statistical Analysis") optimised straight line, we calculated the BRAI (Cecchi et al. 2010b) of the probiotics that represents the mass of gallic acid (mg) equivalent per 10^9 cells.

From Fig. 1 it is clear that the antioxidant activity is different for different portions of the cells: the cell membrane and wall are the portions less involved in the antioxidant capacity. This is not true only for *L. acidophilus*-5®, even if the difference between the antioxidant activity of the extract and of the cell membrane and wall is not statistically significant. The antioxidant capacity of the cell extract of the pathogens seems to be higher than that of the whole cell, while the opposite occurs for probiotics. *L. rhamnosus* IMC 501® and *L. paracasei* IMC 502® have the strongest antioxidant activity as whole cells, and *L. rhamnosus* IMC 501® seems to be slightly superior to *L. paracasei* IMC 502®. It is important to underline that the antioxidant properties of the combination of these two strains, named Synbio®, have been demonstrated in a previous in vivo study on athletes (Martarelli et al. 2011). Surprisingly, the *L. casei* 431® extract shows the strongest antioxidant capacity, and this result deserves further consideration. From the results of this work, is evident that all *Lactobacillus* tested showed varying degrees of antioxidant activity, also for different portions of the cells. This is in accordance with two of the main characteristics of bacteria which are species specificity and, even more important, strain specificity. Although some properties may be shared by various microbial strains, others for sure do not. There is clear evidence that some genotypic and phenotypic properties differ between strains of bacteria even when these strains belong to the same species.

It is particularly interesting to observe that other probiotics and pathogens share the antioxidant activity almost to a similar extent. Actually, a comparison between them as regards radical scavenging ability was never performed before; hence, our findings are interesting since they support the hypothesis

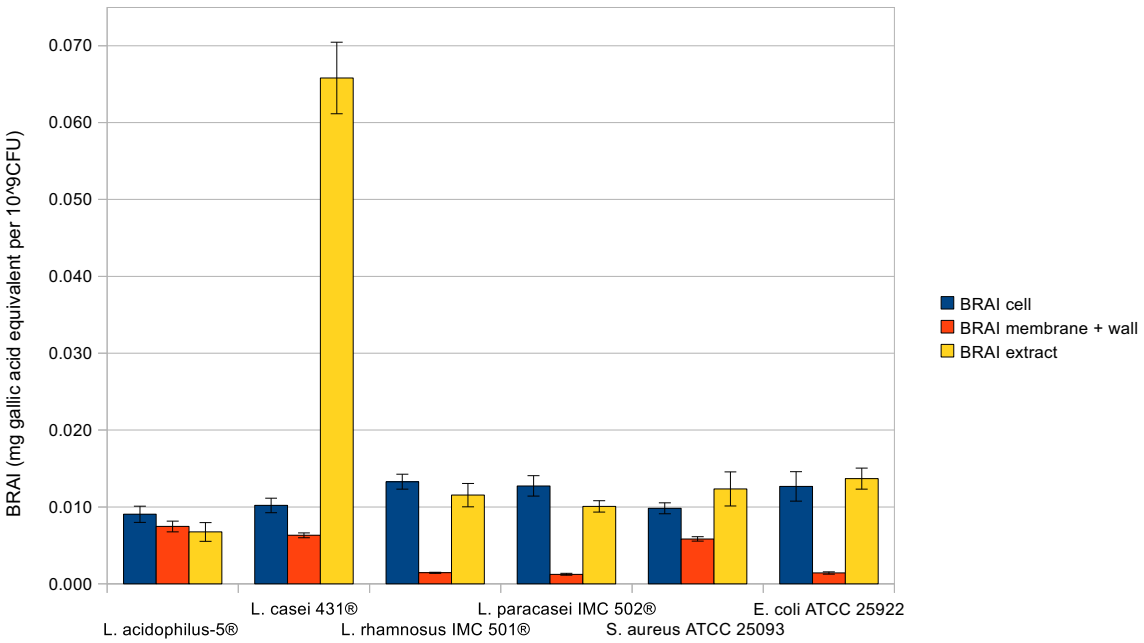

Fig. 1 Antioxidant activity associated to whole cells, cell membrane and wall and cellular extract of tested microorganisms estimated via the BR method

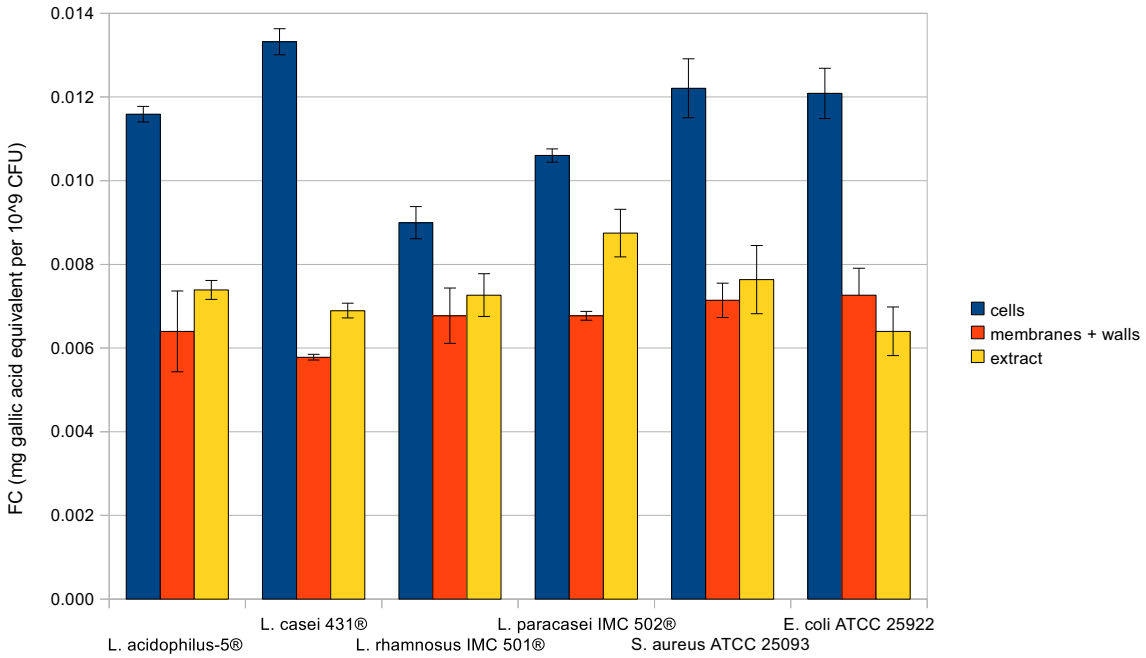

Fig. 2 Antioxidant activity associated to whole cells, cell membrane and wall and cellular extract of tested microorganisms estimated via the FC method

that microorganisms all have to devise some biochemical paths to face the oxidant capacity of oxygen and to get rid of free radicals produced during cellular respiration.

The results of the Folin-Ciocalteu assay (Fig. 2) are interesting; since we decided to use gallic acid as the standard antioxidant molecule, they are expressed as the mass of gallic acid (mg) equivalent per 1E+9 CFU cells; hence, a full comparison with the results obtained via the BR method (Fig. 1) is possible. Firstly, it is rewarding to observe that the order of magnitude of the two alternative quantitative estimates of the antioxidant activity is the same; hence, the two methods do agree from a quantitative point of view. However, it is clear that in this case, for all the tested bacteria, both probiotics and pathogens, the strongest antioxidant activity is shown by the intact cell, at variance with results in Fig. 1. The already discussed (i) strongest reliability of the BR test to mimic in vivo conditions and its other advantages and (ii) the drawbacks of the FC method let us regard the results in Fig. 1 as more reliable than those in Fig. 2.

As a matter of fact, the mechanisms of protective action of probiotic LAB are a hotly debated issue. It is well known from in vitro studies that LAB strains inactivate ROS via enzymatic mechanisms, e.g. by a coupled NADH oxidase/peroxidase system, superoxide dismutase and catalase (Koller et al. 2008). It is, however, worth noting that the antioxidant activity of cell-free extracts of probiotic strains was present even if the superoxide dismutase was negligible (Zanoni et al. 2008). One study concerning the resistance of both intact cells and intracellular cell-free extracts of *L. casei* KCTC 3260 to ROS (Lee et al. 2005, 2006) confirmed that the antioxidant capacity may be caused by chelating metal ions instead of SOD activation. It was also recently demonstrated that the ability of lactobacilli to prevent hydroxyl radical production was related to their ability to chelate 'free' ferrous ion that is able to initiate Fenton's reaction (Sun et al. 2010) and to scavenge Mn^{++} (Koller et al. 2008), that actually plays a crucial role also in the BR mixture: this issue further lends support to the use of the BR method to test the antioxidant activity. To sum up, the defence of microorganisms composing microbiocenosis from the toxic effect of ROS is due to the production of bacterial metabolites which inactivate radicals or prevent their formation (Sgibnev et al. 2009): the BR method is able to take into account both mechanisms of the antioxidant activity, and in this respect, it is actually superior to other assays.

The BRAI indexes of *L. casei* 431®, *L. rhamnosus* IMC 501® and *L. paracasei* IMC 502® in Fig. 1 confirm the higher antioxidant activity of the intact cells and intracellular cell-free extracts compared to the cell membrane and wall as already observed for *L. casei* KCTC 3260; again, for that strain, the antioxidant activity was related to the metal chelating activity (Lee et al. 2005) instead of superoxide dismutase activation.

Conclusions

As a matter of fact, it is interesting to conclude that the antioxidant activity is not a characteristic of probiotics since this feature is shared by other microorganisms, even potential pathogens, even if to a different extent: oxidative stress that may lead to oxidation of nucleic acids, proteins and lipids is caused by an imbalance between the production of ROS and the ability of the biological system to neutralise them.

Obviously, all living organisms put forward their own armoury against oxidative stress. Superoxide anion and hydrogen peroxide are always in vivo degraded to harmless species by superoxide dismutase (that transforms two superoxide molecules to oxygen and hydrogen peroxide) and catalase (that transforms two molecules of hydrogen peroxide to water and oxygen), while no enzymatic action in living organisms is known to scavenge HOO·, ROO· and HO·. The armoury against them relies on a variety of non-enzymatic antioxidants and free radical scavengers, but since HO· is an extremely reactive and short-lived species, its direct scavenging in any biological system is unrealistic as the cellular concentration of any antioxidant is negligible compared with that of other biological molecules (Huang et al. 2005). These considerations may rationalise the absence of a strong difference between the antioxidant capacity of probiotics and pathogens that we fairly reported in this work.

This study may be useful as it allows the European Food Safety Authority to verify the soundness of health claims made for probiotics as regards their antioxidant capacity, a very fashionable characteristic of theirs. The method here described is particularly important for screening probiotic *Lactobacillus* strains characterised by the highest antioxidant activity so that the food industry could take an evidence-based step towards the production of effective functional foods (Silvi et al. 2003).

Compliance with Ethics Requirements This article does not contain any studies with human or animal subjects.

Conflict of Interest Teresa Cecchi has no conflict of interest; Michele Savini has no conflict of interest; Stefania Silvi has no conflict of interest; Maria Cristina Verdenelli has no conflict of interest; Alberto Cresci has no conflict of interest; and the research was not sponsored.

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