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Khalid Hussain Salaria
Natural Products-cum-Nano Laboratory, Division of Biochemistry, Faculty of Basic Sciences, Sher-e-Kashmir University of Agricultural Sciences and Technology of Jammu, Main Campus Chatha, Jammu, Jammu and Kashmir, India

Sanjay Guleria
Natural Products-cum-Nano Laboratory, Division of Biochemistry, Faculty of Basic Sciences, Sher-e-Kashmir University of Agricultural Sciences and Technology of Jammu, Main Campus Chatha, Jammu, Jammu and Kashmir, India

Proloy Sankar Dev Roy
Natural Products-cum-Nano Laboratory, Division of Biochemistry, Faculty of Basic Sciences, Sher-e-Kashmir University of Agricultural Sciences and Technology of Jammu, Main Campus Chatha, Jammu, Jammu and Kashmir, India

Neha Sharma
Natural Products-cum-Nano Laboratory, Division of Biochemistry, Faculty of Basic Sciences, Sher-e-Kashmir University of Agricultural Sciences and Technology of Jammu, Main Campus Chatha, Jammu, Jammu and Kashmir, India

Aasiya Majeed
Natural Products-cum-Nano Laboratory, Division of Biochemistry, Faculty of Basic Sciences, Sher-e-Kashmir University of Agricultural Sciences and Technology of Jammu, Main Campus Chatha, Jammu, Jammu and Kashmir, India

Shubham Kailas Padekar
Natural Products-cum-Nano Laboratory, Division of Biochemistry, Faculty of Basic Sciences, Sher-e-Kashmir University of Agricultural Sciences and Technology of Jammu, Main Campus Chatha, Jammu, Jammu and Kashmir, India

Faiqa Aiman
Natural Products-cum-Nano Laboratory, Division of Biochemistry, Faculty of Basic Sciences, Sher-e-Kashmir University of Agricultural Sciences and Technology of Jammu, Main Campus Chatha, Jammu, Jammu and Kashmir, India

Corresponding Author
Khalid Hussain Salaria
Natural Products-cum-Nano Laboratory, Division of Biochemistry, Faculty of Basic Sciences, Sher-e-Kashmir University of Agricultural Sciences and Technology of Jammu, Main Campus Chatha, Jammu, Jammu and Kashmir, India

Fermentation based augmentation of polyphenol content and bioactive properties of *Malus domestica* fruit juice using probiotic *Bacillus subtilis*

Khalid Hussain Salaria, Sanjay Guleria, Proloy Sankar Dev Roy, Neha Sharma, Aasiya Majeed, Shubham Kailas Padekar and Faiqa Aiman

Abstract
In the present study, probiotic bacteria *Bacillus subtilis* (MTCC-2389) was used for augmenting/ enhancing total polyphenol content and antioxidant activity of *Malus domestica* fruit juice (MDFJ). Different assays were performed for determining the antioxidant activity viz., ferric reducing antioxidant power (FRAP), chelating effect on ferrous ions and superoxide radical scavenging activity. Results indicated that the total polyphenol content and antioxidant activity was significantly enhanced at 36h over a period of 48h fermentation. The fermentation process increased the total phenolic content by 39.81%, total flavonoid content by 35.37%, ferric reducing antioxidant power by 33.34% and metal ion chelation activity by 42.61% over non-fermented control. Moreover, the IC_{50} value of superoxide radical scavenging activity dropped by 60.60%. These results provide the foundation for further exploration of the functional benefits of *Bacillus subtilis* (MTCC-2389) induced fermentation of *Malus domestica* fruit juice (MDFJ). Furthermore, the study can be helpful in developing probiotic juices with superior nutritional properties.

Keywords: fermentation, *Malus domestica*, *Bacillus subtilis*, probiotics, bioactive constituents, antioxidant activity

1. Introduction
Apple belongs to the genus *Malus* of the family Rosaceae. Thousands of cultivars a of *Malus domestica* are grown all around the world (Zhang, L.*et al.*, 2019 and Li, Y. *et al.*, 2020) [22, 15].
People are consuming *Malus domestica* across the world as this fruit and its products are very popular owing to their good taste, juiciness, color, texture and nutritional qualities. *Malus domestica* has a good storage efficiency and are found year-round in markets, at relatively low prices (Bars-Cortina, D. *et al.*, 2020; Mohebbi, S. *et al.*, 2020 and Bílkova, A. *et al.*, 2020) [1, 17, 5].
Fermentation is a process of transforming one substance into another which is carried out by microorganisms, such as bacteria and fungi under controlled conditions. The fermentation can be carried out under aerobic and/or anaerobic conditions. The formation of a specific product during fermentation depends upon the type of microorganisms used and the fermentation parameters (Katz, S.E. *et al.*, 2012 and Couto, S.R. *et al.*, 2006) [12, 7].
A number of strains of lactic acid bacteria have been engineered to augment/enhance the phenolic or bioactive components of *Malus domestica* fruit juice responsible for enhanced and promising antioxidant activity (Rossi *et al.*, 2013 and Pereira-Caro *et al.*, 2015) [20, 19].
In view of above facts, a research problem was planned with the objective to investigate the potential of *Bacillus subtilis* induced fermentation to enhance total polyphenol content and antioxidant activity of *Malus domestica* fruit juice (MDFJ)

2. Materials and Methods
2.1 Collection of plant material
The *Malus domestica* fruits were purchased in the month of June (2021) from fruit complex Narwal Mandi, Jammu, Jammu and Kashmir, India.

2.2 Preparation of *Malus domestica* fruit juice (MDFJ)
Malus domestica fruits were first washed with tap water followed by double distilled water (ddw). The fruits were cut down into small slices/pieces and then crushed in order to get fresh juice using a cold press extractor.

The juice so obtained was filtered by using four folds of muslin cloth in order to get pulp free juice.

2.3 Pasteurization of *Malus domestica* fruit juice (MDFJ)
The *Malus domestica* fruit juice was pasteurized using a heating water bath (70°-80 °C) for 20 minutes followed by immediate cooling and stored at 4 °C in a refrigerator until further use.

2.4 *Bacillus subtilus* probiotic inoculum preparation
Bacillus subtilis strain (MTCC-2389) was obtained from the Institute of Microbial Technology (IMTECH) Chandigarh, The culture was activated at 37 °C for 24 h in 150 mL Erlenmeyer flasks containing 100 mL nutrient broth. Cell cultivation was carried out statically in an incubator until the cell density reached 1.000 which corresponds to 9.18 log colony forming units (CFU) per millilitre. Cell density was spectrophotometrically determined at 600 nm and this culture was used as inoculum source in the fermentation process.

2.5 Total cell counts of the inoculum load
Total cell counts of inoculum load (*Bacillus subtilis*) were obtained by serial dilution with 0.7% NaCl solution till 10^{-6} dilutions. Aliquots of 0.1 mL of dilution were plated in triplicates in plates containing nutrient agar (spread plate method). The plates were incubated for 24 h at 37 °C and plates containing 20–300 colonies were measured or recorded as Log CFU/mL.

2.6 pH
The pH of both non-fermented and fermented MDFJ was adjusted using an electronic pH meter to 6.5±0.1 before the start of fermentation process.

2.7 *Malus domestica* fruit juice (MDFJ) fermentation process
Fermentation process was conducted in sealed 150 mL Erlenmeyer flasks (covered with cotton plugs), each containing 25 mL of pasteurized MDFJ without supplementary nutrients or water. 2% of inoculum, containing 9.18 Log CFU/mL of *Bacillus subtilis* (MTCC-2389) strains, were added to 150mL Erlenmeyer's flasks containing pasteurized MDFJ. The fermentation process was performed at 37 °C for 48h. Aliquots were taken at 0, 12, 24, 36 and 48h for analysis of total phenolic content.

2.8 Optimization of incubation time
The optimum incubation time of the fermentation process was evaluated by estimating the total phenolic content (TPC) (Chang and others 2001) [66] of non-fermented and fermented *Malus domestica* fruit juice (MDFJ). The TPC was determined using double beam UV-visible spectroscopy after every 12h upto 48h.

2.9 Determination of total phenolic (TPC) content
Total phenolic content of non-fermented and fermented MDFJ was determined according to Folin–Ciocalteu method with slight modifications (Chang and others 2001) [6]. Briefly, 1mL of MDFJ was mixed with 1mL of 1N Folin–Ciocalteu's phenol reagent. The mixture was kept for 5 minutes followed by the addition of 1mL of 20% Na_2CO_3. After 30 minutes of incubation at room temperature, the absorbance was measured at 730nm using double beam UV-VIS spectrophotometer. The total phenolic content was expressed as mg gallic acid equivalent/mL (mg GAE/mL) of fresh weight.

$y = 0.0395x + 0.0879$: $R = 0.998$

2.10 Determination of total flavonoid (TFC) content
Flavonoid content of both non-fermented and fermented MDFJ was determined using the method of (Jia *et al.*, 1999) [10] with slight modifications. 1ml of MDFJ was mixed with 75µL of 5% $NaNO_2$ solution and incubated for 6 minutes. 150µL of 10% $AlCl_3 \cdot 6H_2O$ was thoroughly mixed with the reaction mixture and incubated for 5 minutes. 0.5mL of 1M NaOH was added to the reaction mixture. Final volume of the reaction mixture was raised to 2.5mL with double distilled water. The mixture was vigorously vortexed and the absorbance was recorded at 510nm after 10 minutes of incubation. Results were expressed in mg quercetin mL^{-1} fresh weight.

$y = 0.0208x + 0.0395$: $R = 0.997$

2.11 Metal ion chelation activity
The chelating effect on ferrous ions of non-fermented and fermented MDFJ was estimated by the method of Dinis and others (1994) [8] with slight modifications. Briefly, 200µL of different concentrations of MDFJ and 740µL of methanol were added to 20µL of 2mM $FeCl_2$. The reaction was initiated by the addition of 40µL of 5mM ferrozine into the mixture, which was then left at room temperature for 10 minutes. The absorbance of the mixture was recorded at 562 nm. The ratio of inhibition of ferrozine-Fe^{2+} complex formation was calculated using the equation:

% inhibition = ([absorbance of control – absorbance of test sample]/absorbance of control) × 100.

2.12 Superoxide scavenging activity assay
Superoxide scavenging activity of the bio active compounds present in non-fermented and fermented fruit juice was determined by monitoring the competition of those with nitroblue tetrazolium chloride (NBT) for the superoxide anion generated by the phenazine methosulphate-nicotinamide adenine dinucleotide reduced (PMS-NADH) system (Nishikimi and others 1972) [18]. The reaction mixture contained 1mL of NBT solution (312µM prepared in phosphate buffer pH 7.4), 1mL of NADH solution (936µM prepared in phosphate buffer pH 7.4), and different concentrations of fermented and non-fermented extracts. The reaction was initiated by the addition of 100µL PMS solution (120µM prepared in phosphate buffer pH 7.4) to the mixture. The reaction mixture was incubated at 25 °C for 5 minutes Blue chromogen, formed due to NBT reduction, was recorded at 560nm. Results were expressed as percentage inhibition of superoxide radicals.

2.13 Ferric reducing antioxidant power (FRAP) assay
Ferric reducing antioxidant power of non-fermented and fermented fruit juice was measured according to the method of Benzie and Strain (1996) [2]. The principle of this method is based on the reduction of ferric-tripyridyltriazine complex to its ferrous, coloured form, in the presence of antioxidants. 132mL of FRAP reagent was prepared by mixing 100mL of 300mM sodium acetate buffer (pH 3.6), 10mL of 10mM TPTZ [2,4,6-tri-(2-pyridyl)-1,3,5-triazine] solution of 40mM HCl plus 10ml of 20mM $FeCl_3$ and 12mL of distilled water.

The samples, including both fermented and non-fermented MDFJ were taken from their respective stock solutions (1mg/mL) and mixed with 3mL of FRAP reagent. The absorbance was taken at 593nm after incubating the reaction mixture for 10 minutes. The FRAP activity was calculated from the calibration curve of ferrous sulphate ($FeSO_4.2H_20$). FRAP value was determined for each sample and expressed as mM $FeSO_4.2H_2O$ eq/mL of fresh weight.

3. Statistical analysis
All experiments were carried out in triplicates. The results were expressed as mean ± S.D. (standard deviation). Analysis of variance (single factor) was used to analyze the experimental data.

4. Results and Discussion
Fermentation has been used in the food industry for improving organoleptic and nutritive properties (Kantachote *et al.*, 2008) [11]. Thus, the application of probiotic based fermentation can induce effective microbial modifications of naturally occurring plant compounds and produce bioactive secondary metabolites, resulting in the detoxification of undesirable constituents and the enhancement of biological activities (Kim *et al.*, 2009) [14].

The fermentation of *Malus domestica* fruit juice (MDFJ) with *Bacillus subtilis* (MTCC-2389) at 37 °C and 150 rpm significantly enhanced total polyphenol content upto 36h over a period of 48h of fermentation. Thus, optimum incubation time for fermentation of *Malus domestica* fruit juice with *Bacillus subtilis* was 36h at 37 °C and 150 rpm. The total phenolic content increased from 153.28±1.73 to 254.63±3.10 GAE's mg/mL of fresh weight and flavonoid content increased from 49.21±7.78 to 76.13±13.02 QE's mg/mL of fresh weight as shown in table 1 and fig. no. 1 and 2. Similar results were obtained by Kaprasob *et al.*, (2017) [23] where total phenolic content (TPC) increased during fermentation of *Malus domestica* by *L. acidophilus, L. casei* and *L. plantarum* at 12h over a period of 48h of fermentation. In the present study, there was significant enhancement in the ferric reducing antioxidant power and metal ion chelation activity of fermented *Malus domestica* fruit juice as shown in fig. 3 and 4 respectively. The Ferric reducing antioxidant power increased from 1188.66±33.39 to 1783.33±5987mM Fe^{2+}/mL of fresh weight and metal ion chelation activity increased from 15.27±1.03 to 26.61±4.91 (%). The IC_{50} value of superoxide radical scavenging activity of non-fermented MDFJ was 23.96±2.70 (µg/ml) and 9.44±1.31 (µg/ml) of fermented MDFJ as shown in table no.3 and fig. no. 5. In a similar study which was carried out by Thakur and Joshi (2018) [21] where fermentation of *Malus domestica* fruit juice using probiotic *Lactobacillus plantarum* and *streptococcus thermophilus* was carried out for 72h and significant enhancement was observed in antioxidant and antimicrobial activity.

Table 1: Total phenolic (TPC) and total flavonoid (TFC) content of non-fermented and fermented MDFJ by probiotic *Bacillus subtilis*.

Fruit juice	GAEs mg/mL of fresh weight	QEs mg/mL of fresh weight
NF	153.28±1.73	49.216±7.78
F	254.631±3.10	76.13±13.02

Table 2: Ferric reducing antioxidant power (FRAP) and metal ion chelation activity of non-fermented and fermented MDFJ by probiotic *Bacillus subtilis*.

Fruit juice	FRAP (mM Fe^{2+}/mL of fresh weight)	Metal ion chelation activity (%)
NF	1188.66±33.29	15.27±1.03
F	1783.33±59.87	26.61±4.91

Table 3: Superoxide radical scavenging activity of non-fermented and fermented MDFJ by probiotic bacteria *Bacillus subtilis*.

Fruit juice	Superoxide radical scavenging activity IC_{50} (µg/ml)
NF	23.96±2.70
F	9.44±1.3

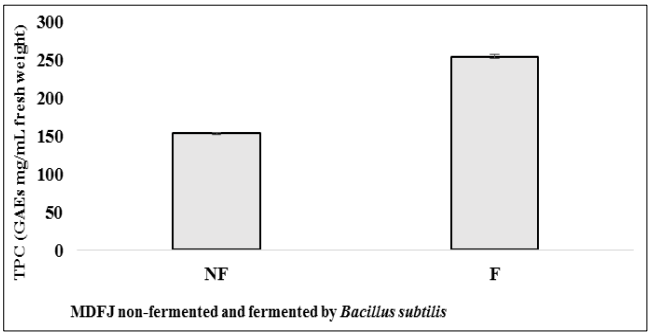

Fig 1: Difference between total phenolic content (GAEs mg/mL fresh weight) of *Malus domestica* fruit juice, non-fermented and fermented by *Bacillus subtilis* at optimum time of incubation.

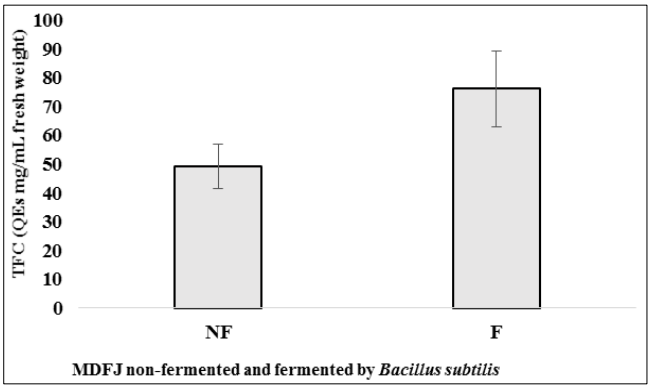

Fig 2: Difference between total flavonoid content (QEs mg/mL fresh weight) of *Malus domestica* fruit juice, non-fermented and fermented by *Bacillus subtilis* at optimum time of incubation.

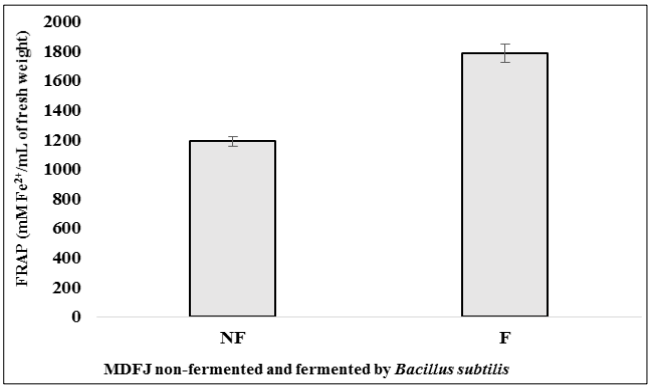

Fig 3: Difference between ferric reducing antioxidant power (mM Fe^{2+}/mL of equivalent extract) of *Malus domestica* non-fermented and fermented fruit juice by *Bacillus subtilis* at optimum time of incubation.

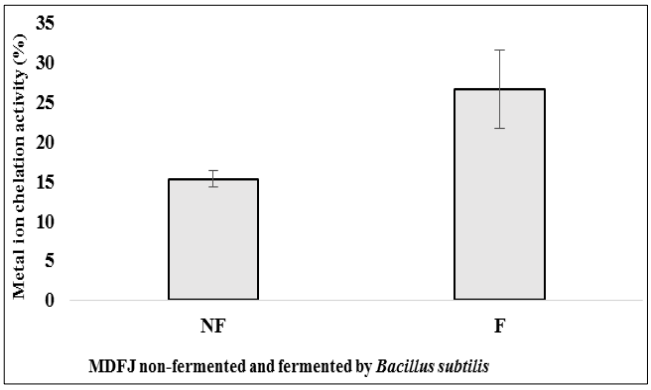

Fig 4: Difference between metal ion chelation activity (%) of both non-fermented and fermented *Malus domestica* fruit juice using *Bacillus subtilis* at optimum time of incubation.

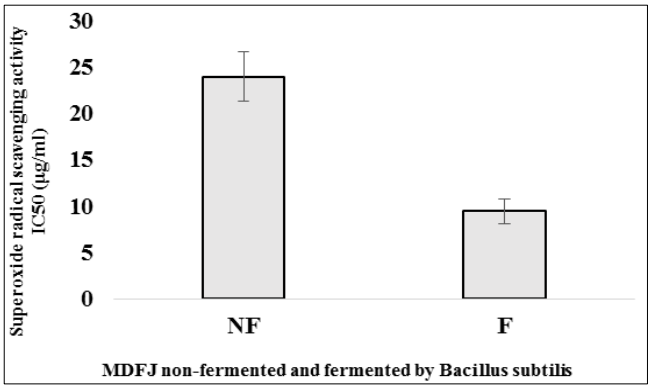

Fig 5: Difference between superoxide radical scavenging activity (IC_{50} (µg/ml) of *Malus domestica* fruit juice, non-fermented and fermented by *Bacillus subtilis* at optimum time of incubation.

5. Conclusion

The present study showed that MDFJ is an excellent substrate for fermentation based biotransformation to augment value added bioactives and beneficial constituents using *Bacillus subtilis*. The study indicated that bioactive constituents such as polyphenolic compounds and associated antioxidant activity can be beneficially enhanced to higher levels using probiotic based biotransformation. Overall this study provides a sound foundation for further value addition to MDFJ through *Bacillus subtilis* induced fermentation.

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