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Highlights

CAA may be a better choice for the detection of the antioxidant activity of lactobacilli

Cellular model to assess the antioxidant activity of lactobacilli

Jiali Xing[1], Gang Wang[1*], Zhennan Gu[1], Xiaoming Liu[1], Qiuxiang Zhang[1], Jianxin Zhao[1], Hao Zhang[1], Yong Q. Chen[1], Wei Chen[1, 2*]

1 State Key Laboratory of Food Science and Technology, School of Food Science and Technology, Jiangnan University, Wuxi 214122, P.R. China

2 Synergistic Innovation Center for Food Safety and Nutrition, Wuxi 214122, P.R. China

*Corresponding author: Wei Chen and Gang Wang

Phone: (86)510-85912155

Fax: (86)510-85912155

E-mail address: chenwei66@jiangnan.edu.cn and wanggang@jiangnan.edu.cn

Abstract

Lactobacilli are associated with multiple health-protective effects. One beneficial effect is their antioxidant activity, which needs to be measured using efficient biologically relevant assays. In this study, a cellular antioxidant assay (CAA) was used to determine the cellular anti-oxidative properties of 10 *Lactobacillus* strains from 5 species (*L. rhamnosus, L. plantarum, L. acidophilus, L. casei, L. fermenti*) in hepatocellular carcinoma (HepG2) cells and the results were compared with those obtained with the traditional 2, 2-diphenylpicrylhydrazyl (DPPH) radical scavenging assay. The results from these two methods showed no obvious correlation at three concentrations (10^7 - 10^9 colony-forming units/mL). A further study was performed to evaluate the protective effects of the strains against H_2O_2-induced oxidative stress in HepG2 cells and the results showed greater consistency in the data obtained with CAA assay than in those from the DPPH radical scavenging assay. The findings indicate that CAA may be a better choice for the detection of the antioxidant activity of *Lactobacillus* strains.

Key words: *Lactobacilli; Oxidative stress; Antioxidant activity; Cellular antioxidant assay; 2, 2-diphenylpicrylhydrazyl radical scavenging assay*

Introduction

Oxidative stress, is an imbalance in the production of reactive oxygen (ROS) and reactive nitrogen species (RNS) and antioxidant defense. Oxidative stress plays a pivotal role in various pathophysiological conditions [1], causing damage in proteins, mutations in DNA, oxidation of membrane phospholipids and modification of low-density lipoproteins. Excessive amounts of ROS can result in cellular damage, which in turn, promotes chronic diseases such as atherosclerosis, arthritis, diabetes, neurodegenerative diseases, cardiovascular diseases, and cancer [2]. The body synthesizes antioxidant molecules, which together with antioxidants found in food intake, set up a biological antioxidant barrier to counteract the oxidant molecules [3]. However, this defense system still fails to protect the body against oxidative stress in certain circumstances. Consequently, efforts should be focused on increasing antioxidant defenses for the maintenance of human health and disease prevention [4].

Probiotics have recently received significant attention because of their role in the maintenance of human health [5]. Many studies have demonstrated that *Lactobacillus* strains can decrease oxidative stress and accumulation of ROS, which help to prevent diseases [6-8]. In addition, the concentrations of 10^7 colony forming units (cfu)/mL. – 10^9 cfu/mL were the common concentration used in different research for probiotics[9-12]. This evidence led us to investigate the protective effects of *Lactobacillus* strains against oxidative stress at the concentration 10^7 cfu/mL – 10^9 cfu/mL in this study.

Several methods are available to assess the antioxidant activity (AA) of *Lactobacillus* strains. Chemical antioxidant assays of *Lactobacillus* strains could be assigned to ROS scavenging, as well as metal ion chelation and the inhibition of ascorbate or linoleic acid peroxidation [13, 14]. The 2, 2-diphenylpicrylhydrazyl (DPPH) radical scavenging activity assay is an important AA estimating method because it reacts directly and rapidly with antioxidant compounds [15-17]. However, this assay is not perfect as it is affected by light intensity, oxygen concentration, and solvent type [18]. Furthermore, it does not reflect of biological activity because it does not take into account cell uptake, partitioning of antioxidants between aqueous and lipid phases, or phases I and II metabolism [19]. Although the antioxidant molecular mechanisms of probiotics have not yet been elucidated completely, probiotics are live organisms, and as such, are more complicated and differ from pure antioxidant compounds. Considering the complexity involved in their action mechanisms, the obtained antioxidant capacity indices by chemical assays thus reflect their antioxidant effects in vivo insufficiently.

Animal models and human studies are more appropriate but are also more expensive and time-consuming. These aspects render the cellular antioxidant assays (CAA) extremely attractive intermediate testing method [20]. The CAA assay, which quantifies AA in cell cultures, was developed to meet the need for a more biologically representative method than the popular chemistry antioxidant capacity measures [21], and has been applied to evaluate the AA of several natural products

74 including foods, and dietary supplements. It could also be engaged to evaluate
75 whether *Lactobacillus*, as a live organism, can change the redox cellular state [20].

76 In this study, the CAA assay were applied to evaluate the AAs of *Lactobacillus*
77 strains in human hepatocellular carcinoma (HepG 2) cells in order to enhance our
78 knowledge and understanding of their biological activity. The CAA values of 10
79 *Lactobacillus* strains from 5 species (*L. rhamnosus, L. plantarum, L. acidophilus, L.*
80 *casei, L. fermenti*) were compared with those obtained by DPPH radical scavenging
81 assay. In particular, the strains with high AA appraised by CAA assay and DPPH
82 radical scavenging assay respectively were further examined to evaluate their
83 protective effects against hydrogen peroxide (H_2O_2)-induced oxidative stress in
84 HepG2 cells so as to determine which method was more suitable for the assessment
85 AA of *Lactobacillus* strains.

86 **Materials and methods**

87 **Chemicals**

88 Methylene blue, 2', 7'-dichlorofluorescin diacetate (DCFH-DA), 2, 2-azobis
89 (2-amidinopropane) dihydrochloride solution (ABAP), 2, 2-
90 diphenyl-1--picrylhydrazyl (DPPH), and quercetin dihydrate were purchased from
91 Sigma-Aldrich Chemical Co. (St. Louis, MO). Dimethyl sulfoxide (DMSO) and
92 Hanks' Balanced Salt Solution (HBSS) were purchased from Gibco Life Technologies
93 (Grand Island, NY). Dulbecco's minimum essential medium–high glucose (DMEM),

fetal bovine serum (FBS), penicillin, and streptomycin were obtained from HyClone (Logan, UT, USA). DeMan, Rogosa, and Sharpe (MRS) broth were purchased from Qingdao Hopebio Company (China). Ethanol, acetic acid, glycerol, glutaraldehyde and other chemicals were purchased from Shanghai Chemical Reagent Company (China). All chemicals used in this study were of analytical grade.

Cell culture

Human hepatocellular carcinoma HepG2 cells (from the cell bank of the type culture collection of the Chinese Academy of Sciences, Shanghai, China) were grown in DMEM supplemented with 10% FBS, penicillin (100 U/mL), and streptomycin (100 μg/mL) and maintained at 37 °C in an incubator with 5% carbon dioxide. The cells used in this study were at passage 10 to 20.

Bacterial culture

All of the *Lactobacillus* strains used in this study are listed in Table 1. These strains were maintained as frozen stocks (−80 °C) in MRS broth supplemented with 30% (v/v) glycerol. These strains were consecutively reactivated at least three times using 1% (v/v) inoculum in MRS broth at 37 °C for 20 h prior to use.

Preparation of bacterial suspensions

The biomass from the 20 h cultures was harvested by centrifugation (3,000 × g for 10 min at 4 °C), washed three times, and suspended in a phosphate-buffer saline (PBS), as appropriate for the specific assay. Bacterial suspensions used for cell cytotoxicity, the CAA assay, and the DPPH radical scavenging assay were obtained from cell

115 suspensions containing approximately 1×10^9, 1×10^8, and 1×10^7 cfu/mL.

116 **Free radical-scavenging assay**

117 The DPPH radical scavenging method is commonly used to evaluate the AAs of
118 lactobacilli [22]. The scavenging effects of 10 *Lactobacillus* strains on the DPPH free
119 radical were measured in accordance with the slightly modified method of Lin and
120 Chang [23]. Bacterial suspensions (10^7, 10^8, and 10^9 cfu/mL, 1 mL) and freshly prepared
121 DPPH solutions (0.2 mM, 1 mL) were briefly mixed. The mixture was shaken
122 vigorously and allowed to react for 30 min in the dark at room temperature. The
123 control sample contained deionized water instead of the sample solution. The
124 scavenged DPPH was then monitored by measuring the absorbance at 517 nm using a
125 SpectraMax M5 microplate reader (Molecular Devices, Sunnyvale CA, USA). The
126 scavenging activity was defined as follows:

127 $$DPPH\ activity\ (\%) = (A_C - A_S) \times 100$$

128 where A_C and A_S are the absorbance of the control and test samples at 517 nm,
129 respectively.

130 **CAA assay**

131 **Cell cytotoxicity assay**

132 The HepG2 cell cytotoxicity induced by the 10 *Lactobacillus* strains was measured
133 using the modified methylene blue assay[24]. HepG2 cells were seeded at a density of 4
134 $\times 10^4$ cells/well on a 96-well microplate in 100 µL of DMEM-10% FBS for 24 h at
135 37 °C. After rinsing with PBS, the HepG2 cells were treated with 100 µL different

136 concentrations of quercetin or *Lactobacillus* strains (10^7, 10^8, and 10^9 cfu/mL) and
137 then incubated again for 6 h at 37 °C. Untreated cells were used as a control. Washed
138 cells were mixed with 50 μL/well methylene blue (98% HBSS, 0.67% glutaraldehyde,
139 and 0.6% methylene blue) for 1 h at 37°C to assess cell viability. After incubation, the
140 cells were washed with deionized water until the water was clear and 100 μL/well
141 elution solution (49% PBS, 50% ethanol, and 1% acetic acid) were added on a table
142 oscillator (Thermomixer Comfort, Eppendorf, Hamburg, Germany) for 20 min. The
143 absorbance was measured at 570 nm using a SpectraMax M5 microplate reader
144 (Molecular Devices). Different samples were compared with the control. A sample
145 that resulted in more than 10% less absorbance than the control, was considered to be
146 cytotoxic [25].

147 **CAA assay of lactobacilli**

148 The CAA assay used for evaluating the AAs of the 10 *Lactobacillus* strains was that
149 described previously [26] with a minor modification. The HepG2 cells were seeded at a
150 density of 6×10^4 cells/well on black 96-well microplates (with transparent bottoms)
151 in 100 μL of DMEM for 24 h at 37 °C to reach 80% confluence and were then treated
152 with *Lactobacillus* strains at final concentrations of 10^7, 10^8, and 10^9 cfu/mL after
153 rinsing with PBS, including 25 μM of DCFH-DA (dissolved in DMSO) for up to 1 h
154 at 37°C. Quercetin (final concentrations of 2, 4, 8, 16, 32 and 64 μM) was used as
155 antioxidant control. Subsequently, the cells were washed with PBS and treated with
156 100 μL of 600 mM ABAP (dissolved in HBSS). Fluorescence was measured using a

SpectraMax M5 microplate reader (Molecular Devices) for 13 cycles at 5 min intervals (λex = 485 and λem = 538). The area under the curve of fluorescence versus time was integrated to calculate the CAA value of each sample after a blank subtraction from the fluorescent readings.

$$CAA\,(unit) = \left\{1 - \left(\int S_A / \int C_A\right)\right\} \times 100$$

where SA is the area of the sample and CA is the integrated area in the control curve.

Cells treated with DCFH-DA were used as blank, whereas cells treated with DCFH-DA and ABAP were used as controls. Quercetin was used in each experiment as a standard, and the CAA values (units) for *Lactobacillus* strains were expressed as the equivalent amount of quercetin with the same AA (μM).

Protective effect of *Lactobacillus* strains against H_2O_2 induced oxidative stress in HepG2 cells

The antioxidant indicators were measured according to a method reported previously [27] with some modifications. HepG2 cells that received different treatments were designated as groups G 1, G 2, G 3, G 4, and G 5, respectively. G 1, with no H_2O_2 treatment, served as the control group. Oxidation was induced by exposing HepG2 cells to 500 μM H_2O_2 in DMEM for 6 h in G 2. In G 3 (quercetin + H_2O_2) and G 4, G 5, (*Lactobacillus* strains + H_2O_2), HepG2 cells were pretreated for 12 h with 100 μM quercetin or different *Lactobacillus* strains, respectively, and subsequently incubated with 500 μM H_2O_2 for an additional 6 h. Quercetin was dissolved in absolute ethanol and then diluted in DMEM to achieve the final concentration (100 μM). Each

178 treatment was carried out in triplicate wells. After the 18 h incubation, cells were
179 washed and collected separately for the measurement of antioxidative indicators. The
180 activities of total antioxidant capacity (T-AOC), superoxide dismutase (SOD),
181 glutathione peroxidase (GSH-PX), catalase (CAT), and peroxidase (POD) were
182 determined using the detection kit provided by Nanjing Jiancheng Bioengineering
183 Institute (Nanjing, China).

184 **Statistical analysis**

185 All of the tests were performed in triplicate. All of the data are presented as mean ±
186 standard deviations (SD). One-way analysis of variance was performed using SPSS
187 (Version 13.0, SPSS Inc., Chicago, IL, USA), followed by Fisher's least significant
188 difference test to verify significant differences between the samples. The results were
189 considered to be significant only when $p < 0.05$. The Pearson correlation test was
190 conducted to determine the correlations between the variables.

191 **Results**

192 **Cell cytotoxicity assay of quercetin and lactobacilli**

193 The cell toxicity of quercetin and the *Lactobacillus* strains was performed on
194 HepG2 cells using a modified methylene blue assay. The cytotoxic effect of quercetin
195 from concentrations of 2 μM to 64 μM caused 7.04 ± 0.09% to 8.50 ± 0.3% cell death,
196 which was lower than 10% cell death, as depicted in the inset graph of Figure 1. The
197 results were consist with previous study[24, 28, 29], which have demonstrated that

198 quercetin has no cytotoxicity to cells, and have protective effect on cells. The
199 cytotoxic effect of *Lactobacillus* strains ranged from 0.65 ± 0.002 % (CCFM237, 10^7
200 cfu/mL) to 7.05 ± 0.41% (CCFM6, 10^9 cfu/mL). All of the 10 *Lactobacillus* strains at
201 three concentrations (10^7 -10^9 cfu/mL) and the different concentrations of quercetin
202 caused less than 10% HepG2 cell death (Figure 1). No significant decrease in cell
203 viability was observed after 24 h of incubation with quercetin or *Lactobacillus* strains
204 ($p > 0.05$), which indicated that neither *Lactobacillus* strains nor quercetin displayed
205 toxicity at the concentrations used in these experiments.

206 **CAA assay**

207 The CAA of quercetin at concentrations of 2–64 μM was determined by
208 monitoring its ability to prevent ABAP induced oxidation of non-fluorescent
209 DCFH-DA to fluorescent 2'-7'-dichlorofluorescin (DCF) in HepG2 cells. A notable
210 dose-dependent inhibition of the ABAP-induced increase in fluorescence was
211 observed following treatment with quercetin (Figure 2A). A decrease in fluorescence
212 intensities ($p < 0.05$) indicated an increase in the CAA value ($p < 0.05$), which
213 corresponded to the increase in quercetin concentration. The fluorescence intensities
214 increased with the extension of incubation time with every test concentration of
215 quercetin. The dose-response curve generated by the CAA values from the data
216 presented from quercetin–fluorescence intensities in Figure 2A are shown in Figure
217 2B. The CAA value increased quickly from 2 μM to 8 μM of quercetin, and the
218 increase trend got slowly from 32μM to 64 μM. DCF formation was inhibited by

219 approximately 90% with 64 μM of quercetin compared with the control based on the
220 fluorescence intensity, which can satisfy the determining of lactobacilli AA. So the
221 concentration range from 2 μM to 64 μM of quercetin was chosen in this study. A
222 standard curve was also generated by the regression equation: $y = 24.53\ln(x) - 7.047$.
223 This equation had a statistically significant correlation coefficient of 0.9774, where *y*
224 (unit) is the CAA and *x* is the concentration of quercetin in units of μM. The good
225 non-linear relationships between the quercetin standard and the CAA value were used
226 to measure the AAs of the *Lactobacillus* strains, i.e., the CAA values for
227 *Lactobacillus* strains were expressed as the equivalent amount of quercetin with the
228 same CAA value.

229 The CAA assay was used to quantitatively evaluate the AAs of the *Lactobacillus*
230 strains in HepG2 cells. The typical time kinetics for the inhibition of peroxyl
231 radical-induced DCFH oxidation in the CAA assay of 10 *Lactobacillus* strains from 5
232 species was tested. The *Lactobacillus* strains reduced the DCF fluorescence in a
233 time-dependent manner (Figure 3). All of the strains inhibited the oxidation, with
234 lower fluorescence intensities ($p < 0.05$) than those of controls over time (Figure 3).
235 Significant differences ($p < 0.05$) in CAA values were found between 10^9 cfu/mL and
236 the two other concentrations (10^7 and 10^8 cfu/mL) for all the strains (Figure 4). The
237 concentration that resulted in the greatest cellular antioxidant activity was 10^9 cfu/mL
238 for the 10 *Lactobacillus* strains, with lower (10^7 cfu/mL and 10^8 cfu/mL)
239 concentrations producing smaller increases in activity (Figure 4).

Evaluation of the AA of lactobacilli using the DPPH radical-scavenging assay

The range of the DPPH radical scavenging activity of 10 Lactobacillus at three different concentrations ranged between 14.93 ± 0.85% DPPH scavenged and 41.28 ± 1.28% DPPH scavenged (Figure 5). CCFM239, CCFM238, CCFM6 and CCFM381 showed a slight increase from 10^7 cfu/mL to 10^9 cfu/mL, but this was not statistically significant. The effects were dose-dependent from 10^7 cfu/mL to 10^9 cfu/mL ($p < 0.05$) for the other strains.

Comparison of the AAs of the 10 Lactobacillus strains

The AAs of the 10 *Lactobacillus* strains were compared using the DPPH radical scavenging assay and the CAA assay. The DPPH radical scavenging activities differed significantly from those of CAA assay from 10^7 cfu/mL to 10^9 cfu/mL for the 10 *Lactobacillus* strains ($p < 0.05$) (Table 2).

The AAs of the 10 *Lactobacillus* strains at a concentration of 10^9 cfu/mL were compared using the CAA assay and the DPPH radical scavenging assay. The 10 *Lactobacillus* strains exhibited different AAs at 10^9 cfu/mL in the two assays (Figure 6). The activity of CCFM9 (13.85 ± 0.81μM) was remarkably higher ($p < 0.05$) than that of other strains in the CAA assay (Figure 6A). However, CCFM237 had a significantly higher AA (41.28 ± 1.28%) than the other strains ($p < 0.05$) in the DPPH radical scavenging assay (Figure 6B). In particular, two strains in each species had significantly different AAs ($p < 0.05$) in the DPPH scavenging assay. The CAA values of the *Lactobacillus* strains (Figure 6A) also established the intra- strain difference

between two strains in each species ($p < 0.05$).

Protective effect of *Lactobacillus* strains against H_2O_2 Induced oxidative stress in HepG2 Cells

The protective effect of *Lactobacillus* strains against oxidative stress in HepG2 cells was also studied. Two strains with high AAs identified by the two different methods (CCFM9 and CCFM237) were used to pretreat the cells. The results showed that the addition of H_2O_2 reduced the activity of SOD, POD, CAT, and T-AOC while the activity of GSH-PX was non-significantly lower than that of the control (Table 3). Analysis of the results revealed that quercetin group (G 3, quercetin + H_2O_2) had high levels of T-AOC, SOD, GSH-PX, CAT, and POD activity ($p < 0.05$) compared with the H_2O_2 group (G 2). Moreover, the activities of GSH-PX and POD were remarkably lower whereas those of T-AOC, CAT and SOD were markedly higher in the quercetin group (G 3, quercetin + H_2O_2) compared with the *Lactobacillus* groups (G 4, CCFM9 + H_2O_2; G 5, CCFM237 + H_2O_2). In addition, the CCFM9 group (G 4, CCFM9 + H_2O_2) had a significantly greater effect on the antioxidant indexes of T-AOC, SOD, GSH-PX, and CAT than did the CCFM237 group (G 5, CCFM237 + H_2O_2). POD concentrations were slightly decreased in G 5 compared with G 4, but this was not statistically significant.

Discussion

Accumulated evidence suggests that lactobacilli provide different health benefits

281 through several mechanisms. Among their different physiological features, the
282 anti-oxidative properties appear to be the most important [30, 31]. The high AAs of
283 lactobacilli improves their growth and survival, and protects the human body from
284 aging, inflammation, and even from developing cancer [3, 32-35]. Thus, the development
285 of effective methods that could be helpful in assessing potential AA, which results in
286 the scavenging of reactive radicals, should be exploited to formulate novel probiotic
287 foods or supplements that can prevent oxidative stress and related diseases.

288 Given the potential of antioxidants to decrease the risk of chronic diseases,
289 measurement of AAs using biologically relevant assays is important [36]. In this study,
290 the CAA assay was first developed to demonstrate cellular anti-oxidative properties of
291 10 *Lactobacillus* strains from 5 species in the HepG2 cells (Figure 4). In this assay,
292 DCFH-DA is taken up by cells and deacetylated to DCFH. Peroxyl radicals generated
293 from ABAP lead to the oxidation of DCFH to fluorescent DCF. The decrease in
294 fluorescence measured after excitation in the presence of *Lactobacillus* strains is
295 proportional to the level of their oxidation-inhibiting activity. It was reported that
296 quercetin quenched peroxyl radicals and inhibits the generation of DCF in a dose and
297 time-dependent manner [29, 37], and showed a high AA in the CAA assay [38]. Thus,
298 quercetin was used as a standard to quantify the CAA value of different samples in
299 this study, and the result is consistent with the previous study[39]. Similar to the
300 anti-oxidative behavior of quercetin, 10 *Lactobacillus* strains at three different
301 concentrations (10^7 - 10^9 cfu/mL) also prevented the ABAP-induced oxidation of

DCFH in the HepG2 cells in a time and dose – dependent manner (Figures 3 and 4). These three different concentrations (10^7, 10^8, 10^9 cfu/mL) of bacteria were also commonly used in previous research for probiotics[9-12]. Both the CAA assay and the DPPH radical scavenging assay in our study showed that 10^9 cfu/mL was a better concentration of *Lactobacillus* strains for scavenging hydroxyl radicals than 10^7 cfu/mL or 10^8 cfu/mL. This result agreed with a previous report that *L. acidophilus* 874 had a more significant effect at 10^9 cfu/mL than at other concentrations on H_2O_2-induced CT-26 cell oxidative damage [40]. We believe that the CAA assay can be used to reflect the AAs of *Lactobacillus*, and all of the 10 *Lactobacillus* strains tested had higher AAs in the DPPH and CAA assays at 10^9 cfu/mL than at 10^7 or 10^8 cfu/mL. The promising results of our study supported those of previous reports, in which the concentration of 10^9 cfu/mL was selected to assay the AAs of different *Lactobacillus* strains [22, 30, 41].

The cytotoxicity assay was performed in this study to confirm that *Lactobacillus* strains had a minimal negative effect on the viability of HepG2 cells using the modified methylene blue assay. Our results agreed well with the findings of Liu and Pan [42], in which no negative effects on cell viability were seen after Intestine 407 cells were incubated with 12 different *Lactobacillus* strains for 48 h. It was also found in another study that the *Lactobacillus* strains tested did not produce toxic effects in IPEC-J2 cells [43].

Chemical assays of the anti-oxidative potential of *Lactobacillus* strains have

mainly involved DPPH - free radical scavenging [41, 42]. The DPPH assay measures the ability of antioxidants to scavenge peroxyl radicals. The CAA assay is an important tool for screening AAs in natural products by evaluating their potential to exert an antioxidant response at the cellular level, and not just their capacity as reducing agents [20]. In this study, we compared the correlation between these two assays and a poor correlation was observed ($R^2 = 0.594$, $p > 0.05$), ($R^2 = 0.627$, $p > 0.05$), ($R^2 = 0.478$, $p > 0.05$) at the concentrations tested (10^7 –10^9 cfu/mL). The poor correlation between the DPPH assay and the cellular assay was probably due to the biological mechanisms of the CAA assay [19]. The CAA index reflects the capacity of antioxidants to decrease intracellular oxidative stress and evaluates the reduction potential, free radical scavenging-like membrane activity, permeability, cell uptake (taking other distributions into account), and metabolism of an antioxidant compound [20]. Huang et al. [44] compared the AAs of Chinese bayberry in assays for 2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid (ABTS), the ferric reducing ability of plasma (FRAP), DPPH, oxygen radical absorbance capacity (ORAC), and CAA. The authors found that the antioxidant values obtained from chemical assays (i.e., for ABTS, FRAP, and DPPH) were not significantly correlated with those from the ORAC and CAA assays. This lack of correlation may be because antioxidant action is not limited to ROS/RNS scavenging but also includes the upregulation of antioxidant and detoxifying enzymes, the modulation of cell signaling, and gene expression [20].

There is evidence that the antioxidant triad comprising SOD, CAT, and GSH-PX

344 constitutes the first line of defense against the adverse effects of ROS [45]. In this study,
345 two *Lactobacillus* strains that were identified as having a high AA in the CAA assay
346 and DPPH radical scavenging assay, acted differently to counteract the oxidative
347 stress in HepG2 cells induced by H_2O_2. The reduction in SOD, CAT, and POD
348 activities in cells exposed to H_2O_2 indicated that oxidative stress might have occurred.
349 Corresponding to the results above, *Lactobacillus* strains showed a potent protective
350 effect against the damage caused by oxidants in HepG2 cells. Our results showed that
351 both quercetin and *Lactobacillus* strains recovered their levels of T-AOC, GSH-PX,
352 SOD, CAT and POD. It should be noted that cells pre-incubated with CCFM 9
353 exhibited higher levels of these five indices except for POD, than those observed in
354 cells pretreated with CCFM 237, indicating that CCFM 9 was more effective in
355 protecting against H_2O_2 induced oxidative stress in HepG2 cells than of CCFM 237.
356 This result exhibited a better consistency with the data obtained by CAA assay than
357 that by DPPH scavenging assay, indicating that CAA assay may be a better choice for
358 the detection of AAs of Lactobacillus strains. Because mammalian cells were used in
359 the CAA assay, the AAs observed in this assay may be more highly correlated with
360 the actual situation in the organisms than those obtained from chemical assays.
361 Meanwhile, the CAA assay avoided the disadvantage of animal models and human
362 studies which being expensive and time-consuming. Therefore, the CAA assay used
363 for detecting the AA of lactobacilli could be an attractive intermediate method
364 between chemical assay and animal model.

Conclusion

Recently, lactic acid bacteria have received much attention from scientists due to their potential for the treatment of many diseases because of their AA. In this study, *Lactobacillus* strains were assessed for their protective effects against radical-induced oxidative damage in chemical assay and in HepG2 cells. The screening resulted in no correlation between the AA of 10 *Lactobacillus* strains detected in the CAA assay and the DPPH radical scavenging assay. CCFM 9 had highest AA (13.85 ± 0.81μM) in the CAA assay, while CCFM237 had the highest AA (41.28 ± 1.28% DPPH scavenged) in the DPPH scavenging assay. Due to the biological relevance of the CAA assay, CCFM 9 showed a greater protective effect, as seen in the expression of cellular antioxidant enzymes. In addition, the AA of *Lactobacillus* strains could be affected by their concentration according to our results. A concentration of 10^9 cfu/mL of the 10 *Lactobacillus* strains was more effective than 10^8 cfu/mL or 10^7 cfu/mL based on the DPPH radical scavenging assay and CAA assay. Thus, this study proposed a method of screening *Lactobacillus* strains in cultured cells, which is as an extremely attractive intermediate method between chemical assays and animal models. Although the biological mechanism of the AA of *Lactobacillus* strains on cells should still be explored, the development of CAA assays to screen highly antioxidant *Lactobacillus* strains could provide a new class of potent methods that are potentially devoid of the side effects of chemical assays and animal models.

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FIGURE CAPTIONS

Figure 1. Cytotoxicity of quercetin (inset) and 10 *Lactobacillus* strains on human hepatocellular carcinoma HepG2 cells. Bars indicate means ± SD of different concentrations of *Lactobacillus* strains: 10^7 cfu/mL (black), 10^8 cfu/mL (dark gray), and 10^9 cfu/mL (dashed).

Figure 2. AAs of quercetin evaluated by the CAA method. (A) Inhibition of peroxyl radical-induced oxidation of DCFH to DCF in HepG2 cells by quercetin (mean ± SD, n = 3); (B) CAA values of quercetin. The CAA value was calculated as the difference in the area under the curve between the tested samples and the control wells; calibration curves with non-linear fitting.

Figure 3. Peroxyl radical-induced oxidation of DCFH to DCF in HepG2 cells and the inhibition of oxidation by the 10 *Lactobacillus* strains over time.

Figure 4. CAA values of the 10 *Lactobacillus* strains. The CAA value was expressed as an equivalent amount of quercetin (μM). Data represent the mean ± SD values that were obtained from six wells in each group. Different letters mean statistically significant differences at $p < 0.05$.

Figure 5. DPPH radical scavenging activity of 10 *Lactobacillus* strains. Bars indicate means ± SD of different concentrations of *Lactobacillus* strains: 10^7 cfu/mL (black), 10^8 cfu/mL (dark gray), and 10^9 cfu/mL (dashed). Means with a common letter are not significantly different within each bacterial group ($p > 0.05$).

Figure 6. DPPH radical scavenging activity (A) and CAA values (B) of the 10

503 *Lactobacillus* strains at 10^9 cfu/mL. Different letters mean statistically significant
504 differences at $p < 0.05$. Bars with no letters in common are significantly different ($p <$
505 0.05).
506

Table 1. Lactic acid bacteria from the Culture Collection of Food Microorganisms of Jiangnan University (Wuxi, China) used in this study

Lactobacillus Strain	Origins
L. rhamnosus CCFM-JU 237	Pickles
L. rhamnosus CCFM-JU 469	Pickles
L. plantarum CCFM-JU 239	Pickles
L. plantarum CCFM-JU 238	Pickles
L. acidophilus CCFM-JU 6	Pickles
L. acidophilus CCFM-JU 137	Pickles
L. casei CCFM -JU 9	Pickles
L. casei CCFM -JU 5	Pickles
L. fermenti CCFM-JU 381	Old leaven dough
L. fermenti CCFM-JU 424	Acid kidney bean

520 Table 2. Correlations between the two assays for antioxidant activity

Correlation coefficient	CAA assay at 10^7 cfu/mL	CAA assay at 10^8 cfu/mL	CAA assay at 10^9 cfu/mL
DPPH at 10^7 cfu/mL	0.594		
DPPH at 10^8 cfu/mL		0.627	
DPPH at 10^9 cfu/mL			0.478

521 * $p < 0.05$

522

523 Table 3: Antioxidant enzyme activities of cell lysate in different treatment groups

Target	G 1 (control)	G 2 (H_2O_2)	G 3 (quercetin + H_2O_2)	G 4 (CCFM9 + H_2O_2)	G 5 (CCFM237 + H_2O_2)
T-AOC (U/mgprot)	5.34 ± 0.01^{b}	3.19 ± 0.012^{a}	8.16 ± 0.035^{e}	6.85 ± 0.014^{d}	5.91 ± 0.023^{c}
SOD (U/mgprot)	24.46 ± 0.024^{b}	23.82 ± 0.01^{a}	39.59 ± 0.042^{e}	32.63 ± 0.036^{d}	28.19 ± 0.031^{c}
GSH-PX (U/mgprot)	1.11 ± 0.012^{a}	1.05 ± 0.016^{a}	3.98 ± 0.013^{b}	6.91 ± 0.04^{d}	5.58 ± 0.064^{c}
CAT (U/mgprot)	2.48 ± 0.13^{b}	1.34 ± 0.11^{a}	3.45 ± 0.13^{e}	3.11 ± 0.13^{d}	3.07 ± 0.13^{c}
POD (U/mgprot)	1.36 ± 0.016^{b}	0.88 ± 0.011^{a}	5.10 ± 0.019^{c}	5.30 ± 0.02^{d}	5.25 ± 0.025^{d}

524 Different letters indicate statistically significant differences at $p < 0.05$.

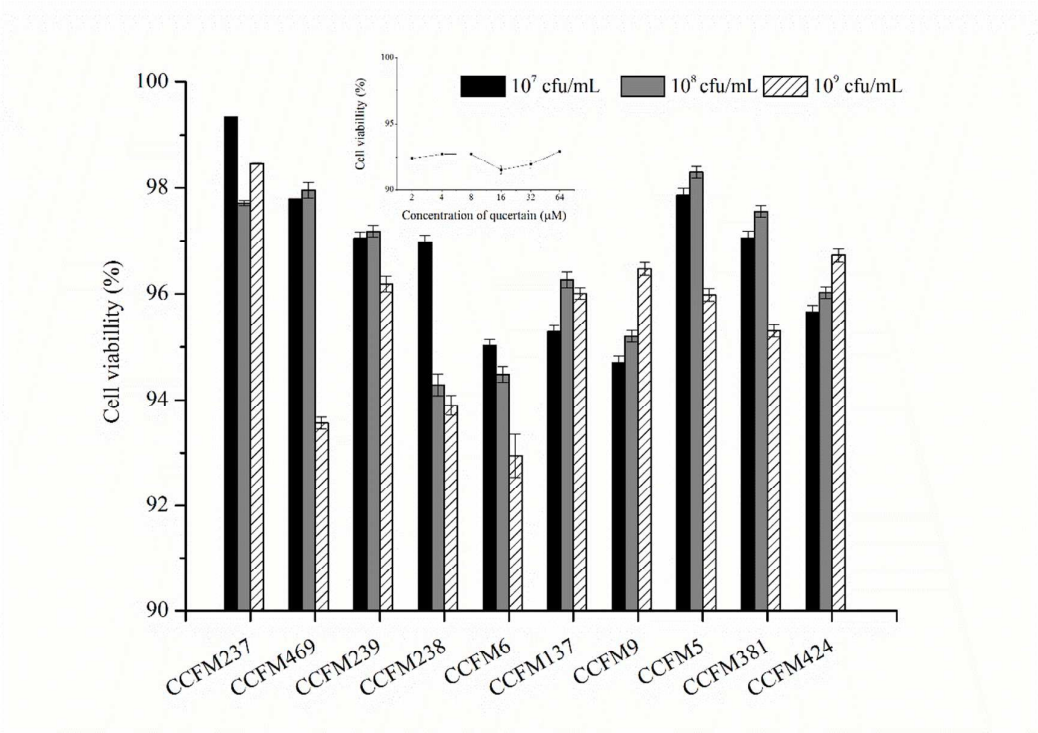
525 T-AOC, total antioxidant capacity; SOD, superoxide dismutase; GSH-PX, glutathione

526 peroxidase; CAT, catalase; POD, peroxidase

527

528 **FIGURE GRAPHICS**

529 **Figure 1.**



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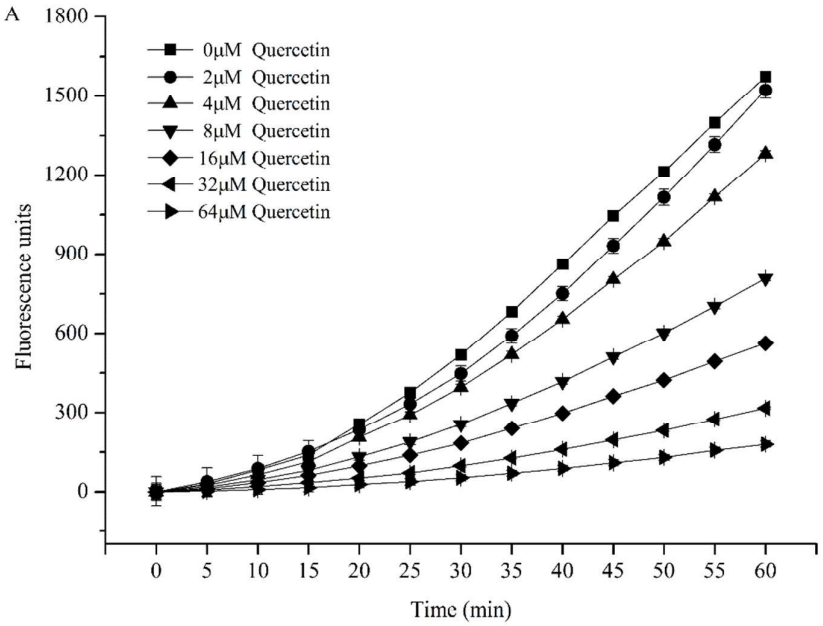
533 **Figure 2A.**



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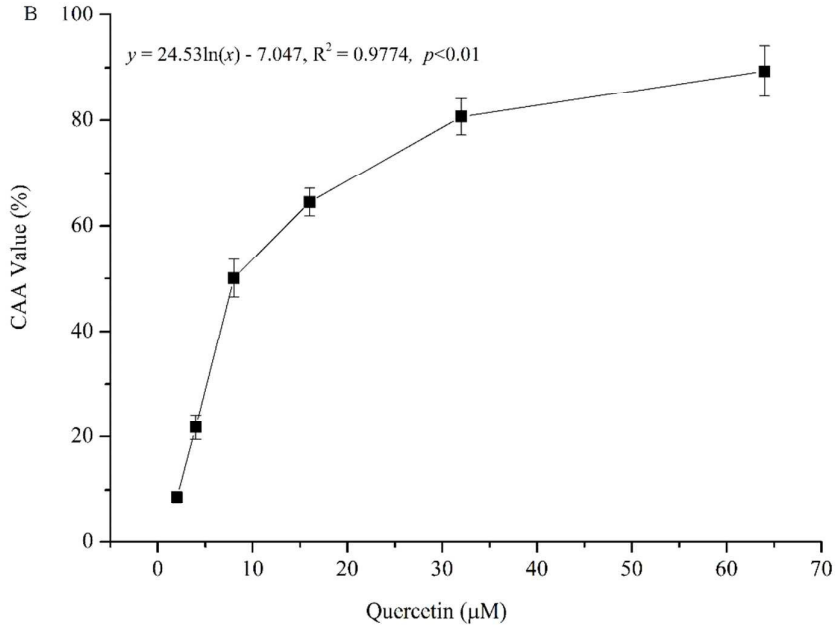
536 **Figure 2B.**



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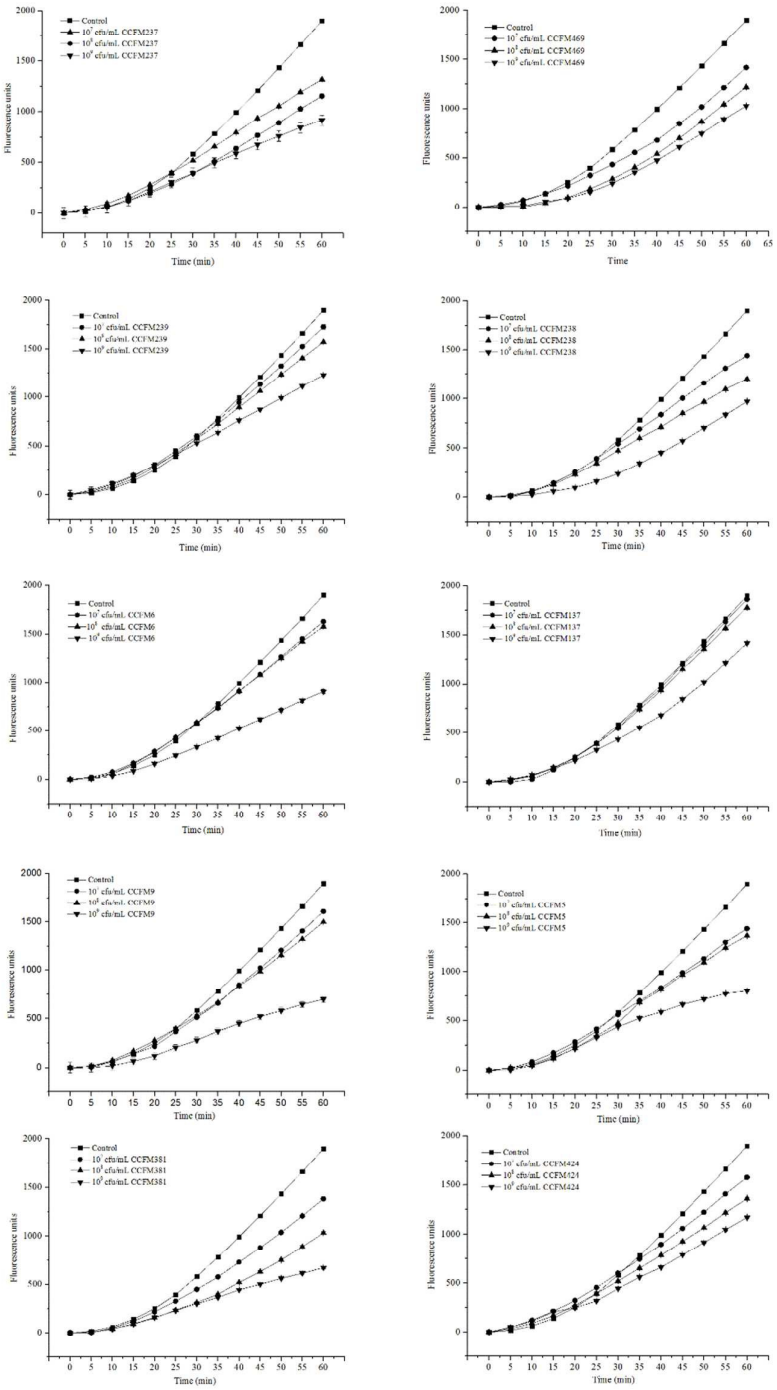
539 **Figure 3**



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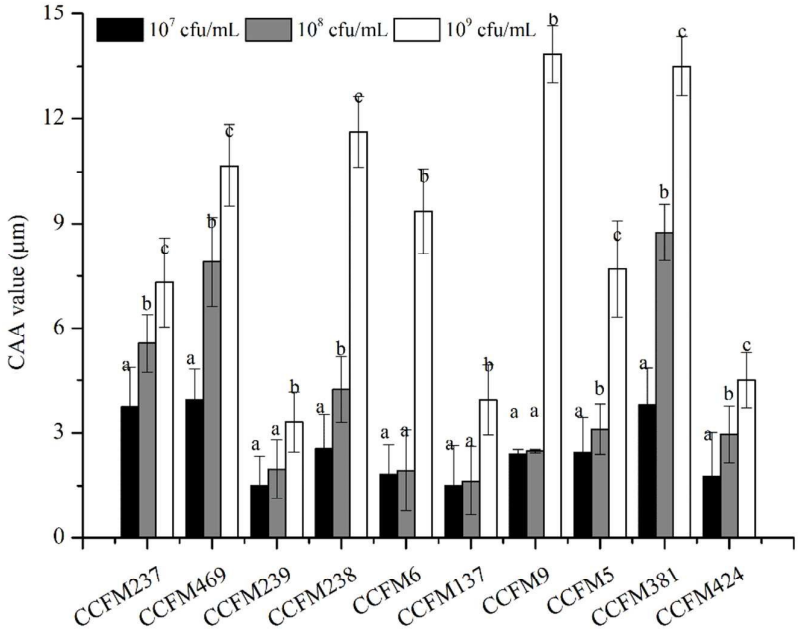
542 **Figure 4.**



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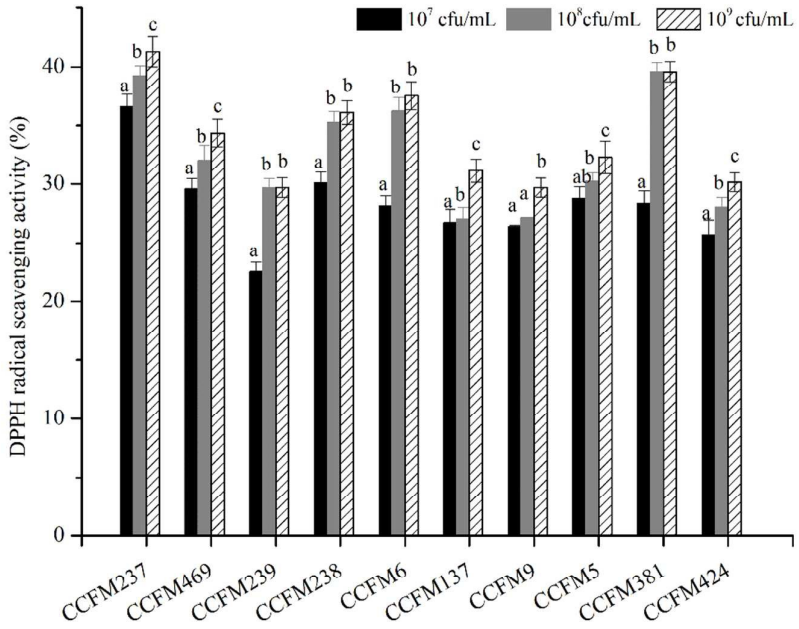
546 **Figure 5.**



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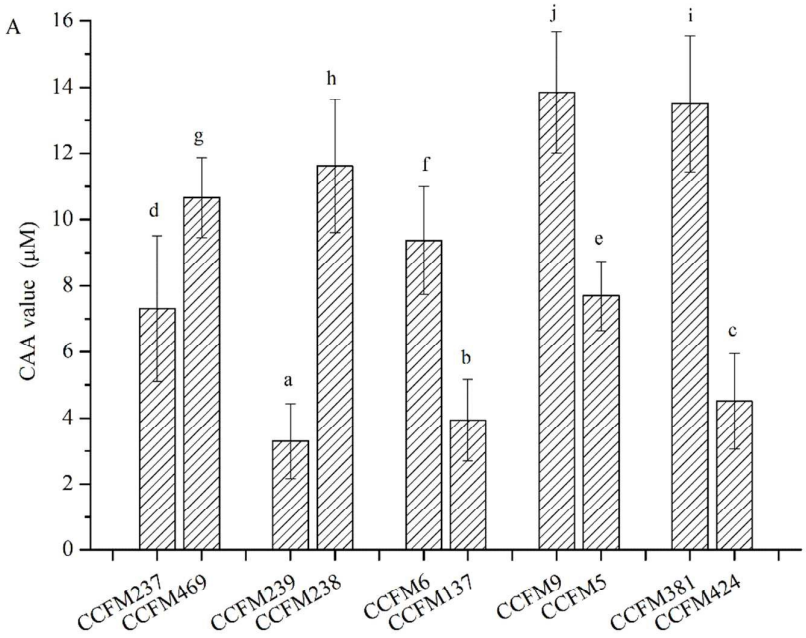
549 **Figure 6A.**



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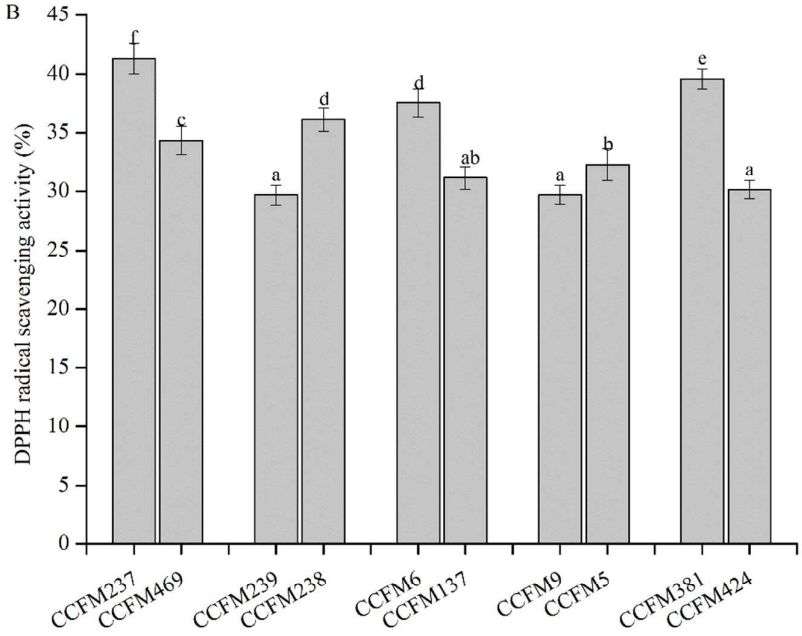
553 **Figure 6B.**



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