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Effect of *Allium parvum* and *Allium cepa* on *Bacillus cereus* isolated from fermented millet porridge

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Abstract
Bacillus cereus is a common and major food borne pathogen that can generate spores and form biofilms which makes them persistence and virulence at even harsh environments. The present study revealed the incidence of *B. cereus* in ragi millet porridge and the antibacterial activity of onion (*A. parvum and A. cepa*) against the *B. cereus* isolates. Contamination was observed in 87.5% of the samples with range of 2.07 ± 1.89 to 4.33 ± 0.45 mean $\log_{10}$ CFU/ml. Total phenol and flavonoid content of *A. parvum* was higher 344.66 ± 17.98 mg GAE/g and 136.66 ± 41.55 mg RE/g respectively. The higher antioxidant activity was observed for *A. parvum* DPPH (54.12%) and FRAP (19.59 mM Fe (II)/mg) when compared to *A. cepa*. The MIC (500µl) of *A. parvum* showed significantly the greater inhibition percentage, 3.19 to 23.38% compared to *A. cepa* which showed 0.67 to 21.42%. Natural antimicrobials, phenols and flavonoids present in the *A. parvum* and *A. cepa* helped in restricting the growth of *B. cereus* isolated from the ragi millet porridge.

Keywords: *B. cereus*, millet porridge, Antimicrobials, phenols

1. Introduction
Millets are one among the cereals apart from rice, maize and wheat. Cereals are the most common plant item in most diets, especially in developing nations, where they can account up to 90% of the overall diet (Amadou *et al*., 2013) [2]. Millets possesses notable amounts of proteins, dietary fiber, carbohydrates, phytochemicals and micronutrients (Annor *et al*., 2017) [3]. Porridge, a traditional fermented millet-based food popular in south india with long history and strongly associated with the local population. This fermented food was usually taken as breakfast diet and was best known for its flavor and nutritional benefits and is becoming popular as alternative source for other carbohydrate foods like wheat and rice (Thirumangaimannan & Gurumurthy, 2013) [24]. It is usually processed and handled at room temperature and can be stored under refrigerated conditions for few days. However, if any psychrotropic *B. cereus* strains are present in porridge samples it may be cause of food borne illness.

Bacillus cereus is a gram positive, facultatively anaerobic bacteria that produces endospores. The organisms spores tolerance to a variety of harsh environments has resulted in their worldwide distribution. The ubiquitous nature of organism makes contamination of foods a common occurrence (Griffiths & Schraft, 2017) [10]. Similarly, *B. cereus* contamination was highly expected in millet porridge samples due to the presence of carbohydrates, starch, micronutrients which are highly responsible for organism's growth (Rajkovic *et al*., 2019) [18]. *B. cereus* can be psychrotropic (grows well below 10 °C) and mesophilic (grows well at 37 °C) and also the bacterial spores do not have any metabolic activity and are resistant to heat, freezing, drying as well as irradiation (Enosi Tuipulotu *et al*., 2021) [7]. Besides this, many studies revealed that *B. cereus* was found to be resistant to ∝-lactam antimicrobials due to production of ∝-lactamases enzymes (Min Park *et al*., 2018) [13]. The acquisition of resistance genes in *B. cereus* which results in a new resistance phenomenon marked by increased levels of resistance, particularly to multiple drugs (Fiedler *et al*., 2019, Zheng *et al*., 2015) [8, 26]. Hence, the natural antimicrobials derived from plants which helps in arresting microbial growth can be used as an alternative to the synthetic drugs.

Onions are presumed to have antibacterial and antifungal properties, which could make them useful against human pathogens. It possesses good amounts of flavonoids, polyphenols, sulfur

compounds and a variety of other secondary metabolites, all of which are responsible for its therapeutic properties (Anyaegbunam *et al*., 2019) [4]. Onion bulbs have anticarcinogenic, antibacterial, hypoglycemic, anti-inflammatory, antiasthamatic, antihelminthic, antithrombotic activities (Golubkina & Caruso, 2020) [9]. Previous research have shown that onion extracts have pharmacological qualities, making them viable therapy for neoplastic, metabolic, immunological and infectious diseases (Obied *et al*., 2018) [16].

The present study aims to isolate the *B. cereus* from the selected ragi millet porridge samples, to characterize the *A. parvum* and *A. cepa* extracts and to study the antibacterial activity against selected *B. cereus* isolates.

2. Materials and Methods

2.1 Sample collection

A total of 40 ragi millet porridge samples were collected at regular intervals from Thanjavur, Tamil Nadu. The samples were collected in the sterile plastic bag and transferred to the laboratory in a cool box for microbiological examination.

2.2 Chemicals

Nutrient agar (NA), Nutrient broth (NB), Muller-Hinton broth (MHB), Muller-Hinton agar (MHA), Mannitol egg yolk polymyxin agar (MYP), Polymyxin B sulphate (FD003), Egg yolk emulsion (FD045), Luria-Bertani broth (LB), Methanol were purchased from Himedia, Mumbai, India.

2.3 Isolation of *B. cereus* from porridge

Isolation of *B. cereus* was performed according to the AOAC method (Tallent *et al*., 2012) [23]. 10g of each sample was mixed with 90 ml of buffered peptone water and the serial dilutions were made upto 10^{-6} from the resulting homogenate. Each dilution (0.1 ml) was spread plated on to the selective agar medium MYP for the isolation of *B. cereus.* Then plates were aerobically incubated at 37 °C for 24h. The colonies of *B. cereus* were identified as pink colonies surrounded by the precipitate zone indicating the lecithinase development. Then individual colonies were enumerated and reported in log CFU/ml.

2.4 Characterization of *A. parvum* and *A. cepa*

2.4.1 Extract preparation

The onion bulbs (*Allium parvum* and *Allium cepa*) were purchased from the local market of Thanjavur, Tamil Nadu and it was peeled, washed in sterile water, dried and weighed. About 25 g of each bulbs were rapidly crushed and soaked in 1:4 w/v in selective solvents methanol, hexane and ethyl acetate (1:1:1 ratio) for 24h in shaking incubator at 120 rpm at room temperature. The crude material was filtered using whatmann no.1 filter paper. After filtration, the filtrate was collected and concentrated on a rotary evaporator (40-43 °C). The dried extracts were stored under -20 °C and utilized for further experiments.

2.4.2 Determination of total phenol content

The total phenol content of *A. parvum* and *A. cepa* extracts were determined by Folin- Ciocalteu method (Chahmi *et al*., 2015) [6]. About 200 µl of extract was mixed with 20% sodium carbonate (2.5 ml) and also 0.5 ml of Folin- Ciocalteu reagent (FCR) was added and incubated for about 40 minutes in room temperature. The absorbance was taken at 723 nm in UV-spectrophotometer (Shimadzu Japan 1800) against the blank which was FCR reagent without the sample. The results were calculated based on the standard curve y=0.332x + 0.0967 = 0.9867; where x is the absorbance and gallic acid equivalents (mg/g) is y.

2.4.3 Determination of total flavonoid content

The total flavonoid content in *A. parvum* and *A. cepa* were measured according to the method described by (Bahorun *et al*., 2004) [5]

]. 500µl of each extract was added with 75 µl of sodium nitrite and 1 ml of distilled water and subjected to incubation at room temperature for 6 minutes. After the incubation, aluminium chloride (75 µl) was added and subjected to incubation for about 6 minutes and finally 1 ml of 4% NaoH was added and again subjected to incubation for about 20 minutes. The absorbance was read against the methanol blank at 510 nm in UV spectrophotometer (Shimadzu Japan 1800). The results were determined based on the calibration curve (y= 0.0022x + 0.0029= 0.9939) where x is the absorbance and rutin equivalence value (mg/g) is y.

2.4.4 Antioxidant assay

2.4.4.1 DPPH radical scavenging activity

DPPH activity was performed with slight modification described by (Kaur *et al*., 2009) [11]. About 25µl of aliquot methanolic extract was added to 2ml of 0.5 mM DPPH in methanol. Discoloration was detected with a UV spectrophotometer at 517 nm. The scavenging activity of DPPH radicals was expressed as the following

$$\text{Inhibition percentage} = \frac{(\text{Absorbance}_{t=0\ \text{min}} - \text{Absorbance}_{t=60\ \text{min}})}{(\text{Absorbance}_{t=0\ \text{min}})} \times 100$$

2.4.4.2 FRAP radical scavenging activity

The ferric reducing power of *A. parvum* and *A. cepa* extracts were measured based on the protocol described by (Shah & Modi, 2015) [21]. The ferric tripyridyl triazine (Fe^{+3}-TPTZ) complex is reduced to the ferrous (Fe^{+2}-TPTZ) form at lower pH in the presence of TPTZ, resulting in the development of a vivid blue color with a maximum absorption at 593 nm. 100 µl of extracts were added with 900µl of FRAP reagent and the blank was FRAP reagent excluding the sample. The mixture was then allowed for incubation in the dark for about 30 mins at 37 °C. The absorbance was taken at 573nm using spectrophotometer.

2.5 Determination of Antibacterial activity by broth dilution method

The minimum inhibitory concentration of *A. parvum* and *A. cepa* extracts were determined by broth dilution method (Manoharan *et al*., 2019) [12]. 5mg/ml concentration of extract was serially diluted in 900 µl Muller Hinton broth. 100 µl of culture was then added to broth and incubated for 24h at 37 °C. Another culture medium without microorganism suspension was used as control. After the incubation the OD values at 570 nm were taken in UV spectrophotometer. From the OD values the inhibition percentage and MIC values were calculated and recorded.

2.6 Statistical analysis

The experimental values were represented as mean ± standard deviation. All statistical analysis (one way ANOVA followed by Duncan's Multiple range test) were carried out by using

IBM SPSS version 26.0 software, IBM Ltd., New York, USA.

3. Results and Discussion

3.1 Prevalence of *B. cereus* in Ragi millet porridge

B. cereus contamination was observed in 87.5% (35 out of 40) of the samples analyzed. The incidence of *B. cereus* in ragi millet porridge was shown in table. About 40 samples from 9 different locations showed the *B. cereus* count in the range of 2.07 ± 1.89 to 4.33 ± 0.45 mean log_{10} CFU/ml. The contamination levels reported in porridge samples were unacceptable. Most of the tested samples showed the *B. cereus* cells as 10^4 CFU/ml, however, most foods have a safety limit or permissible count of *B. cereus* cells of less than 10^4 CFU/ml (EFSA, 2016) [19]. Hence the samples tested in this study were not in the safety limits which could be a great concern for food safety. Isolates from the highest contaminated samples were randomly picked and used for further studies.

Previous studies have been reported that detection rates of *B. cereus* in the ready to eat cooked rice of about 34% (the contamination levels in the range of 3.59 – 5.95 log_{10} CFU/ml) (Navaneethan & Effarizah, 2021) and similarly, in ready to use infant foods the contamination levels were in the average range of 1.896-3.453 log_{10} CFU/g (Sadek *et al*., 2018) [20]. Another study conducted by (saad A *et al*., 2020) reported the 54.2% contamination in meat products in the levels ranging 2.37-5.79 log_{10} CFU/g.

Table 1: Prevalence of *B. cereus* in ragi millet porridge

Sample location	Sample code	*B. cereus* count (CFU/ml)	Log_{10} (CFU/ml)	Mean log_{10} ± SD (CFU/ml)
Location 1	R1	$3.554x\ 10^3$	3.55	3.97 ± 0.56^b
	R2	3.613×10^3	3.57	
	R3	3.745×10^4	4.57	
	R4	3.659×10^3	3.56	
	R5	4.018×10^4	4.60	
Location 2	R6	2.640×10^4	4.42	4.04 ± 0.62^b
	R7	2.213×10^3	3.34	
	R8	3.390×10^4	4.53	
	R9	3.559×10^4	4.55	
	R10	2.318×10^3	3.36	
Location 3	R11	2.331×10^4	4.36	4.03 ± 0.56^b
	R12	2.118 x 103	3.32	
	R13	3.231×10^4	4.50	
	R14	2.872×10^4	4.45	
	R15	3.277x 103	3.51	
Location 4	R16	1.859×10^3	3.26	3.02 ± 1.77^{ab}
	R17	<DL	<DL	
	R18	1.718×10^3	3.23	
	R19	2.159×10^4	4.33	
	R20	2.027×10^4	4.30	
Location 5	R21	2.695×10^3	3.42	2.77 ± 1.55^{ab}
	R22	2.954×10^3	3.47	
	R23	<DL	<DL	
	R24	3.213×10^3	3.50	
	R25	3.054×10^3	3.48	
Location 6	R26	2.695×10^4	4.44	3.15 ± 1.83^{ab}
	R27	2.863×10^3	4.27	
	R28	3.09×10^4	4.47	
	R29	<DL	<DL	
	R30	2.572×10^4	4.37	
Location 7	R31	2.791×10^3	3.44	2.07 ± 1.89^a
	R32	2.862×10^3	3.45	
	R33	<DL	<DL	
	R34	2.954×10^3	3.47	
	R35	<DL	<DL	
Location 8	R36	3.781×10^4	4.57	4.33 ± 0.45^b
	R37	3.613×10^4	4.55	
	R38	3.363×10^3	3.52	
	R39	2.863×10^4	4.45	
	R40	3.445×10^4	4.53	

Note: R- Ragi millet porridge, <DL indicates below detection limit < 2log CFU/ml, values represent mean ± standard deviation. The mean values were statistically significant at $p < 0.05$.

3.2 Characterization of *A. parvum* and *A. cepa*

3.2.1 Extract percentage yield of *A. parvum* and *A. cepa* extracts

The total percentage yield of the *A. parvum* and *A. cepa* extracts were represented in the Table 2. The percentage yield was reported higher for *A. cepa*, 9.88% compared to *A. parvum*, 7.52%. The extraction was performed by conventional maceration process which helps in preventing the loss of volatile compounds when compared to hot extraction methods.

3.2.2 Total phenol and flavonoid content

The phenolic content of *A. parvum* and *A. cepa* extracts were determined and presented in Table 2. The total phenolic content was observed maximum for *A. parvum* extract (344.66 ± 17.98 mg GAE/g) compared to *A. cepa* extract (178.98 ± 10.57 mg GAE/g) and Likewise, the total flavonoid content of *A. parvum* (136.66 ± 41.55 mg RE/g) was recorded higher compared to *A. cepa* (51.66 ± 17.55 mg RE/g). The findings of this study were more satisfied with those of previous investigations (Bahorun *et al*., 2004, Prakash *et al*., 2007) [5, 17]. The values for both the extracts were differing due to the existence of one or more hydroxyl acidic groups connected to an aromatic arene ring in phenolics and flavonoids compounds, It is important to quantify the polyphenolic and flavonoid content in the extracts to benefication to antioxidant activity (Prakash *et al*., 2007) [17].

3.2.3 Antioxidant activity

Antioxidants are organic chemicals that are highly derived from natural sources and are made up of a complex phytocompound combination. Prooxidants produce such high levels of oxidative stress that biological molecules such as proteins, lipids and nucleic acids endure oxidative damage, which can lead to tissue instability. Antioxidants are important for human health because they prevent or slowdown the oxidation process. Hence, the antioxidants present in the natural sources may play an important role in free radical stabilization (Manoharan *et al*., 2019) [12]. The total antioxidant activity of both the extracts of *A. parvum* and *A. cepa* were shown in Table 2 and compared with BHT as control standard. The DPPH free radical which is a stable free radical that has long been used to assess antioxidants free radical-scavenging ability. In this test, the antioxidants convert the stable DPPH radical to yellow-colored diphenyl picryl hydrazine. Antioxidants have the ability to scavenge DPPH radicals because of their hydrogen-donating action (Ye *et al*., 2013). In this study, the *A. parvum* showed highest inhibition 54.12% compared to *A. cepa* which showed 29.69% inhibition and the standard control BHT showed 34.95 % inhibition. The results obtained were convincing with the previous reports (Nuutila *et al*., 2003, Shon *et al*., 2004, Kaur *et al*., 2009) [11] and the FRAP assay revealed the methanolic extract of *A. parvum* (19.59 mM Fe (II)/mg extract) was able to reduce TPTZ-Fe (III) to TPTZ-Fe (II) compared to *A. cepa* (14.55 mM Fe (II)/mg extract). These results suggested that *A. parvum* with good antioxidant content could be beneficial as a dietary supplement and thus *A. parvum* could be useful as potent antioxidant foods.

Table 2: Total phenol, flavonoid content and antioxidant activity of *A. parvum* and *A. cepa* extracts

Samples	Extract yield (%)	Total phenolic content mg GAE/g	Total flavonoid content mg RE/g	Antioxidant activity	
				DPPH % inhibition	FRAP mM Fe(II)/mg
Allium parvum	7.52^{b}	344.66±17.98^{a}	136.66±41.55^{a}	54.12±0.95^{c}	19.59±3.21^{a}
Allium cepa	9.88^{a}	178.98±10.57^{b}	51.66±17.55^{b}	29.69±0.35^{a}	14.55±1.77^{b}
BHT (Standard)	NA	NA	NA	34.95±0.98^{b}	NA

Values were mean of triplicate determination (n=3) ± standard deviation. GAE- Gallic Acid Equivalents, RE- Rutin Equivalents, BHT- Butylated hydroxytoluene; NA-Not applicable.

3.3 Antibacterial activity of *A. parvum* and A. *cepa* extracts

The antibacterial activity of *A. parvum* and *A. cepa* extracts were given in the Table 3. The total 8 *B. cereus* isolates were randomly picked and subjected to antibacterial activity assay. The minimum inhibitory concentration (500 µl) of the extracts revealed the percentage growth inhibition of selected *B. cereus* isolates. For *A. parvum* Isolate R5 showed the greater inhibition percentage (23.38%) followed by R21 (22.99%) and R18 (21.07%) and the least inhibition percentage was observed in isolate R8 (3.19%). Similarly, for *A. cepa* the highest inhibition percentage was observed in isolate R21 (21.42%), R18 (18.61%) and R26 (14.95%) and likewise, the least inhibition percentage was reported for isolate R8 (0.67%).

Table 3: Antibacterial activity of *A. parvum* and *A. cepa* extracts

Isolate	Minimum inhibitory concentration	
	A. parvum	***A. cepa***
R5	23.38 ± 0.92^{a}	12.51 ± 2.01^{d}
R8	3.19 ± 1.29^{a}	0.67 ± 0.52^{a}
R12	18.10 ± 1.15^{c}	7.42 ± 1.24^{bc}
R16	15.25 ± 2.20^{b}	4.91 ± 1.37^{b}
R18	21.07 ± 1.65^{de}	18.61 ± 3.40^{e}
R21	22.99 ± 0.96^{e}	21.42 ± 1.23^{e}
R26	20.24 ± 0.67^{cd}	14.95 ± 1.79^{d}
R29	15.34 ± 1.29^{b}	8.62 ± 2.96^{c}

Note: R- Ragi millet porridge, values represent mean ± standard deviation. The mean values (n=3) were statistically significant at p <0.05.

4. Conclusion

The present study revealed that the incidence of *B. cereus in* ragi millet porridge samples is comparatively high. Contamination rate was 87.5% which concerning to the potential risk of food borne diseases and health hazard to the consumers. However, natural antimicrobials from the *A. parvum* arrested the growth of pathogenic *B. cereus* strains which is more important because the bacterium is major food poisoning pathogen and its control is necessary since many strains of *B. cereus* were found resistant to synthetic drugs due to the existence of resistance genes.

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