

ORIGINAL ARTICLE

# DNA-protection and antioxidant properties of fermentates from *Bacillus amyloliquefaciens* B-1895 and *Bacillus subtilis* KATMIRA1933

E.V. Prazdnova<sup>1</sup>, V.A. Chistyakov<sup>1</sup>, M.N. Churilov<sup>1</sup>, M.S. Mazanko<sup>1</sup>, A.B. Bren<sup>1</sup>, A. Volski<sup>2</sup> and M.L. Chikindas<sup>3,4,5</sup>

<sup>1</sup> Academy of Biology and Biotechnologies, Southern Federal University, Rostov-on-Don, Russia

<sup>2</sup> School of Arts and Science, Rutgers State University, New Brunswick, NJ, USA

<sup>3</sup> Astrabiol, LLC, Highland Park, NJ, USA

<sup>4</sup> Health Promoting Naturals Laboratory, School of Environmental and Biological Sciences, Rutgers State University, New Brunswick, NJ, USA

<sup>5</sup> Center for Digestive Health, New Jersey Institute for Food, Nutrition and Health, New Brunswick, NJ, USA

**Significance and Impact of the Study:** In humans, oxidative stress is a cause or an important component of many serious diseases, as well as being one of the age influencing factors. Environmental stresses lead to the increase in levels of reactive oxygen species (ROS). Oxidative DNA damage is a side effect of nonspecific inflammation. These human health challenging factors trigger the search for health-promoting bacteria capable of production of antioxidants and DNA-protectors. In this study, two *Bacillus* strains of interest were shown to produce noticeable DNA protective and antioxidant activities.

## Keywords

antioxidant activity, *Bacillus amyloliquefaciens*, *Bacillus subtilis*, DNA damage, genotoxicity, *Lux* biosensors, probiotic.

## Correspondence

Evgeniya V. Prazdnova, Research Institute of Biology, Southern Federal University, Prospect Stachki, 194/1, Rostov-on-Don, Russia.

E-mail: prazdnova@sfedu.ru

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## Abstract

DNA protective and antioxidant activity of *Bacillus amyloliquefaciens* B-1895 and *Bacillus subtilis* KATMIRA1933 were evaluated by *Escherichia coli*-based *Lux* biosensors. Two biosensor strains of *E. coli*, MG1655 (pColD-lux) and MG1655 (pSoxS-lux), which react on DNA damage and superoxide-anion radical activity, were used. SOS-response and Sox-response were stimulated by addition of dioxidine (2,3-Quinoxalinedimethanol,1,4-dioxide) and paraquat (*N,N'*-dimethyl-4,4'-bipyridinium dichloride) respectively. Preparations of both *Bacillus* fermentates demonstrated DNA protective and antioxidant (superoxide scavenging) activity (up to 60.19%). The strain K1933 is, in general, characterized by higher DNA protective activity (28.85%), with parameters of antioxidant activity of both bacilli strains being statistically not significantly different. Sporogenous potential probiotic micro-organisms with antioxidant and DNA protective activities can become an effective tool for compensation of various negative oxidative stress processes in humans.

## Introduction

According to the WHO definition, probiotics are live micro-organisms which, when administered in adequate amounts, confer a health benefit on the host (FAO/WHO 2001; FAO/WHO 2002). While immunomodulation and protection against various infections are the most commonly known and desired functions of probiotics (Floch 2014), there are other features such as stimulation of muscular tissue growth in animals, control of obesity (Kadooka *et al.* 2010), and even impact on beha-

viour (Putignani *et al.* 2014; Stilling *et al.* 2014; Cox and Dalloul 2014). Probiotics are effective in addressing health conditions which are not directly associated with infections, in particular allergies, toxicoses of different aetiology, etc. (Priebe *et al.* 2002; Vanderhoof and Mittermeyer 2010). A wide range of adaptogenic activities can be related to release of metabolites, which protect eukaryotic host cells from most harmful consequences of stress: reactive oxygen species production and DNA damage (Marnett 2000; Masood *et al.* 2014; Skulachev 2005).

Oxidative stress underlies action of many health-threatening factors (Fridovich 1999; Wells *et al.* 2005; Sedelnikova *et al.* 2010). Among these are hypoxia/ischaemia, inflammatory and autoimmune disorders, and poisonous effect of the sitotoxins, in particular mycotoxins (Atroschi *et al.* 2002).

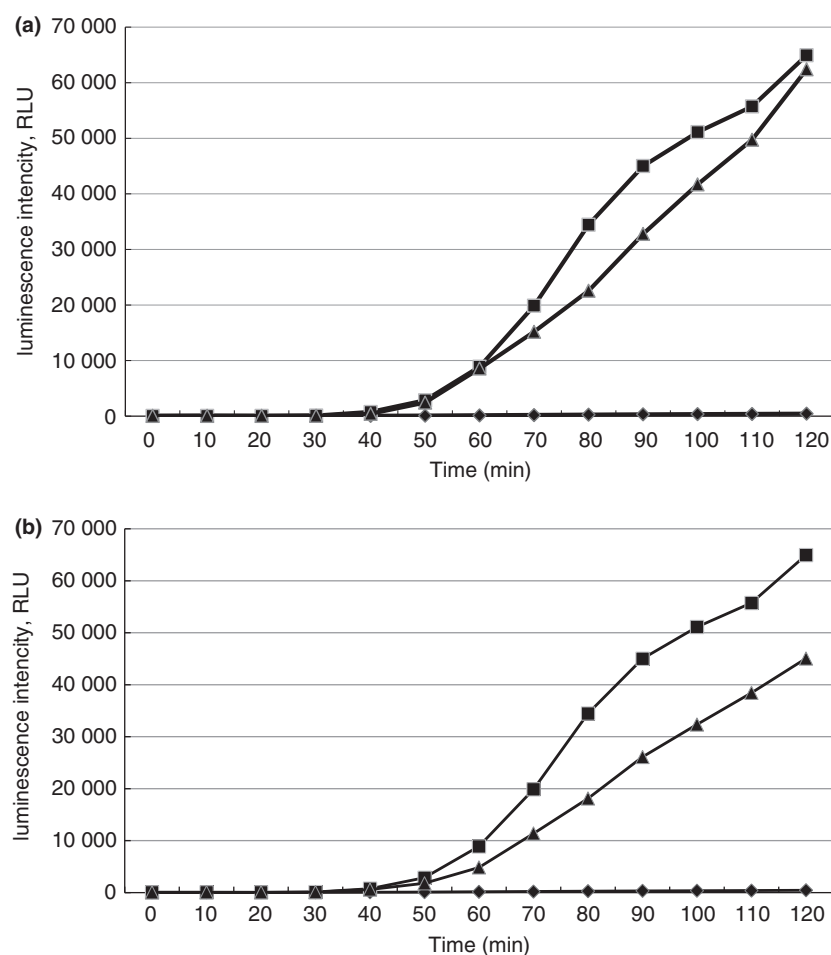
Various environmental stresses lead to the increase in levels of reactive oxygen species (ROS) and damage of the eukaryotic organism's DNA (Ames 1999; Ames and Gold 2000; Halliwell 2005). These human health challenging factors justify the emerging search for probiotic strains capable of production of antioxidants and DNA protectors (Kodali and Sen 2008).

The majority of micro-organisms used as probiotics belong to the lactic acid bacteria (Burgain *et al.* 2014). However, sporeforming health-promoting bacteria are broadly used in agricultural practice (Quigley *et al.* 2013) and are on the rise in various human applications, with the *Clostridium* and *Bacillus* genera being among them (Cutting 2011; Bader *et al.* 2012).

*Bacillus amyloliquefaciens* B-1895 (soil isolate) is a nonpathogenic micro-organism with reported high level

proteolytic activity. Probiotic properties of B-1895 manifest in stimulation of growth and intolerance to fish and bird pathogens (Chistyakov *et al.* 2015; Karlyshev *et al.* 2014). *Bacillus subtilis* KATMIRA1933 was found in a fermented dairy product and was isolated from several independent batches of this product over the period of 3 years (Sutyak *et al.* 2008a). The strain KATMIRA1933 produces the ribosomally synthesized antimicrobial protein (bacteriocin) subtilisin (Zheng and Slavik 1999), previously isolated from *B. subtilis* 168, a laboratory derivative of a Marburg strain ATCC 6051 of unidentifiable origin (Lamanna 1954). The subtilisin preparation obtained from the strain KATMIRA1933, was confirmed as being safe for human tissues, having spermicidal activity (Sutyak *et al.* 2008b), and activity against foodborne (Amrouche *et al.* 2010) and vaginal (Noll *et al.* 2011) pathogens.

Here, we report on DNA protective and antioxidant activity of supernatants of two bacilli strains, *B. amyloliquefaciens* B-1895 and *B. subtilis* KATMIRA1933 evaluated with *Escherichia coli*-based *Lux* biosensors.



**Figure 1** Response of *Escherichia coli* MG1655 pColD-lux to dioxidine with and without B 1895 strain metabolites (Part a), and K 1933 strain metabolites (Part b), where ◆ is the usual level of luminescence, ■ is the response to dioxidine, and ▲ is the response to dioxidine in the presence of the studied bacilli fermentates.

## Results and discussion

Data on the protective activity of the supernatants of two probiotic strains' fermentates (cell-free supernatants) as determined based on biosensors tests, are shown in Figs 1 and 2 and Table 1. Preparations of both fermentates demonstrate DNA protective and antioxidant activity. The strain K1933 is characterized by higher DNA protective activity, with parameters of antioxidant activity of both probiotic strains being statistically not significantly different. Hundred-fold dilution does not result in considerable loss of activity. This may be indicative of the protector's concentration being much higher than saturation level. The observed protective effect became significantly lower only at a thousand-fold dilution of the supernatants. Data on dose-response are presented in Fig. 3.

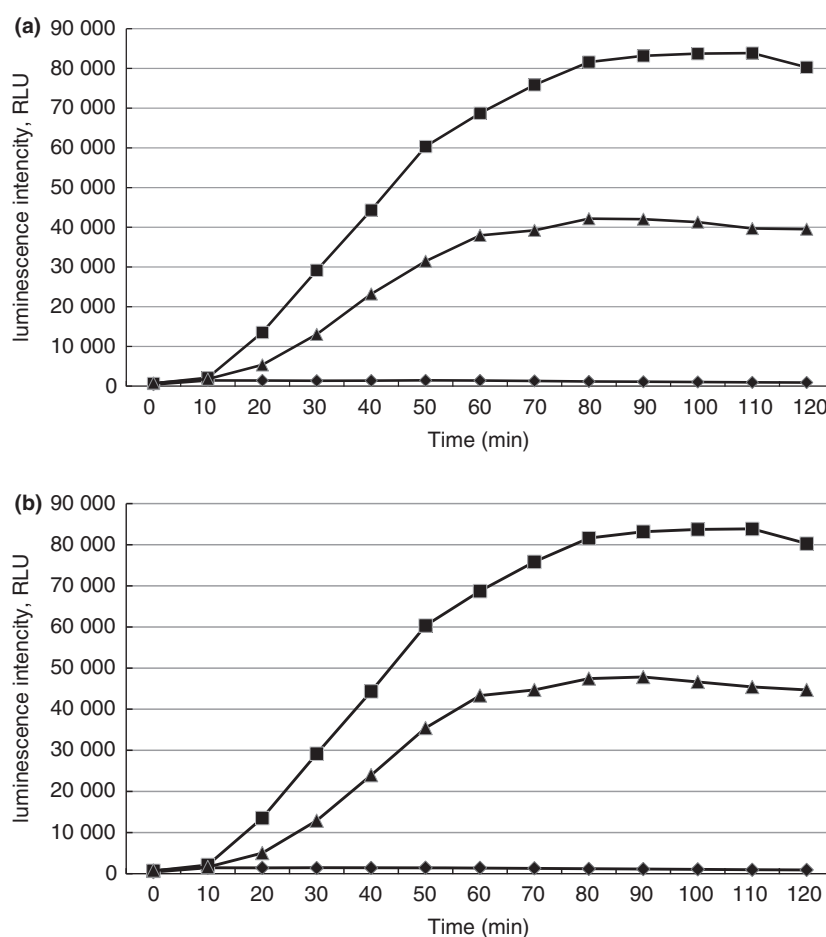
In addition, a series of experiments to examine thermostability of active components was performed. It was shown that heating of supernatant for 30 min decreases protective (antioxidant) effect of the strain B-1895 by 31.47%, and heating for 10 min decreases it by 7.57%.

The strain's K-1933 supernatant heating for 10 min produces a decrease of 7.79%, whereas heating for 30 min completely removes the antioxidant activity.

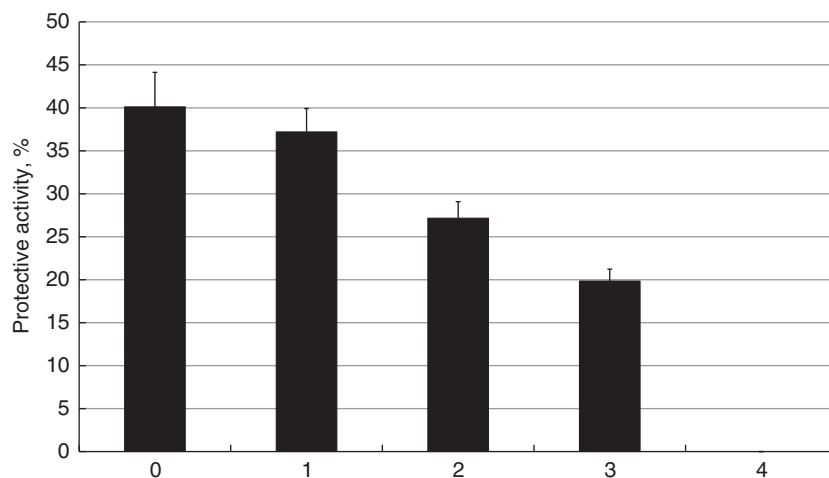
Dioxidin (2,3-Quinoxalinedimethanol,1,4-dioxide) is a mutagenic substance widely used for treatment of infectious diseases (Piopov *et al.* 2013). Its mutagenicity has been demonstrated for a broad range of prokaryotes and eukaryotes. It was shown to act by the reactive oxygen species (ROS) production mechanism (Ordzhonikidze *et al.* 2011). Paraquat (*N,N'*-dimethyl-4,4'-bipyridinium dichloride) is a commonly used reference chemical inducer of ROS generation. It triggers oxidative stress, switch-

**Table 1** DNA protective and superoxide-scavenging activity of *Bacillus* strains

| <i>Bacilli</i> strain/<br><i>Escherichia coli</i> strain | Protective activity (P), %                              |                                                  |
|----------------------------------------------------------|---------------------------------------------------------|--------------------------------------------------|
|                                                          | <i>E. coli</i> MG 1655<br>pColD-lux (DNA<br>protection) | <i>E. coli</i> MG1655<br>pSoxS-lux (Antioxidant) |
| B1895                                                    | 19.53 ± 4.48                                            | 42.79 ± 13.45                                    |
| K1933                                                    | 28.85 ± 3.82                                            | 60.19 ± 3.69                                     |



**Figure 2** Response of *Escherichia coli* MG1655 pSoxS-lux to paraquat with and without fermentates of the studied strains B 1895 (Part a) and K 1933 (Part b), where  $\blacklozenge$  is the usual level of luminescence (control),  $\blacksquare$  is the level of luminescence in the presence of paraquat, and  $\blacktriangle$  is the level of luminescence in the presence of paraquat and fermentate.



**Figure 3** Dose-effect relationship of antioxidant activity of *Bacillus subtilis* KATMIRA1933 cell-free supernatant, where 0 is nondiluted supernatant, 1 is the ten-fold dilution, 2 is the hundred-fold dilution, 3 is thousand-fold dilution, 4 is ten-thousand-fold dilution.

ing cell's bioenergetics to the superoxide-anion generation instead of ATP synthesis (Liochev *et al.* 1994; Miller *et al.* 2007).

Ability of health-promoting lactobacilli and bifidobacteria to produce DNA protective agents has been known for more than two decades (Renner and Münzner 1991). These bacteria generate agents which lower genotoxicity of substances such as 4-nitroquinoline-1-oxide, nitrosoguanidine, 2-amino-3,4-dimethyl, imidazo[4,5-f]quinoline, polycyclic aromatic hydrocarbons, aflatoxins etc. (McBain and MacFarlane 2001; Lo *et al.* 2004). Also, high antimutagenic activity of sporogenous bacilli was recently described (Cenci *et al.* 2008; Piopov *et al.* 2013).

The reports on the connection between antimutagenic activity of probiotic bacteria and their production of antioxidants are scattered (Gotteland *et al.* 2006; Shen *et al.* 2011; Achuthan *et al.* 2012), and so far it has not been reported for sporogenous probiotics. Probiotic micro-organisms that generate substances with antioxidant and DNA protective activity can become an effective tool for compensation of the aforementioned negative processes.

We assume the bacterial model is acceptable for this study because antioxidant mechanisms are quite general for all living organisms. Nevertheless, it should be taken into account that there is a considerable gap between the *in vitro* assays performed on *E. coli* here and the antimutagenic activity in humans.

## Materials and methods

Two bacilli strains were grown in Luria–Bertani (LB) broth (Difco, Detroit, MI) at 37°C on the circular shaker Biosan (Riga, Latvia) during 14 h until early stationary growth phase had been achieved (OD = 0.7, approx.  $3.5 \times 10^9$  CFU ml<sup>-1</sup>).

The strains' cell-free supernatants were collected by centrifugation (Minispin-plus; Eppendorf, Leipzig, Germany) at 6000 g for 7 min. The DNA protective and antioxidant activities of these supernatants were evaluated with bacterial *Lux* biosensors. Supernatants were not filter sterilized, assuming that for a rapid biosensor test it is not necessary.

*Escherichia coli* strains MG1655 (pColD-lux) and MG1655 (pSoxS-lux) (obtained from Manukhov, State Scientific Center Genetika, Moscow, Russia) were used as *Lux* biosensors, identifying induction of *ColD* gene and *Sox* operon, which are involved in SOS-repair and serve as a part of the cellular antioxidant defence system respectively (Zavilgelsky *et al.* 2007).

DNA protective and antioxidant activity was evaluated by ability of the secreted bacterial metabolites to reduce SOS-response and *Sox*-response, stimulated by addition of dioxidine (2,3-Quinoxalinedimethanol, 1,4-dioxide, Biosintez, Penza, Russia) up to  $2.25 \times 10^{-5}$  mol l<sup>-1</sup> and paraquat (*N,N'*-dimethyl-4,4'-bipyridinium dichloride, Sigma-Aldrich, Saint-Louis, MO) up to  $10^{-3}$  mol l<sup>-1</sup> concentrations respectively (Chistyakov *et al.* 2012). The methodology for *Lux* biosensors bioluminescence detection was thoroughly described by Manukhov *et al.* (1999) and Zavilgelsky *et al.* (2007). Thirty-minute pre-incubation of supernatant with culture was performed. For luminescence measurements, an LM-01A automatic microplate luminometer (Immunotech, Praha, Czech Republic) was used. Measurements were carried out every 10 min for 120 min. For evaluation of the influence of the studied factors on *Sox* operon expression, the induction factor ( $I_s$ ) was calculated according to the formula:

$$I_s = (L_e/L_k) - 1, \quad (1)$$

where  $L_k$  and  $L_e$  are luminescence intensities of control and experimental samples respectively. A statistically sig-

nificant excess of  $L_e$  over  $L_k$  estimated with the  $t$ -criterion was considered as a sign of significant influence on the induction effect.

Protective activity ( $P$ , %) was calculated taking induction into account in the presence of corresponding protector concentrations:

$$P = (1 - (I_a/I_p)) \times 100\%, \quad (2)$$

where  $I_a$  and  $I_p$  are induction factors of the SOS-response with the investigated influence in the presence of protector and in the control sample respectively.

To characterize the protective activity of the studied concentration of the substance, the mean value of  $P$  during the whole duration of measurements was used.

Each experiment was conducted at least three times in triplicate and the statistical analysis was performed using Student's  $t$ -test. Confidence intervals were calculated by program MICROSOFT EXCEL (Microsoft Corporation, Redmond, WA) for  $P = 0.05$ .

To study the thermostability of cell-free supernatants, preparations were heated in a water bath to 85°C.

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## Conflict of Interest

The authors declare no conflict of interests.

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