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Research paper

Strain-Specific Modulation of Oxidative Stress, Carbohydrate Metabolism and Adipogenesis by Probiotic *Bacillus* spp. in 3T3-L1 cells

Zayeem Firoz Hussain [1], Sujan Antony [1], ShyamuKaverappa [1], Akhina Tom [2], Shreya Shivashankara [2], Shreya Shreya [2], Varadharajan Thiyagarajan [3], Priyashree Kumar [3], Vidya ShivamoggaMuddappa [4], Manjunatha Bukkambudi Krishnaswamy [1 *], FirozHirehal HussainMirza [2], Sadashiva ChannangihalliThimmegowda [2 *]

[1] Department of Biotechnology, Oxford College of Engineering, Bangalore, Karnataka, India.
[2] R&D Department, Star Hi Herbs Pvt. Ltd., Jigani,Bangalore, Karnataka, India.
[3] Department of Cell and Molecular Biology, Radiant Research, Peenya, Bengaluru, Karnataka 560058
[4] NMAM Institute of Technology (NMAMIT) Department of Biotechnology Engineering, Nitte 574110, Karnataka, India
**Corresponding author E-mail: research@starhiherbs.com*

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Abstract

Background: Abnormal adipocyte growth, marked by increased cell number and differentiation, is a significant pathological aspect of obesity. Thus, inhibiting adipocyte differentiation is a potential approach for obesity prevention. Recent studies indicate that specific probiotic strains can influence lipid metabolism both *in-vitro* and *in-vivo*. In the present study, the cytotoxic, antioxidant, and anti-adipogenic potential of four *Bacillus* strains - *B.coagulans, B.subtilis, B.clausii*, and *B.paralicheniformis* was investigated.
Methods: Anti-adipogenic activity was tested in 3T3-L1 preadipocyte cells through treatment with selected strains during differentiation. Cytotoxicity was measured using the MTT assay to evaluate cellular metabolic activity. Antioxidant activity of *Bacillus* strains was assessed *in-vitro* via DPPH scavenging, and anti-adipogenic protein expression was analyzed using the ELISA assay. Additionally, the strains' inhibitory effects on carbohydrate digestion and starch hydrolysis were tested through α-amylase and α-glucosidase assays.
Results: All tested strains demonstrated anti-adipogenic potential by reducing lipid accumulation in 3T3-L1 cells, as evidenced by Oil Red O staining. The results revealed that all *Bacillus* strains exhibited a comparable reduction in lipid accumulation relative to MDI-induced control cells. The modulation of adipogenic differentiation was further confirmed by the PPAR-γ assay, which demonstrated that all strains influenced adipogenesis-related protein expression. Among the tested *Bacillus* strains, *B.paralicheniformis* and *B.subtilis* exhibited the strongest anti-adipogenic effects, while *B.coagulans* showed moderate activity. *B.clausii* had the least anti-obesity potential. Additionally, all strains demonstrated significant free radical scavenging activity, indicating their antioxidant potential.
Conclusion: These findings suggest that certain *Bacillus* probiotic strains could be effective functional ingredients for addressing obesity and related metabolic disorders.

Keywords:*Alpha-Amylase; Alpha-Glucosidase; Adipogenic Differentiation; Probiotics; 3T3-L1 Cells.*

1. Introduction

Obesity is a complicated and long-term metabolic condition marked by the excessive buildup of fat tissue due to a persistent imbalance between energy consumption and expenditure. Its substantial correlation with type 2 diabetes mellitus, dyslipidemia, insulin resistance, cardiovascular disease, and other obesity-related comorbidities has made it one of the most significant global public health concerns. Adipogenesis, the process by which preadipocytes differentiate into mature adipocytes and hypertrophy of already-existing adipocytes both contribute to the growth of adipose tissue at the cellular level [1].
Probiotics are thought to be living, non-pathogenic bacteria that can improve the balance of microorganisms in the human gut, favoring the colonization of beneficial microorganisms. They serve as prophylactics for humans and aquaculture, growth promoters in feed additives, functional foods and dietary supplements, and antibiotic substitutes in animal husbandry [2]. The *Bacillus* genus possesses enzymatic capacity and possibly valuable metabolic compounds. *Bacillus* species are known for their spore production, which prevents them from passing through the rumen and allows them to germinate in various pH environments. They can colonize the intestines, in contrast to other transient probiotics [3]. By secreting antibiotic compounds, these bacteria can directly and indirectly prevent the growth of potentially dangerous microbes by competitive exclusion [4], [5]. Furthermore, bovine intestinal epithelial cells' toll-like receptor 3, which triggers innate immune responses, allows immune system stimulation by *Bacillus* species [6]. Additionally, by increasing food digestibil-

ity [7] and encouraging intestinal eubiosis and secreting short-chain fatty acids, they may aid in the colonization of beneficial bacteria [5]. Unlike the more common probiotic species, they are resistant microbes that generate endospores and can thrive in a range of food products [8]. It has been demonstrated that certain strains of rod-shaped, Gram-positive, catalase-positive, spore-forming *Bacilli*, such as *B. laterosporus, B. subtilis, B. clausii, B. paralicheniformis, B. cereus, B. coagulans,* and *B. pumilus*, have probiotic qualities [9].
The rod-shaped, endospore-forming, Gram-positive bacteria *B. coagulans, B. clausii, B. subtilis*, and *B. paralicheniformis* are frequently utilized in industries and as probiotics. Their spores may effectively survive and colonize the gut due to their great tolerance to heat, acidity, and gastrointestinal conditions. These species are significant possibilities in probiotic research and medicinal applications since they have been shown to promote gut microbial balance, boost immunological responses, and improve digestive health [10], [11].They can withstand rigorous processing conditions and the acidic GI environment because of their capacity to produce extremely resistant spores. Species of *Bacillus* are known to produce a diverse array of extracellular enzymes, antimicrobial substances, and bioactive metabolites, which help maintain gut microbial balance, enhance nutrient digestion, and promote overall host health [12], [13]. *B. subtilis* is thought to synthesize vitamin K2, boost the immune system, and have anti-cancer qualities [14].
Probiotics have been widely studied for their potential benefits in biotechnology, food science, and health-related research. *Bacillus* species have drawn a lot of interest from the food, feed, and pharmaceutical industries due to their safety profile and functional versatility. Therefore, the purpose of this study is to evaluate the biological activity of *B. coagulans, B. clausii, B. subtilis*, and *B. paralicheniformis in vitro*.

2. Materials and Methods

2.1. Preparation of sample

Probiotic strains of *Bacillus* species, including *B. coagulans*(MTCC 25308), *B. subtilis*(MTCC 25840), *B. clausii*(MTCC 25605), and *B. paralicheniformis*(MTCC 13272), were obtained from Star Hi Herbs Pvt. Ltd. (India). Spray-dried powder of *B. coagulans, B. subtilis, B. clausii*, and *B. paralicheniformis* with potencies of 390×10^9 CFU/g, 230×10^9 CFU/g, 400×10^9 CFU/g, and 390×10^9 CFU/g, respectively, were used in this study. The cultures were kept under conventional microbiological conditions and activated prior to experimental use. The powdered samples were added into selective broth and incubated in the 18-24hrs range. Following incubation, the samples were subjected to centrifugation at 6000 rpm at 4ºC, and the resulting supernatant was collected after filtration for further analysis.

2.2. Chemical and reagents

All the chemicals and reagents such as PBS, DMEM, antibiotics and biochemical assay reagents were purchased from Sigma or GIBCO (USA).

2.3. Cell line and cell culture

3T3-L1 preadipocytes (3T3-L1 cells) were procured from the National Centre for Cell Science, Pune. 3T3-L1 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum under standard conditions (37 °C, 5% CO_2). Upon reaching confluence, cells were maintained for an additional 48 h to achieve growth arrest. Adipogenic differentiation was initiated by treating the cells with the induction medium containing 167 nM insulin, 0.5 M isobutylmethylxanthine, and 1 µM dexamethasone for 2 days. This was followed by incubation in DMEM supplemented with 10% FBS and 167 nM insulin for another 2 days. Subsequently, cells were maintained in DMEM with 10% FBS for an additional 4 days to allow full maturation. At the end of the differentiation period, approximately 90% of cells exhibited lipid droplet accumulation, indicating successful adipocyte formation.

2.4. Cytotoxicity assay

The cytotoxic effects of the selected probiotic strains were evaluated in 3T3-L1 cells using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay. Cells were seeded into 12-well plates at a density of 5×10^4 cells per well in 1 mL of DMEM supplemented with 10% fetal bovine serum and allowed to attach for 24 h. Following this, cells were treated with different concentrations of probiotic strains (0–1000 µg/mL) and incubated for an additional 48 h. After treatment, the medium was removed and replaced with fresh medium containing MTT reagent (final volume ratio 4:1; medium: MTT, sourced from Sigma-Aldrich). The plates were incubated for 4 h at 37 °C in a humidified 5% CO_2 atmosphere, protected from light to facilitate the formation of formazan crystals. Subsequently, the supernatant was carefully discarded, and cells were gently washed with phosphate-buffered saline (PBS). The insoluble formazan product was then solubilized using 1 mL of dimethyl sulfoxide (DMSO) per well. The absorbance was recorded at 570 nm using a microplate reader (Thermo Fisher Scientific), and cell viability was expressed relative to untreated controls [15].

$$\text{Cell viability (\%)} = \frac{\text{Absorbance of sample}}{\text{Absorbance of control}} \times 100 \quad (1)$$

2.5. Antioxidant activity using DPPH assay

The free radical scavenging potential of the samples was evaluated using the DPPH (2,2-diphenyl-1-picrylhydrazyl) assay. In brief, 2 mL of fermentation supernatant or bacterial suspension was combined with 2 mL of DPPH solution (0.2 mmol/L prepared in methanol). The reaction mixtures were incubated at 37 °C for 30 min under dark conditions to prevent photodegradation. Following incubation, the reduction in DPPH was monitored by measuring the absorbance at 517 nm using a spectrophotometer [16].
The radical scavenging activity was calculated using the following equation:

$$IC_{50} = 1 - \frac{(A_1 - A_0)}{(A_c)} \times 100 \quad (2)$$

Where A_1 denotes the absorbance of the sample mixed with DPPH solution, A_0 represents the absorbance of the sample without DPPH (blank), and A_c corresponds to the absorbance of the control containing DPPH solution with deionized water.

2.6. Anti-adipogenesis activity

Intracellular lipid accumulation in differentiated 3T3-L1 cells was assessed using Oil Red O staining. After aspirating the culture medium, cells were gently rinsed twice with phosphate-buffered saline (PBS, pH 7.4). The cells were then fixed with 3.7% formaldehyde (1 mL per well) for 15 min at room temperature. Following fixation, the fixative was removed, and the cells were washed three times with PBS. A working solution of Oil Red O was prepared by diluting the stock solution with distilled water in a 6:4 ratio and filtering it through a 0.2 µm membrane to remove precipitates. Subsequently, 500 µL of the staining solution was added to each well and incubated for 30 min at room temperature. Excess stain was removed by washing the cells three times with PBS. For quantitative analysis, the retained dye was eluted with 100% isopropanol, and the absorbance was recorded at 540 nm using a microplate reader [1].

2.7. *In vitro* activities of α-amylase

The inhibitory effect of the samples on α-amylase activity was evaluated using a modified colorimetric assay based on the hydrolysis of p-nitrophenyl α-D-maltohexaoside (pNPM, 5 mmol/L) as the substrate. Briefly, 50 µL of pNPM solution was mixed with 50 µL of the test sample prepared in 0.02 M sodium phosphate buffer (pH 6.9) at the desired concentrations. Subsequently, 50 µL of porcine pancreatic α-amylase (PPA; 50 mg/mL) dissolved in the same buffer was added to initiate the reaction. The reaction mixture was incubated at 37 °C for 30 min. Enzymatic activity was determined by measuring the release of p-nitrophenol, which was quantified by recording the absorbance at 405 nm using a microplate reader [17].

2.8. α - Glucosidase inhibitory activity

The inhibitory activity of the samples against α-glucosidase was evaluated using a modified colorimetric assay based on p-nitrophenyl glycoside hydrolysis. The spray-dried samples were prepared over a range of concentrations (0–20 mg/mL) in 50 mM Tris–HCl buffer (pH 8.2). Alternatively, samples were dissolved in 0.02 M sodium phosphate buffer (pH 6.9) at concentrations of 0–40 mg/mL. For the assay, 50 µL of the sample solution was added to each well of a 96-well plate, followed by 50 µL of substrate solution containing p-nitrophenyl glycoside (10 mM) prepared in 0.1 M sodium phosphate buffer (pH 6.9). The reaction was initiated by adding 50 µL of α-glucosidase enzyme solution (0.2 U/mL). The plate was incubated at 37 °C for 30 min, after which the enzymatic release of p-nitrophenol was determined by measuring absorbance at 405 nm using a microplate reader (Thermo Fisher Scientific). Acarbose was included as a positive control and tested under identical conditions [17].

2.9. The protein PPAR-γcontent detection

The expression level of PPAR gamma in differentiated 3T3-L1 cells was quantified using a commercially available ELISA kit, following the manufacturer's instructions. Briefly, differentiated adipocytes were harvested and resuspended in phosphate-buffered saline (PBS). Cell lysis was achieved through repeated freeze–thaw cycles to release intracellular contents. For the assay, 50 µL of the prepared samples at different concentrations was added to each well of a 96-well plate, followed by 100 µL of enzyme-linked detection reagent. The plate was incubated at 37 °C for 60 min. After incubation, the wells were emptied and washed twice using the provided wash buffer to remove unbound components. Subsequently, 100 µL of chromogenic substrates (solutions A and B) were added to each well, and the plate was incubated for 15 min at 37 °C in the dark to allow colour development. The reaction was terminated by adding 50 µL of stop solution. Absorbance was measured at 450 nm using an ELISA microplate reader, and the PPARγ levels were calculated based on the standard curve [18].

2.10. Statistical analysis

Statistical comparisons among experimental groups were carried out using one-way analysis of variance (ANOVA). All data were processed using GraphPad Prism and are presented as mean ± standard deviation (SD). A p-value less than 0.05 was considered to indicate statistical significance.

3. Results

3.1. Cytotoxicity and cell viability on 3T3L1 cell line

The cytotoxic effects of all the *Bacillus* strains on the 3T3-L1 cell line, following treatment with different concentrations, are graphically presented in Fig. 1. The 3T3-L1 cell line was used to study the effects of *Bacillus* strains. The results showed that *B. clausii* and *B. coagulans* exhibited higher cell viability among the four tested strains. In contrast, *B. subtilis* and *B. paralicheniformis* showed comparatively reduced cell viability. These findings suggest that the tested *Bacillus* species are generally safe at the cellular level, potentially exhibit cytoprotective effects at lower concentrations, and are not associated with significant cytotoxicity under the experimental conditions.

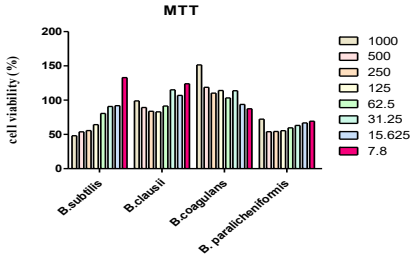

Fig. 1: Effect of different concentrations of *Bacillus* species in 3T3L1 cell line for cell viability. Results are expressed as Mean±SD of the mean (n=3)

3.2. Anti-adipogenic activity of *Bacillus* species

All *Bacillus* species were tested for their anti-adipogenic properties during the 3T3L1 cell line differentiation. Oil Red O staining is a direct indicator of adipogenesis and offers a quantitative assessment of intracellular neutral lipid deposition such as triglycerides and cholesteryl esters. The results showed that all the *Bacillus* strains displayed a comparable reduction in oil red O staining compared to MDI-induced controls. *B. paralicheniformis* (3.9 µg/mL & 7.8 µg/mL) and *B. subtilis* (62.5 µg/mL) showed a significant reduction in color intensity and absorbance over MDI as compared to other strains. Furthermore, *B. paralicheniformis* (3.9 µg/mL & 7.8 µg/mL) and *B. subtilis* (62.5 µg/mL) exhibit significant percentage inhibition of 25.72%, 26.87%, and 25.71%, respectively. These observations reveal that *Bacillus* strains possess effective anti-adipogenic effects on the 3T3L1 cell line (Fig. 2.).

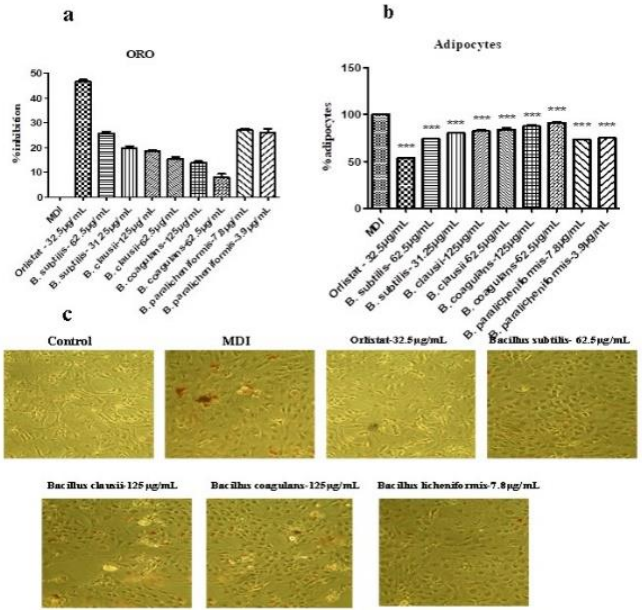

Fig. 2: Oil red O staining. A) Percentage inhibition of lipid accumulation on 3T3L1 cell line. B) Percentage of adipocytes over MDI control group on 3T3L1 cell line. C) Photograph of oil red O staining. The cells were stained with oil red O and observed with a microscope (original magnification ×200) compared with control. Results are expressed as Mean ± SD of the mean (n=3). Statistical significances of adipocytes are compared between MDI versus treatment group (*** P Value < 0.001).

3.3. Alpha-amylase inhibitory activity of *Bacillus* species

All four *Bacillus* strains were evaluated for their possible α-amylase inhibitory activities, and the percentage inhibition is shown in Fig. 3a. Acarbose is a potent α-amylase inhibitor and has been utilized as a known positive control in this study and showed an IC_{50} of 125.81 µg/mL. The study shows that *B. paralicheniformis* (7.8 µg/mL & 3.9 µg/mL) exhibits significant α-amylase inhibition of 28.51% and 22.24%, respectively. Also, *B. subtilis* at a concentration of 62.5 µg/mL showed significant inhibition of 25.18%. *B. coagulans* shows comparable inhibition towards the α-amylase enzyme. *B. clausii* did not show any marked response to inhibition. Hence, the study reveals that *B. paralicheniformis* exhibits a potent inhibition for amylolytic enzymes, and a similar response was observed in *B. subtilis*.

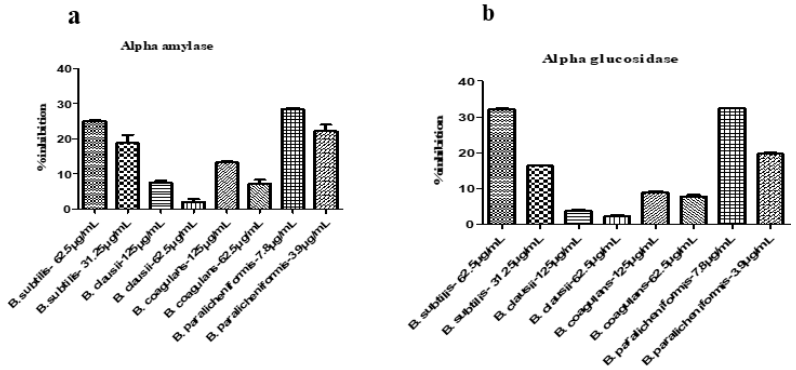

Fig. 3: a) Inhibitory alpha amylase activity of *Bacillus* species. b) Inhibitory alpha glucosidase activity of *Bacillus* species. Results are expressed as Mean ± SD of the mean (n=3)

3.4. Alpha-glucosidase inhibitory activity of *Bacillus* species

The ability of *Bacillus* species to block α-glucosidase was assessed. Acarbose is the standard drug used in this study, which is particularly efficient against the glucoamylase enzyme. Among the *Bacillus* species, *B. paralicheniformis* (7.8 µg/mL) and *B. subtilis* (62.5 µg/mL) exhibit higher inhibitory action against the α-glucosidase enzyme. Both showed a significant effect compared against standard acarbose at 62.5 µg/mL.*B. clausii* and *B. coagulans* did not show any marked effect on inhibitory action. Hence, the study reveals that *B. paralicheniformis* and *B. subtilis* possess effective α-glucosidase inhibitory action (Fig. 3b.).

3.5. Inhibition on PPAR- γexpression

The result shows the effect of *Bacillus* species against PPARγ expression in 3T3-L1 adipocytes. *B. paralicheniformis* and *B. subtilis* exhibit significant inhibitory action on PPARγ expression. Also, *B. clausii* and *B. coagulans* showed comparable PPARγ inhibition. When compared with the standard drug Orlistat, *B. paralicheniforms* and *B. subtilis* showed substantial antagonist activity. Hence, the study reveals that *B. paralicheniformis* and *B. subtilis* significantly downregulated PPARγ expression, indicating a potential inhibitory effect on adipocyte differentiation (Fig. 4.).

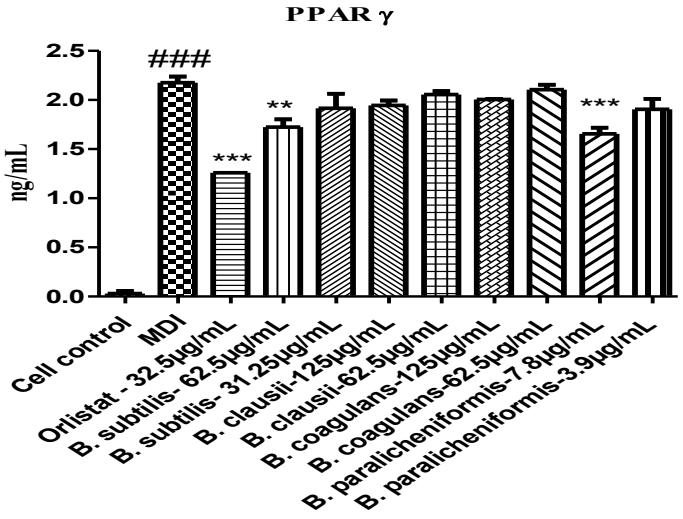

Fig. 4: PPAR gamma expression. Results are expressed as Mean±SD of the mean (n=3)**.** Statistical significances are compared between Control with MDI group ($^{###}$ P Value < 0.001). Statistical significances are compared between MDI versus treatment group (** P Value < 0.01, *** P Value < 0.001).

3.6. Antioxidant activity of probiotic strains

The DPPH radical scavenging assay was used to assess the probiotic strains' antioxidant capacity. The results demonstrated a considerable difference in antioxidant activity compared to the standard, ascorbic acid, as shown in Fig. 5. The *Bacillus* strains showed a notable dose-dependent and gradual increase in DPPH scavenging as the concentration increases from 10 to 100 µg/ml. Among the probiotic strains, *B. coagulans* exhibited the most potent antioxidant activity, with the lowest IC_{50} value (54.19 µg/mL), followed by *B. subtilis* (63.2 µg/mL), *B. clausii* (67.3 µg/mL), and *B. paralicheniformis* (69.6 µg/mL) as compared to the IC_{50} value of ascorbic acid (21.97 µg/mL). All probiotic strains demonstrated free radical scavenging activity, suggesting their potential as effective probiotics with significant antioxidant properties. Although *Bacillus* strains have lower antioxidant activity than ascorbic acid, they nonetheless exhibited significant, dose-dependent radical scavenging capabilities.

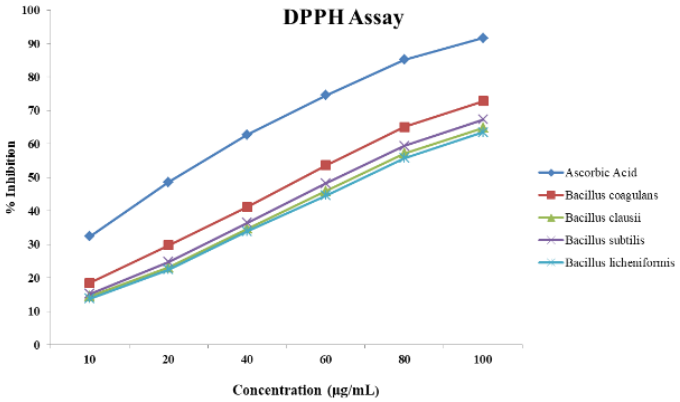

Fig. 5: Antioxidant activity and IC_{50} value of *Bacillus coagulans, Bacillus clausii, Bacillus subtilis,* and *Bacillus licheniformis*, compared with standard Ascorbic Acid.

4. Discussion

Probiotics confer numerous health benefits, including enhancing nutrient bioavailability, modulating the immune system, and reducing insulin intolerance [19], [16]. Probiotics have been suggested as a possible treatment approach for metabolic ailments such as obesity and nonalcoholic fatty liver disease (NAFLD) [20]. In addition to lactic acid bacteria, which have well-established scientific and commercial utility [21]. *Bacillus* species have drawn interest as possible probiotic strains and have been marketed as various health supplements [12]. While recent research has suggested that supplements containing *Bacillus* can alleviate metabolic dysfunctions [22], the mechanisms behind these improvements are still unclear. Probiotics are typically regarded as safe, but new formulations must undergo testing for adverse effects and toxicity. *In vitro* toxicity studies play an essential role in assessing the safety of these probiotic formulations [23].

Recent studies indicate that adipogenesis is governed by transcription factors like C/EBPα, PPARγ, and SREBP-1c, with the MAPK family, especially ERK, playing a crucial role in preadipocyte differentiation. Activated ERK enhances adipogenic transcription factors, while CREB phosphorylation by P-AKT activates C/EBPβ, promoting C/EBPα and PPARγ expression [24]. AMPK phosphorylation leads to ACC inactivation, reducing lipogenesis through suppression of SREBP-1c, FABP4, and FAS [25]. Additionally, adipose tissue secretes ADP, whose expression diminishes with weight gain. ADP may promote adipogenesis by increasing cAMP levels, and it has beneficial effects on obesity and metabolic syndromes, such as regulating adipose tissue expansion, lowering inflammation, and improving insulin sensitivity [26].In addition, obesity is linked to elevated levels of oxidative stress as a result of the overproduction of reactive oxygen species (ROS) and the malfunction of antioxidant systems [27]. By oxidatively damaging cellular membranes, proteins, and DNA, excessive ROS in obesity worsens adipogenic dysfunction. ROS further disrupts lipid homeostasis and adipocyte development by impairing important transcriptional regulators such as PPARγ. A feedback loop is created by this oxidative imbalance, which exacerbates metabolic instability and adipocyte dysfunction [28]. Adipogenesis inhibitors and anti-obesity drugs work by boosting energy expenditure, decreasing fat accumulation and focusing on pre-adipocyte development. This is mostly accomplished by activating cellular energy pathways (like AMPK) and down-regulating important adipogenic transcription factors (including PPARγ and C/EBPα) [29].

Alpha-glucosidase and alpha-amylase are enzymes that digest carbohydrates, contributing to hyperglycemia and fat storage in obese individuals [30]. Inhibiting α-glucosidase is effective in lowering blood glucose levels by delaying digestion, which helps prevent late diabetic complications [31], [32]. Similarly, blocking α-amylase reduces the risk of obesity and hyperglycemia by slowing the digestion of carbohydrates and the absorption of glucose [33]. Bacteriocins, organic acids, short-chain fatty acids (SCFAs), and peptides are among the bioactive substances that probiotics like *B. coagulans* can produce. These substances may impede the activity of α-amylase and α-glucosidase. The functional activity of the enzymes may be reduced as a result of these metabolites' direct interactions with them [34]. In our study, *B. paralicheniformis* and *B. subtilis* exhibit excellent inhibition on α-amylase and α-glucosidase enzymes. *B. coagulans* and *B. clausii* showed minimal inhibitory action. Hence, the results indicate that these inhibitions aid in controlling obesity-related pathways by reducing the amount of energy available for adipogenesis.

PPARγ is a key regulator of adipogenesis [35], playing a prominent role in the metabolism of lipids and glucose, as it promotes adipocyte differentiation, supports energy balance, enhances insulin sensitivity, and aids glucose utilization [36], [37]. PPARγ is considered to control the early stages of preadipocyte development in mammalian cells [38]. Previous research demonstrated that a variety of natural products could block differentiation and lipid buildup in 3T3-L1 adipocytes by down-regulating PPARγ's mRNA and protein levels [39]. Our study reveals that *B. paralicheniformis* and *B. subtilis* significantly inhibit PPARγ expression, showing substantial antagonist activity compared to the standard drug orlistat. Additionally, *B. clausii* and *B. coagulans* exhibited comparable inhibition. The findings suggest a potential inhibitory effect of these *Bacillus* species on adipocyte differentiation by downregulating PPARγ expression.

In cytotoxicity assay, all the *Bacillus* species showed cytoprotective activity at lower concentrations. *B. coagulans* and *B. clausii* showed <95% cell viability; in particular, *B. coagulans* possesses potential proliferation with enhanced viability. *B. subtilis* and *B. paralicheniformis* showed moderate cell viability on the 3T3L1 cell line. Hence, the result showed that all the *Bacillus* species possess cytoprotective action in the fibroblast cell line at lower concentrations. The antioxidant activity of *B.coagulans*, *B. subtilis*, *B.clausii*, and *B.paralicheniformis* was evaluated using the DPPH radical scavenging assay. All strains demonstrated measurable free radical scavenging activity, indicating their capacity to reduce oxidative stress. Relatively higher activity was observed in *B. subtilis* and *B.paralicheniformis*, whereas *B.coagulans* and *B.clausii* showed moderate effects. This activity is likely associated with the production of antioxidant metabolites and enzymes such as superoxide dismutase and catalase [40]. These findings suggest that *Bacillus* species possess functional antioxidant potential and could be beneficial to health as probiotics.

5. Conclusion

The present study provides a comprehensive evaluation of the probiotic potential of novel strains of *B. coagulans* (25308), *B. subtilis* (25840), *B. clausii* (25605), and *B. paralicheniformis* (13272) using *in vitro* cell-based models to assess cytotoxicity and anti-obesity activity. The results demonstrated that these *Bacillus* species effectively inhibited the differentiation of 3T3-L1 pre-adipocytes via inhibiting the PPARγ pathway, thereby exhibiting significant anti-adipogenic potential. Furthermore, all strains showed notable cytoprotective and antioxidant activities. Among the tested strains, *B. paralicheniformis* (13272) exhibited the most pronounced anti-adipogenic effect by significantly reducing the degree of adipocyte differentiation. It also demonstrated a potent inhibitory effect against the enzymes α-amylase and α-glucosidase, which may contribute to reduced carbohydrate digestion and glucose absorption. Overall, these findings suggest that the studied *Bacillus* strains possess promising probiotic attributes and could be considered as potential functional dietary supplements or therapeutic agents for the management of obesity by effectively inhibiting preadipocyte differentiation. Further *in vivo* and clinical studies are needed to investigate the possible therapy of *Bacillus* strains. More research is needed to determine the *Bacillus* strain's anti-obesity efficacy via interfering with adipocyte formation, as this could be a useful probiotic for generating multifunctional therapeutic agents.

Ethical Approval

Not Applicable.

Author Contributions

Conceptualization: FHM; Data curation: VT, PK, SCT, AT; Formal analysis: SCT, VT, AT; Investigation: SCT; Methodology: SCT, VT, BKM; Supervision: BKM; Validation: SCT, VSM; Writing-original draft preparation: AT, ZFH, SA, SK, SS, S; Writing- review and editing: SCT, AT, SS, S. All authors read and approved the final version of the manuscript.

Conflict of Interest

The authors declare no conflict of interest.

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