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Bacillus subtilis CBR05 for Tomato (*Solanum lycopersicum*) Fruits in South Korea as a Novel Plant Probiotic Bacterium (PPB): Implications from Total Phenolics, Flavonoids, and Carotenoids Content for Fruit Quality

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Abstract: Plant growth-promoting rhizobacteria (PGPR) are naturally occurring soil biota which benefit plants by improving plant productivity and immunity. The aim of the present work was to evaluate the effect of the inoculation of PGPR strain, *Bacillus subtilis* CBR05 on the quality of tomato fruits produced under greenhouse conditions. Results were compared with mock-inoculated control and market sample. We found a significant increase in total phenol and flavonoid contents of tomato fruits in PGPR strain *B. subtilis* CBR05 inoculated plants compared to those of mock-inoculated control and market sample. Moreover, *B. subtilis* CBR05 inoculation stimulated antioxidant activities and levels of carotenoid (β carotene and lycopene) content in plants. In addition, the inoculation of the strain *B. subtilis* CBR05 produced the highest content of lycopene (21.08 μg/g FW) in tomato fruits as compared to mock-inoculated plants. Our results show that the PGPR strain *B. subtilis* CBR05 is a versatile soil bacterium that enhances tomato production by elevating antioxidant activities and carotenoid (β carotene and lycopene) levels in fruit.

Keywords: *Bacillus subtilis*; tomato; antioxidant activity; carotenoids; probiotics; PGPR

1. Introduction

Tomato (*Solanum lycopersicum*) is regarded as the second most vegetable crop next to potato in the agricultural implications of human consumption. According to agricultural statistics, tomatoes along with sweet corn and snap beans constitute 93% of crop production and processing strategies (Agricultural Statistics, United States Department of Agriculture (USDA), 2016). The positive benefits of tomato consumption have been rigorously proved against a variety of diseases like chronic degenerative diseases, owing to the escalated content of significant phytochemicals with potent health benefits,

like the carotenoids (β-carotene and lycopene), the glycoalkaloids (dehydrotomatine and α-tomatine), ascorbic acid, tocopherols, and many phenolic and flavonoid compounds [1–3]. Tomato also contributes as a major dietary ingredient for Vitamin A and C which implies increased per individual consumption in the United States and many Western countries [2,4]. Fruit ripening in tomato comprises a cascade of events on biochemical, physiological, and structural perspectives involving the influence of secondary metabolites that confer flavor, aroma, texture, and appearance of the tomato [5,6]. Accumulation of large quantities of pigments, especially lycopene and β carotene, inside the plastoglobules of chromoplast provides a visual indication that the fruit is mature and suitable for consumption [4].

Agronomic practices are recognized as a vital factor in determining the nutritional quality of tomato crops [7,8]. The nutrient contents in tomato fruits depend on the environment in which they grow [9,10]. Nowadays, the use of crop modeling to identify effective farmer strategies to counteract adverse future climatic conditions has become a standard in climate change impact assessments [11–13]. Over the past few years, a variety of methods have been proposed to comprehensively assess fruit quality and its relationship with water, including principal component analysis (PCA), analytic hierarchy process (AHP), gray relational analysis (GRA), and technique for order preference by similarity to ideal solution (TOPSIS) [14–17]. The challenge of producing fresh fruits and vegetables is increasing both yield and quality to satisfy consumers as the environment changes in ways that are deleterious to crop species [18]. The quality of agricultural products is affected by many pre- and postharvest factors [9]. The utilization of biofertilizers that mitigate these adverse environmental effects has become a feasible and beneficial production practice. Plant growth-promoting rhizobacteria (PGPR) may be considered as preharvest biotic factors that mitigate adverse environmental effects and promote improved crop yield and quality [19,20].

Among various PGPR approaches, *Bacillus* species are considered as likely candidates due to their broad-spectrum antagonistic activity against phytopathogens, production of long-lived and stress-tolerant spores, secondary metabolites, lytic enzymes, resistance to adverse environments, and plant growth promotion [19,21–23]. *Bacillus subtilis* plays a significant role in improving plant growth and tolerance to both biotic and abiotic stresses. PGPR strains also act as bio-stimulants of phytohormones and peptide synthesis [20,24], but studies of the PGPR strain, *B. subtilis* CBR05 on tomato have not yet appeared. Preharvest factors that directly affect crop yield and quality can be summarized into biological factors comprising pathological, entomological, and animal issues, which was found to be nullified upon increased usage of PGPR. The dire need for assessment of tomato and tomato-based products is given significant attention concerning nutrition and quality relying on the nature of the variety, maturity at harvest, effective transport, and storage [25]. Characterization of the carotenoids, mainly β-carotene and lycopene during storage and various ripening stages, shows drastic developments in sustainable yield and quality parameters of tomato [26]. This information adds to our understanding of temporal differentiation of nutritionally significant phytochemicals during ripening of tomato fruits. The objective of this study was to evaluate the effects of *B. subtilis CBR05* on the quality of tomato fruits under greenhouse conditions.

2. Material and Methods

2.1. Chemicals and Reagents

Authentic standards of carotenoid, all-*E*-lutein, was purchased from Cayman Chemical Company, Michigan, USA. All-*E*-β-carotene were purchased from Sigma-Aldrich, St. Louis, MO, USA. Only the HPLC grade of organic solvents was employed in carotenoid extraction (Daejung, Siheung-*si*, Korea).

2.2. Bacterial Strain and Culture Conditions

B. subtilis CBR05 isolated from our lab maintained on tryptic soy agar (TSA) plates. For long-time storage, bacterial cultures were maintained in tryptic soy broth at −80 °C. For experimental purposes, the cultures were transferred to TSA (MBCell, Seoul, Korea) and incubated at 30 °C for 24 h.

The inoculum mixture of the strain was prepared by culturing in nutrient broth and incubating at 28 °C with constant shaking at 130 rpm. The bacterial cells were centrifuged at 10,000 g for 10 min at 4 °C. The cell precipitate was resuspended in 10 mM $MgCl_2$ and the cell concentration of bacterial suspensions of *B. subtilis* CBR05 was adjusted to 10^8 colony-forming unit (CFU)/mL (OD_{600} = 1.0) for further studies.

2.3. Plant and Growth Conditions

Tomato seeds (Korean cultivar, Kwangbok) utilized for this study were obtained from a Korean seed resource center, Seoul, South Korea. They were surface sterilized in sodium hypochlorite, rinsed several times with distilled water, and planted onto pots containing sterilized growth media (Peat moss with perlite in a ratio of 3:1). Two sets of plants (three plants per set) were maintained, one without PGPR (mock-inoculated control) inoculation and the other with PGPR strain, *B. subtilis* CBR05 inoculum. All treatments were placed randomly in the greenhouse and replicated 3 times. Plant growth-promoting *B. subtilis* CBR05 were applied under sterile conditions to the base of the plants close to the roots to ensure better colonization. Plants were maintained under greenhouse conditions at a temperature of 25 °C with watering carried out every alternate day, to make a better availability for nutrition and plant growth promotion.

2.4. Antioxidant Assays

2.4.1. DPPH Assay

1,1-diphenyl-2-picrylhydrazyl (DPPH) assay was performed to assess the fractions exhibiting scavenging property of free radicals in vitro [27]. Then, 0.2 mM solution of DPPH in ethanol was added to the fraction of aliquot at concentrations (100 µg/mL). The mixture was allowed to stand for 30 min and the absorbance was measured at 517 nm using a UV-Visible spectrophotometer. The percent scavenging activity was determined and Trolox was used as the standard.

2.4.2. ABTS (2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) Assay

A Trolox equivalent antioxidant capacity (TEAC)/ABTS assay was conducted based on the method of Ramos et al. [28]. The ABTS solution (7 mM) was oxidized with potassium peroxodisulfate (2.45 mM) for 16–18h at room temperature. The ABTS solution was diluted with solvents. An aliquot (100 µg/mL) was mixed with diluted ABTS solution and the absorbance was read at 734 nm. Trolox and ascorbic acid were used as reference standards.

2.5. Determination of Total Phenolic Contents

The Folin–Ciocalteu method was used to estimate various concentrations concerned with the total phenolic content. The extracts were dissolved in absolute methanol and later 200 µL of the extract was mixed with 800 µL of 1 N Folin–Ciocalteu reagent (1: 10). After 5 min at room temperature, 3 mL of sodium carbonate (15%) was added to the extracts. Following incubation for 30 min at room temperature, the absorbance was read at 765 nm using a UV-spectrophotometer. A standard curve for gallic acid equivalents (GAE) (milligrams per gram of extract (mg GAE/ge)) was utilized to evaluate the concentration of total phenolic compounds. Analyses were performed in triplicate per each extract.

2.6. Determination of Total Flavonoid Contents

Screening the content of flavonoids was done by a modified protocol as reported previously [29]. The extracts were dissolved in absolute methanol. In a 15 mL conical tube, 1 mL of a sample was mixed with 0.3 mL of 5% sodium nitrite, followed by incubation for 5 min. After incubation, 0.3 mL of aluminum chloride (10%) and 2 mL of sodium hydroxide (1 mol/L) were added to the reaction mixture, and the absorbance was read at 496 nm with a UV-spectrophotometer, using catechin as the

standard. Quercetin equivalents (QE) present per g of extract (mg QE/ge) was used to quantify the expression levels.

2.7. Extraction and Quantification of Carotenoids

Carotenoids were extracted in triplicates and quantified according to previously established protocol with minor modifications [30,31]. All the preparations were performed in low light conditions to avoid the degradation of carotenoids. Three independent biological samples were extracted separately. Briefly, one whole tomato fruit was finely chopped and mixed thoroughly. Five grams of chopped fruits (exact to 0.001 g) from each treatment were separately transferred into test tubes containing 20 mL of acetone and 0.1% butylated hydroxytoluene (BHT: *w/v*). The samples were homogenized with a mechanical homogenizer and centrifuged at 5000× *g* (5 min at 4 °C temperature). The supernatant was recovered and pelleted samples were repeatedly extracted until the pallets became colorless. Supernatants from all extractions were pooled and vacuum-dried in a rotary evaporator (Temperature < 35 °C) (Büchi RE 111, Switzerland).

2.8. HPLC Analysis

The extract was recovered with 10 mL of methylene chloride (CH_2Cl_2) containing 0.1% BHT and transferred to an amber color HPLC vial for HPLC analysis. The chromatographic separation was achieved using an Agilent Model 1100 HPLC instrument (Agilent Technologies Canada Inc., Mississauga, ON, Canada) equipped with a degasser, autosampler, dual pump, and diode array detector (DAD). Samples were scanned (200–800 nm) with 0.05 min (1 s) response time, 8.0 mm slit width, and a detection wavelength of 450 (for most of the carotenoids) and 470 nm (for lycopene). The bandwidth was ±16 nm for all detection wavelengths. Similarly, 600 nm was used as a reference wavelength with ±50 nm bandwidth in all detections. The column used was a YMC, C30 carotenoid column, 250 × 4.6 mm, 5 µm (YMC, Wilmington, NC, USA), and the chromatographic data were recorded with ChemStation LC 3D software. The column thermostat was maintained at 25 °C temperature. Then, 20 µL of standards and samples were injected with an autosampler. The solvent system consisted of Methanol: methyl tertiary butyl ether (MTBE): water (81:15:4) (Mobile phase A) and MTBE: Methanol (91:9) (Mobile phase B). The gradient elution was 0%–100% B in 90 min, and 5-min post-run at a flow rate of 1 mL/min.

2.9. Statistics

All of the experiments were conducted in triplicate and results were tabulated as the Mean ± standard deviation (SD). Statistical significance of the data was determined using one-way analysis of variance (one-way ANOVA) followed by Fisher LSD (Least Significant Difference) test. Data analyses were performed using Sigmastat v8.02 (Systat Software Inc., San Jose, CA, USA). A p value of ≤ 0.05 was considered significant.

3. Results

In the present investigation, the effects of the PGPR strain, *B. subtilis* CBR05 inoculation on the maintenance of carotenoids, total phenolics, flavonoid contents, and antioxidant properties were evaluated. The results revealed that the PGPR strain, *B. subtilis* CBR05 has the capacity to improve the plant growth and change some of the tomato fruit quality characteristics under greenhouse conditions. Tomato plants treated with PGPR strain, *B. subtilis* CBR05 showed significantly higher biomass compared with mock-inoculated controls. Significant increases in root length and dry weight, over mock-inoculated controls, were achieved in green-house conditions (data not shown). In addition, antioxidant activities of three tomato fruits (mock-inoculated, *B. subtilis* CBR05 inoculated, and market fruit) were determined using both DPPH and ABTS radical scavenging method. The extract of fruits from plants inoculated with *B. subtilis* CBR05 strain was the most active against DPPH and ABTS radical

and that of fruits from the market, the differences being significant when compared to mock-inoculated control (Figure 1).

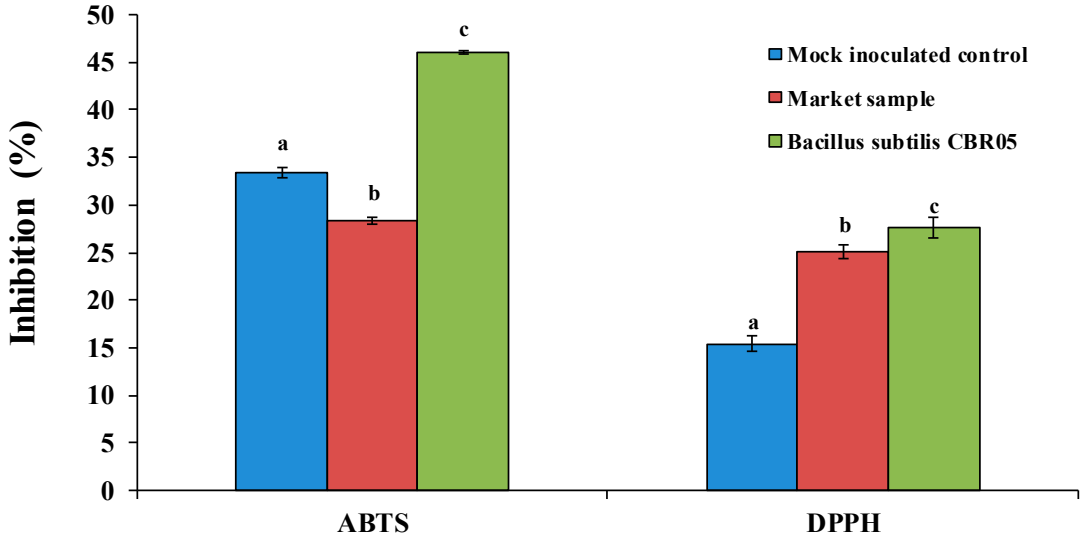

Figure 1. Antioxidant assays ((2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and 1,1-diphenyl-2-picrylhydrazyl (DPPH)). Values are mean ± standard deviation from three replicates. Bars followed by the same letter(s) are not significantly different ($p \leq 0.05$).

The total phenolic content of the fruit extracts is shown in Figure 2. Among the fruits, the PGPR strain, *B. subtilis* CBR05 inoculated plants had the highest phenolic content followed by market fruits. Moreover, the flavonoid contents were also elevated in PGPR treated tomato fruits as compared to those of both mock-inoculated control and market fruits (Figure 2). We did not find any significant differences between mock-inoculated control and market fruit. The results of this study show that *B. subtilis* CBR05 is an effective probiotic agent for the promotion of tomato fruit quality.

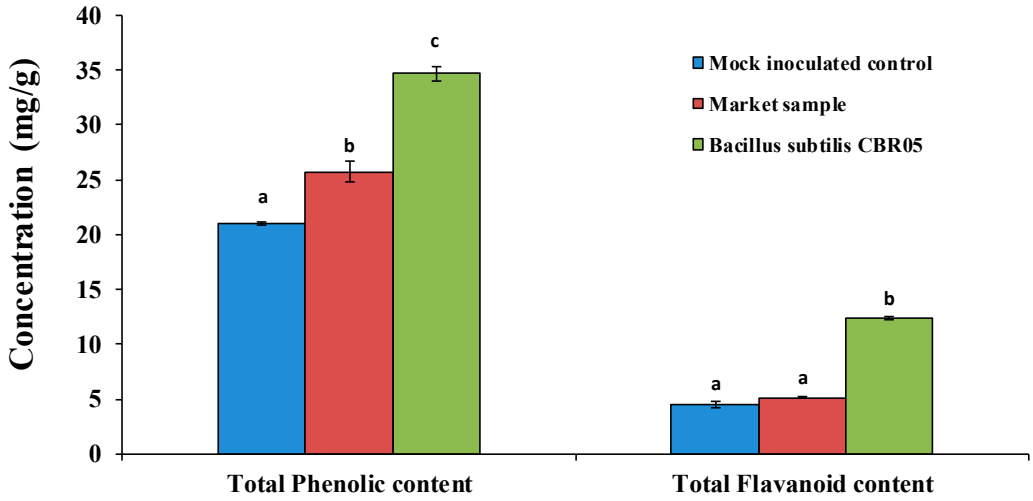

Figure 2. Total phenol and flavonoids contents. Values are mean ± standard deviation from three replicates. Bars followed by the same letter(s) are not significantly different ($p \leq 0.05$).

The carotenoids, such as lycopene and β-carotene, were extracted and quantified (Table 1). The contents and composition of carotenoids were found to differ among control, *B. subtilis* CBR05 inoculated, and market fruits (Figure 3). Carotenoid levels (lycopene) in tomato fruits in plants treated with PGPR strain, *B. subtilis* CBR05 were higher than both the control and market fruits (Table 1; Figure 3).

Table 1. Contents of carotenoids in tomato fruits.

Treatment	Carotenoids	Content (μg/g FW)
Mock inoculated control	All-*E*-β-Carotene	5.63 ± 0.28 [a]
	All-*E*-Lycopene	7.48 ± 1.96 [a]
Market tomato	All-*E*-β-Carotene	4.65 ± 0.96 [a]
	All-*E*-Lycopene	10.51 ± 3.34 [a]
Bacillus subtilis CBR05	All-*E*-β-Carotene	3.53 ± 0.12 [a]
	All-*E*-Lycopene	21.08 ± 0.32 [b]

In each experiment, means followed by different superscript letters are significantly different among the treatment groups ($p \leq 0.05$). Values are mean ± standard deviation from three replicates.

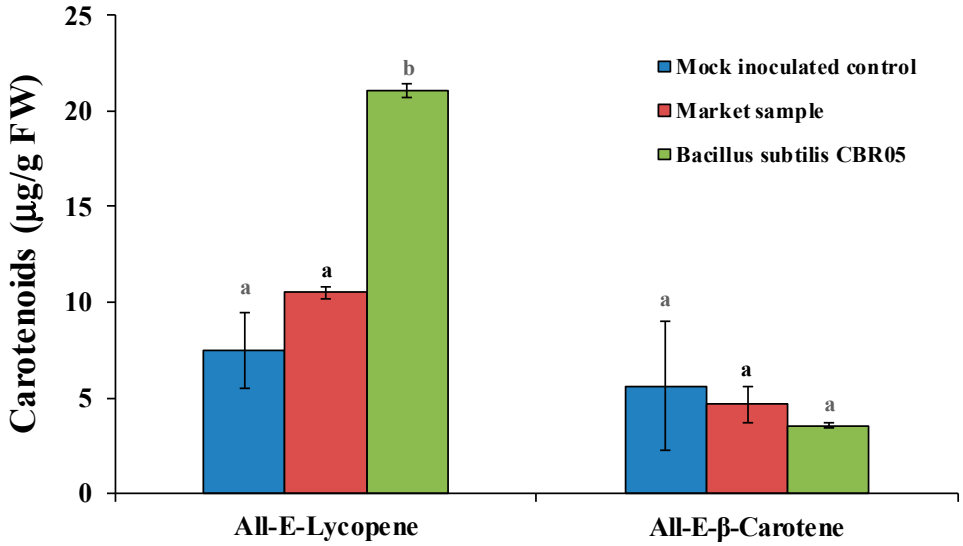

Figure 3. Carotenoids' contents (lycopene and β-carotene). Values are mean ± standard deviation from three replicates. Bars followed by the same letter(s) are not significantly different ($p \leq 0.05$).

The present investigation extends their results by comparing the contents of carotenoids from the fruits using HPLC–DAD (Figure 4). Using this methodology, all-E- β -carotene and all-E- lycopene were identified as the major carotenoids in tomato fruits, based on retention time with standards and by comparing the peak spectra recorded with a DAD during the analysis. The chromatograms (470 nm) and the peak spectra of major identified peaks were shown in Figure 4. The other minor carotenoids were not quantified due to the unavailability of standard compounds. We did not find any significant differences in β-carotene. However, in the present study, we have recorded a significantly higher amount of lycopene (All-E-lycopene) in fruits during ripening in PGPR strain, *B. subtilis* CBR05 inoculated plants than those of both mock-inoculated control and market fruits. These results validate the productive roles of the *B. subtilis* CBR05 in enhancing the nutritional potential of tomato fruits.

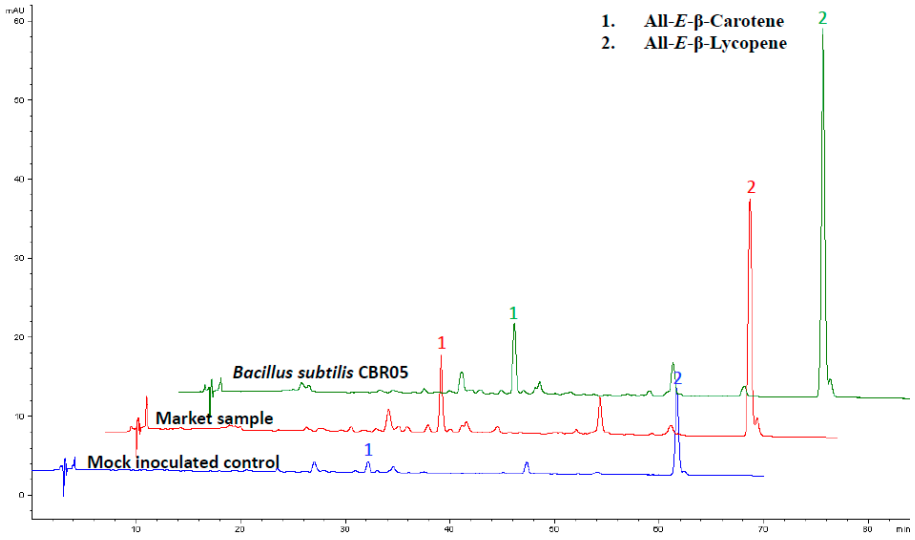

Figure 4. HPLC chromatogram of carotenoids.

4. Discussion

The use of PGPR is increasing in agriculture and may offer an attractive alternative to synthetic chemicals and fertilizers. Plant growth-promoting microorganisms are efficient microbial competitors that can promote plant growth by producing phytohormones and/or by increasing available nutrients through production of secondary metabolites or act as biocontrol agents to protect plants from infection by phytopathogens [19,21–24]. There have been many reports on PGPR and their effective roles [19–24]. Insufficient experimental work has been reported to speculate on the mechanisms of PGPR effects on fruit quality. In the present study, PGPR strain, *B. subtilis* CBR05 isolated from rice were used as inoculants for tomato plants grown under greenhouse conditions. Our results showed that PGPR inoculations significantly increased the total biomass and root length compared to those in the control. *B. subtilis* CBR05 appears to impart plant growth promotion effects that are distinct from other commercial biocontrol agents. Tomato fruits are a good source of antioxidant compounds that can reduce harmful oxidation reactions in the human body, thus preventing various diseases associated with free radical oxidation, such as cardiovascular and neurological disorders and cancer [1–4].

Antioxidant activity has been widely used to test the ability of plant extracts to act as free radical scavengers [32]. In the present investigation, *B. subtilis* CBR05 had a net positive effect on the antioxidant activity measured by the DPPH and ABTS scavenging capacity, which seems to indicate that the bacteria acted as a regulator of the synthesis of antioxidant compounds in the plant (Figure 1). Strong scavenging of ion radical was exhibited by the inoculated tomato fruits, thus showing that *B. subtilis* CBR05 inoculation increased the radical scavenging capacity of tomato fruits. In a previous study, *B. licheniformis* inoculated plants had increased antioxidant profiles in tomato plants under greenhouse conditions [32]. Similar results concerned with enhanced fruit quality and marketable grade have been reported for other crops under the influence of PGPR [33] but this report is the first of its kind to specifically address PGPR strain, *B. subtilis* CBR05 in improvement of fruit quality. Moreover, PGPR enhances fruit characteristics based on the mediation of increased availability of nutrients to plants like phosphorous and iron, enhancing the nutritional status of the plants in the rhizosphere [33–35].

Phenolic contents perform an essential role in plant resistance and defense against phytopathogens, which are closely linked with reactive oxygen species (ROS). This study reveals that among the selected tomato fruits, PGPR strain, *B. subtilis* CBR05 inoculated tomato fruits had the highest amount of phenolics (Figure 2). Some phenolic compounds may prevent oxidative damage in vivo and thus protect against the development of the disease such as cardiac disease and cancer [1,2]. This might be considered as useful for health purposes. In addition, inoculation of *B. subtilis* significantly increased

flavonoid content compared to those of both control and market fruits. Similarly, increases in total flavonoids content by *B. licheniformis* have been reported for tomato fruits [32]. In our previous studies, we also found that *B. subtilis* CBR05 inoculation enhanced the accumulation of peroxidase and polyphenol oxidase enzymes, which are involved in the metabolism of phenols and flavonoids [22,23]. Hence, this shows that *B. subtilis* colonization induces resistance against biotic and abiotic stress agents. The results of antioxidant assays also revealed that tomatoes are a rich source of antioxidants, thus their habitual consumption can potentially help to combat the oxidative stress.

Regulation of carotenoid biosynthesis and high-accumulation lycopene during tomato fruit development is widely studied [36–38]. In the present study, we found a significantly higher amount of lycopene (All-E-lycopene) in *B. subtilis* CBR05 inoculated tomato fruits (Figures 3 and 4). Lycopene possesses the highest antioxidant potential among the carotenoids and several other antioxidants found in fruits and vegetables [39]. Thus, the addition of PGPR enhances lycopene content in tomato fruits and can potentially contribute to antioxidant levels of diets. This potent antioxidant activity of lycopene protects from a variety of ROS and reactive nitrogen species (RNS), thus helping in preventing chronic diseases in humans [31,36]. Similar to the lycopene contents, the DPPH and ABTS antioxidant activity of *B. subtilis* CBR05 inoculated tomato fruits was much higher than both the control and market fruits. Earlier, we also reported that defense-related enzymes in tomato after treatment with *B. subtilis* CBR05 efficiently combated *X. campestris* pv, *vesicotoria* [40] and induction of defense-related enzymes like superoxide dismutase, catalase, peroxidase, and polyphenol oxidase assessment revealed the up-regulation of glucanase and phenyl ammonia lyase indicating induced systemic resistance (ISR) in tomato. The earlier results established that the antioxidant capabilities of tomatoes are naturally present. Further, it was specifically proved that *B. subtilis* CBR05 mechanism of disease resistance against *X. campestris* pv, *vesicotoria* was confirmed for the involvement of the de novo pathway involved in Vitamin B6 biosynthesis [41]. In addition, known bacterial elicitors of ISR are microbial associated molecular patterns triggering immunity. When this fails, microbial effector-triggered immunity is induced and leads to programmed cell death. It increases the plant's systemic resistance to subsequent pathogen challenge by PGPR. Moreover, plant probiotic bacterium (PPB) could be used to reduce the use of chemicals (fertilizers, pesticides) in agriculture. This could lead to improved quality at reduced costs and could provide the basis for more sustainable agriculture [42]. Organic agriculture has been widely promoted and adopted to establish better sustainability in food production and crop protection. *Micromonospora* has been regarded as a PPB due to rhizobia helper bacteria properties in *Medicago sativa* L. [43]. Similarly, *Phyllobacterium* and *B. licheniformis* also show promising benefits for increasing vitamin C content across various functional foods and have been considered as PPB, devoid of economic loss [32,44].

In the present investigation, we extracted and quantified phenols, flavonoids, and carotenoids, in the mock-inoculated control, *B. subtilis* CBR05 inoculated plants and market fruits. Among them, PGPR strain, *B. subtilis* CBR05 inoculated tomato fruits were found to have the richest source of lycopene, total phenolics, and flavonoids contents. Additionally, the PGPR strain, *B. subtilis* CBR05 inoculated tomato fruits showed potent antioxidant activities. The significantly higher lycopene content and radical-quenching activity of PGPR strain, *B. subtilis* CBR05 inoculation confer tomato fruits with more nutritional value, and their consumption can minimize oxidative stress-mediated chronic diseases. Thus, biofertilizers based on PGPR may be a viable alternative to improve the nutraceutical quality of greenhouse-produced tomato fruits.

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