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In vitro Antioxidant and Cytoprotective Potentials of Postbiotic ProbioMETA G55

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Abstract: Studies have demonstrated the favorable beneficial impact of probiotic metabolites on the skin. However, the use of postbiotics for skincare is a relatively new field despite their high safety and stability, which guarantee a longer shelf life. The postbiotic ProbioMETA G55(G55) is a skincare raw material, but none of the studies have evaluated the antioxidant capacity of G55 in skin care products so far. Thus, this study aimed to investigate the antioxidant activity and cytoprotective effect of G55 using B16-F10 melanoma cells. The antioxidant activity of G55 was determined using the 2,2-diphenyl-1-picrylhydrazyl free radical method. Additionally, the effects of G55 on B16-F10 cytoprotection and cell viability were evaluated using the lactate dehydrogenase and WST-8 assays, respectively. Moreover, cell suspension pH, among other parameters, was analyzed using the i-Smart 300 analyzer. The results showed that G55 exhibited promising antioxidant activity and preserved B16-F10 cell viability. G55 also decreased melanin levels in B16-F10 cells. These findings imply that G55 is a skin-safe substance and antioxidant postbiotic that can be used in anti-aging, whitening, and other cosmetic formulations.

Keywords: Antioxidants, Cytoprotection, Microbial Fermentation Component, Postbiotics, ProbioMETA G55

Introduction

Postbiotics are, biologically active compounds produced by the fermentation of probiotics. They exhibit various important biological activities, including immunomodulatory, antimicrobial, anticancer, antioxidant, and anti-diabetic effects, and the ability to reduce food allergies (Aghebati-Maleki *et al*., 2021). Probiotic cosmetic products do not often include live bacteria, but rather bacterial lysates, extracts, or fermentation byproducts. These are referred to as postbiotics (Puebla-Barragan and Reid, 2021). Postbiotics are highly safe and stable owing to the absence of active microbes which prolongs shelf life. They also offer various health advantages (Ciardiello *et al*., 2020). For instance, in the ongoing COVID-19 pandemic, research has demonstrated that Lactobacillus plantarum Probio-88 could possibly be utilized as an integrated medicinal strategy in conjunction with the vaccine to slow down the amplification of the highly transmissible disease and its possible variants (Rather *et al*., 2021).

Microorganisms create extracellular metabolites as a byproduct of metabolic activity in a particular environment (Pinu and Villas-Boas, 2017). Metabolites, such as peptides, biosurfactants, fatty acids, exopolysaccharides, and vitamins are replacing conventional chemicals in several cosmetic formulations (Gupta *et al*., 2019). Bacterial metabolites, pieces of cell walls, and even dead bacteria cause the skin to mount an immunological response, which enhances the skin barrier function (Szántó *et al*., 2019).

Although probiotic metabolites beneficially impact the skin (Lew and Liong, 2013; Matsumoto *et al*., 2014), the application of postbiotics in skincare is still in its infancy (da Silva Vale *et al*., 2023). Probiotics form a protective barrier on the skin surface and prevent bacterial infections, aging, acne, and rosacea by healing connective tissues, improving cell regeneration, and reducing microbial growth via acidifying the skin environment. Thus, probiotics exert anti-aging effects, reduce dryness and improve skin clarity (Patil *et al*., 2020). For example, partially purified extracellular metabolite lactosporin obtained from the probiotic strain Bacillus coagulans MTCC 5856 has been previously investigated for potential skin-protective activity *in vitro*. This metabolite can potentially delay the onset of accelerated aging by exerting significant antioxidant activity, protecting

against UV-induced cell damage, and reducing collagenase activity (Majeed *et al*., 2020).

B16-F10 cell line, derived from the skin tissue of a melanoma-bearing mouse, is spindle-shaped and has epithelial cell-like morphology (Fidler, 1973). Melanin is produced by melanocytes via a process called melanogenesis. The melanin in skin, eyes, and hair colors is produced and distributed differently. Melanin is essential for shielding the skin from harmful substances, including UV rays, melanocyte-stimulating hormones, Reactive Oxygen Species (ROS), and 3-isobutyl-1-methylxanthine (Lee *et al*., 2018). Antioxidants and free radical scavengers lower melanin levels, thereby decreasing hyperpigmentation and oxidative stress-induced melanin synthesis (Kao *et al*., 2013).

The postbiotic ProbioMETA G55(G55), derived from patented strain *Lactobacillus brevis* J2K-55 (KCTC 13620BP), is a skincare raw material that helps soothe the skin and relieve stress. ProbioMETA G55 is made up of 98% Lactobacillus ferment lysate and 2% 1,2-Hexanediol. None of the research studies have evaluated the antioxidant and melanin-inhibiting potential of G55 so far. Given that antioxidants can inhibit melanogenesis (Panich *et al*., 2011), this study aimed to investigate the antioxidant activity and cytoprotective effect of G55 on B16-F10 melanoma cells. To the best of our knowledge, this is the first report on the antioxidant and cytoprotective activities of G55.

Materials and Methods

Materials

G55 was provided by J2KBIO Corporation (Cheongju, Korea). B16-F10 cells were procured from the Korean Cell Line Bank (Korean Cell Line Bank, Seoul, Korea). Dulbecco's Modified Eagle's Medium (DMEM) was obtained from Gibco BRL (Life Technologies, Grand Island, NY, USA). According to the manufacturer's instructions, the OxiTec™ 2,2-Diphenylpicrylhydrazyl (DPPH) Antioxidant Assay Kit (Biomax, Seoul, Korea) was used to evaluate the antioxidative effects of G55. The pH and partial carbon dioxide (pCO_2) levels in response to different G55 concentrations (1, 5, 10, 25, and 50 µL/mL) were measured using the i-Smart 300 gas analyzer (i-SENS, Seoul, Korea). A Quanti-LDHTM PLUS Cytotoxicity Assay Kit (Biomax) was used to analyze Lactate Dehydrogenase (LDH). The WST test (Biomax) was used to assess cell viability.

Instruments

We used Flex Station 3 Multi-Mode Microplate Reader (Molecular Devices Korea, Seoul, Korea) for antioxidant activity assay, cytotoxicity assay, and cell viability assay. I-Smart 300 gas analyzer (i-SENS, Seoul, Korea) was used for performing gas analysis.

Cell Culture

The experiment conducted in this study is shown in the schematic diagram (Fig. 1). B16F10 cells were used for *in vitro* experiments.

B16-F10 cells (Korean Cell Line Bank) were cultured for no more than 15 passages in DMEM supplemented with a 1% penicillin-streptomycin combination at 36°C in a humidified environment containing 5% CO_2.

G55 Antioxidant Activity Assay

First, we prepared various concentrations of G55 (1, 5, 10, 25, and 50 µL/mL).

DPPH, which is purple at 517 nm, changes color upon receiving electrons from antioxidants. Therefore, the DPPH free radical test was conducted to evaluate the antioxidant activity of G55 using 10 µg/mL vitamin C solution (vitC) as the positive control. Initially, 100 µL DPPH solution was mixed with 100 µL G55 in a 96-well plate and incubated for 30 min in the dark. Next, the absorbance of the mixture was measured using a Flex Station 3 Multi-Mode Microplate Reader (Molecular Devices Korea) at 517 nm; 200 µL DPPH solution was used as the standard. The DPPH radical-scavenging activity was determined using the following equation:

$$DPPH\ scavenging\ activity\,(\%) = [(DPPH\ absorbance - sample\ absorbance)/DPPH\ absorbance] \times 100\%$$

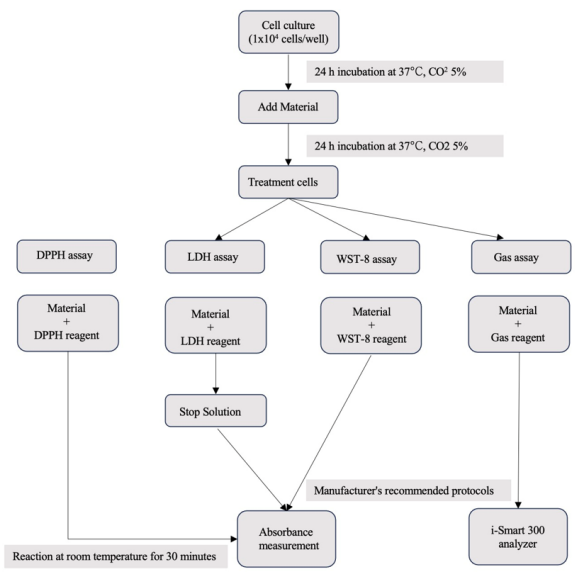

Fig. 1: Flow Chart of the Study. Cell: B16F10; Material: Material by concentration (G55)

G55 Gas Analysis

A suspension of B16-F10 cells (5×10^4 cells/mL), was prepared, plated into 96-well flat bottom plate (100 µL/well), and incubated for 24 h. Cells were then left untreated in a fresh medium (control) or treated with various concentrations of G55 (1, 5, 10, 25, and 50 µL/mL). For every concentration, 100 µL vitC (10 µg/mL), ethanol, or culture medium was added to the wells. At 0 or 24 h after incubation, 200 µL aliquots were obtained from each well and analyzed using a point-of-care gas analyzer i-Smart 300 (i-SENS) to measure pH and pCO_2.

G55 Cytotoxicity Assay (LDH Assay)

A suspension of B16-F10 cells (1×10^4 cells/mL) was prepared, seeded in 96-well microplates (50 µL/well), and incubated at 37°C under 5% CO_2 for 24 h. Next, the medium was replaced and 50 µL G55 (1, 5, 10, 25, and 50 µL/mL) was added into each well. Control groups were prepared for both the low and high G55 concentration groups. The cells were incubated in a CO_2 incubator at 37°C for 12 h. To measure G55 cytotoxicity, an LDH assay kit was used according to the instruction manual (Biomax). Briefly, 100 µL LDH reaction mixture was added into each well; 10 µL cell lysis solution was added to the positive control group. The contents of the microplate were incubated for 30 min in the dark at 27°C. Finally, 10 µL stop solution was added to each well and gently mixed before absorbance was measured at 490 nm using a Flex Station 3 Multi-Mode Microplate Reader (Molecular Devices Korea).

The following equation was used to determine LDH activity:

$$LDH\ release(\%) = (Absorbance\ test\ sample - low\ cont.) / (high\ cont. - low\ cont.) \times 100\%$$

G55 Cell Viability Assay (WST-8 Assay)

B16-F10 cells (1×10^4 cells/mL) treated with serum free DMEM containing 10 µL/mL G55 or the controls (vitC or ethanol [E. cont.]) were plated in 96-well flat-bottomed plates (10 µL/well) and incubated at 37°C under 5% CO_2 for 24 h. Cell viability was determined using the Quanti-MaxTM WST-8 cell viability test kit, as directed by the manufacturer. The plates were shaken gently after incubation in a CO_2 incubator for 24 h. Using a flex station 3 multi-mode microplate reader (Molecular Devices Korea), absorbance was measured at 450 nm.

The following equation was used to evaluate cell viability:

$$Viability(\%) = (Absorbance\ test\ sampple / E.cont.) \times 100$$

Statistical Analysis

The Analysis of Variance (ANOVA) was used for data analysis in the SPSS program. To determine statistical significance, one-way ANOVA with Duncan's multiple range test was used. Results were considered statistically significant at $p<0.05$.

Results

G55 Antioxidant Activity Assay (DPPH)

The DPPH free radical test was performed using different G55 concentrations (1, 5, 10, 25, and 50 µL/mL); 10 µg/mL vitC was considered a positive control treatment. The DPPH radical-scavenging activity was maximal (57%) at 50 µL/mL G55. The radical-scavenging activity of DPPH was comparable to that of vitC at 1 µL/mL and 50 µL/mL G55. The antioxidant capacities of the different G55 concentrations (1, 5, 10, 25, and 50 µL/mL) were comparable (49-57%) (Fig. 2). The potency of G55 in terms of antioxidant activity was inferior to that of vitC. However, the antioxidant activity of G55 was greater than the free radical-scavenging capacity of DPPH.

G55 Gas Analysis

According to the i-Smart 300 analysis, the pH of the control and experimental groups decreased after 24 h treatment with G55. The 25 µL/mL G55 group exhibited the highest decrease, with the pH dropping by 0.133 units (7.803-7.670), followed by the 5 µL/mL G55 group, with the pH dropping by 0.117 units (7.731-7.614). For vitC (10 µg/mL), pH decreased by 0.055 units (7.567-7.512).

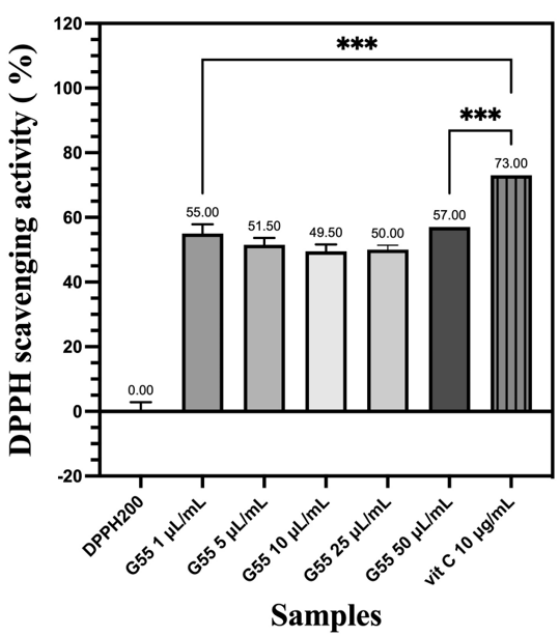

Fig. 2: Antioxidant activity of ProbioMETA G55. G55, ProbioMETA G55; vitC, vitamin C; DPPH200, 200 µL of 2,2-diphenyl-1-picrylhydrazyl

Table 1: Results of the precision analyses of the samples and controls

Parameters	Time (h)	G55 1 µL/mL	G55 5 µL/mL	G55 10 µL/mL	G55 25 µL/mL	G55 50 µL/mL	vitC 10 µg/mL
pH	0	7.653	7.731	7.704	7.803	7.941	7.567
	24	7.637	7.614	7.641	7.670	7.606	7.512
pCO_2 (mmHg)	0	17.200	14.700	18.600	11.300	6.000	14.900
	24	29.800	26.800	21.400	19.500	18.300	15.300

G55, ProbioMETA G55; vitC, vitamin C solution. pH and pCO_2, *Group 0 h vs. group 24 h; p = ns

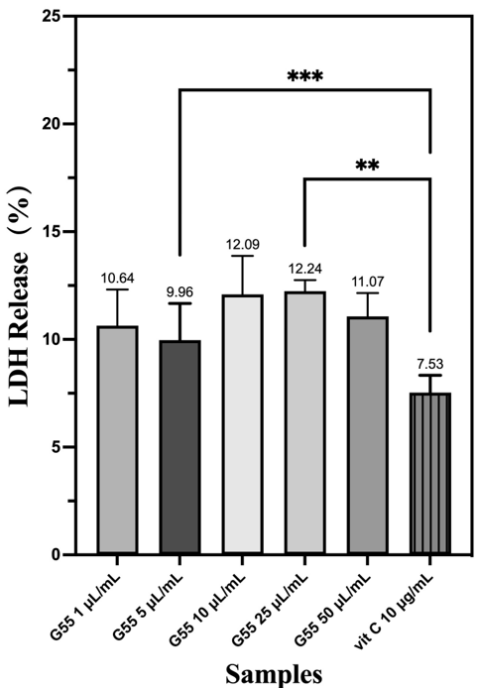

Fig. 3: Cytoprotective effect of G55 on B16-F10 cells. G55, ProbioMETA G55; vitC, vitamin C. ** p≤0.01, *** p≤0.001 vs vitC group

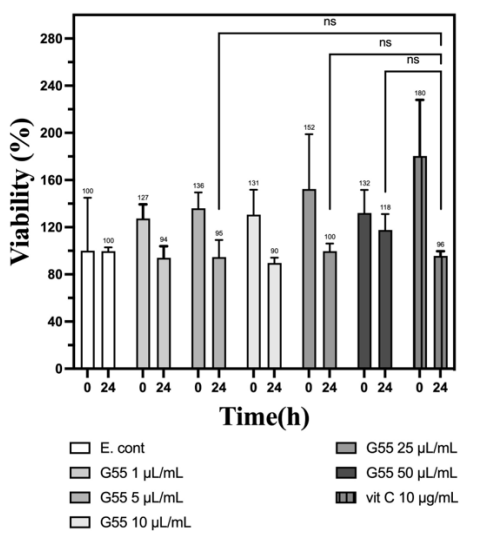

Fig. 4: Effects of various G55 concentrations on B16-F10 cell viability. E. cont (ethanol); G55, ProbioMETA G55; vitC, vitamin C solution

This was similar to that observed for 10 µL/mL G55, which too had the smallest decrease in pH, with a decrease of 0.063 units (7.704-7.641).

Moreover, all G55 samples exhibited increased pCO_2 levels over 24 h (Table 1); the 1 µL/mL G55 group exhibited the highest pCO_2 increase, with a difference of 12.6 mmHg between 0 and 24 h, and the 10 µL/mL G55 group the least increase, with a difference of 2.8 mmHg between 0 and 24 h.

G55 Cytotoxicity Assay (LDH Assay)

The cytotoxic effects of G55 were determined at various concentrations (1, 5, 10, 25, and 50 µL/mL) using B16-F10 cells. After 24 h incubation, 25 µL/mL G55 exerted a maximum cytotoxic effect on B16-F10 cells (12.09%). The cytotoxicity of 5 µL/mL G55 was just 9.96% after 24 h, indicating the highest survival rate for cells incubated with this concentration (Fig. 3). The cytotoxic effect differed significantly between 5 µL/mL and 25 µL/mL G55 and vitC (p≤0.01); G55 (5 µL/mL) and vitC showed comparable cytoprotective effects on B16-F10 cells; however, none of the experimental groups showed a higher cytoprotective effect than vitC.

G55 Cell Viability Assay (WST-8 Assay)

All tested G55 concentrations (1, 5, 10, 25, and 50 µL/mL) decreased the cell viability of B16-F10 cells to a similar extent. Among various groups, cells treated with 50 µL/mL G55 showed the highest viability (118%), followed by 25 µL/mL G55 (100% viability) and 10 µL/mL G55 (95% viability). However, the cell viability in response to vitC treatment decreased most rapidly after 24 h, from 180.08-95.78%, reaching 84.30% (Fig. 4). Although no significant difference was observed (p>0.05), 10, 25, and 50 µL/mL G55 exhibited stronger cytoprotective effects than vitC.

Discussion

There is a heightened interest in determining the possible applications of postbiotics due to their various beneficial effects (Duarte *et al*. 2022). ROS are free radicals that are generated in various cell types and are a crucial factor in skin aging. Increased free radical production can cause wrinkles, photoaging, elastosis, dryness, and pigmentation of the skin (Juan *et al*., 2021). Postbiotic extracts are a rich source of antioxidants, which can help reduce intracellular oxidative stress and delay the skin aging process. Therefore, postbiotic extracts may be used to treat and prevent skin aging (Cao *et al*., 2020).

A previous study reported that *Limosilactobacillus fermentum* JNU532 can scavenge DPPH by approximately 60% (Meng and Oh, 2021). This is comparable to the results of 50 µL/mL G55 in the present study, where the free radical-scavenging rate was approximately 57%. Plant extracts containing natural antioxidants, such as polyphenols and terpenes, are commonly incorporated into cosmetic formulations to act as topical antioxidants. These antioxidants act by removing free radical intermediates and suppressing other oxidation reactions (Ribeiro *et al*., 2015). Vitamin C is an effective natural antioxidant (Carocho and Ferreira, 2013). Therefore, it is widely used in skincare products to help reduce free radical damage and protect the skin. Our study provides evidence that G55 exerts remarkable antioxidative effects and is a potential skin-protecting agent that can be used in topical skincare formulations.

The i-Smart 300E analyzer provides precise measurements for the pH, PCO_2, and electrolytes (Lee and Park, 2022). We performed a gas analysis of G55 using i-Smart 300. The pH alterations of B16-F10 cells and pCO_2 levels were also determined. Melanin is produced in melanosomes in an acidic environment. The difference in pH between melanosomes and culture medium is largely responsible for the former's ability to quench the fluorescence of 9-aminoacridine. A decrease in pH or increase in the salt concentration precipitates melanin, which partially or completely quenches the fluorescence (Bhatnagar *et al*., 1993). Tyrosinase activity and melanin concentration are markedly reduced in melanocytes treated with vitC or its derivatives, magnesium ascorbyl phosphate, and 3-O-ethyl-L-ascorbic acid via cytoplasmic acidification (Miao *et al*., 2019). Lowering intramelanosomal pH promotes tyrosinase breakdown (Zeng *et al*., 2017).

In our study, the pH in the G55 experimental groups decreased between 0 and 24 h, and G55 inhibited tyrosinase activity in the cytoplasm of the B16-F10 cells. A decrease in pH inhibited melanin production in melanocytes at 0 h and after 24 h. Lowered pH leads to acidosis and elevated p CO_2 is a manifestation of acidosis (Rani, 2022; Martín-Rodríguez *et al*., 2023). In our study, pCO_2 levels rose to varying degrees after 24 h. Thus, our results collectively show, that G55 can decrease melanin levels in B16-F10 cells.

Studies have revealed that cell-free supernatants of *Lactobacillus griseus* BNR17 exhibit antioxidant properties by inducing the expression of genes encoding various antioxidants. These supernatants also inhibit melanin production (Lee *et al*., 2022). This dual effect of inducing antioxidant gene expression and reducing melanin production provides evidence.

Cytoprotective assays offer a quick approach to identifying natural and artificial compounds with possible anticancer properties (de Oliveira *et al*., 2013). The cytotoxic effects of plants on melanoma cells have been documented *in vitro* as well as *in vivo* (Spera *et al*., 2019). For example, fermented maca root extract inhibits free radicals to exert antioxidant effects and exhibits a high rate of tyrosinase inhibition, thereby inhibiting melanin production. Moreover, the viability of B16-F10 cells was >95% after 24 h treatment. This indicates the biosafety of fermented maca extracts (Yang *et al*., 2023) and is consistent with that of G55 at various concentrations (1, 5, 10, 25, and 50 µL/mL); in our study, B16-F10 cell viability was >90% after 24 h treatment with various concentrations of G55. Although G55 and vitC exhibited similar cytoprotective effects, G55 led to higher cell viability than vitC. Taken together, these observations suggest that G55 with its high antioxidant properties can be used in formulations focused on mitigating the effects of skin aging and wrinkle formation.

However, this study had certain limitations. First, only the DPPH assay was used to assess the antioxidant effect of G55 and the results should be validated using different methods. Second, we tested a limited range of the G55 concentrations and there is a need to determine, whether even lower G55 concentrations may exhibit clinically beneficial effects on the skin.

Future research should focus on evaluating more concentrations of G55 for their potential skin-protecting effects. Alternatively, G55 can also be used in skin soothing and moisturizing cosmetic products that require high safety levels, such as skin care products for children. Although G55 may also be used in skin whitening cosmetics, this should be investigated in future studies. Moreover, the optimal G55 concentrations for use in clinical formulations and their targeted molecular pathways remain to be elucidated.

Conclusion

G55 is a potential agent for use in anti-aging, anti-wrinkle, and other cosmetic formulations. G55 exhibits antioxidant and cytoprotective properties via mitigating oxidative stress and inhibiting melanin production by regulating pH. Therefore, G55 is a potentially valuable ingredient for developing clinically effective skin care as well as whitening products. Further studies are warranted to determine the clinically optimal G55 concentration and the targeted molecular pathways by which these beneficial effects of G55 are exerted.

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Author's Contributions

Xinyi Song and Ga Yeon Kim: Made substantial contributions to the conception and design of the study. These authors contributed equally to this study.

Young Ki Lee: Contributed to data collection and analysis.

Jae Kyung Kim: Contributed to the acquisition and analysis of data.

Ethics

The Institutional Review Board at Dankook University approved this study (IRB FILE No. DUK NON2022-002-002).

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