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Bacillus Clausii : An Antibiotic Resistant Strain with Tolerance to Gut Environment Stress

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Abstract

The gut microbiota is intrinsically linked to human health; disturbances in microbial homeostasis are implicated in both intestinal and extra intestinal disorders. Probiotics are "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host. Many commercial preparations comprising a diverse range of probiotic microorganism are available. A spore mixture of four bacterial strains of B. clausii, characterized by an extended pattern of resistance to many antibiotics , however these antibiotics are currently less prescribed. A preparation containing mixture of 4 strains of Bacillus clausii (ACC0075, ACC0076, ACC0077 and ACC0079) is used in formulation of medicinal supplement used for humans taking antibiotic treatment for various health conditions and for chronic gastrointestinal disorder. These four strains show resistance against different class of antibiotics. We have extensively studied the antibiotic susceptibility profile of these four-resistance phenotype against different class of antibiotics that were highly prescribed in India. Also understanding its tolerance to different gut conditions, a study was conducted on four Bacillus clausii strains (ACC0075, ACC0076, ACC0077 and ACC0079). The formulation containing all four strains showed remarkable tolerance to pH variations, gastric and osmotic stress, in both vegetative and spore form thereby making it a promising candidate for gastrointestinal health promotion and therapeutic interventions. Studies conducted for antibiotic resistance and gastrointestinal tolerance of the formulation have opened the possibility of a combined therapy with antibiotics and Bacillus clausii strains (ACC0075, ACC0076, ACC0077 and ACC0079).

Keywords: Antibiotic resistance, Beneficial microbes, *Bacillus clausii*, Probiotics, Gastrointestinal conditions.

Introduction

Antibiotic usage in India has increased by about 30% over the past decade, with studies showing that 71.9% of patients in tertiary care hospitals receive antibiotics. This surge is driven by high prescription rates, unregulated sales, and self-medication, exacerbating antimicrobial resistance (AMR). A significant volume of broad-spectrum antibiotics and unapproved fixed-dose combinations (FDCs) is consumed; in 2019, 5071 million defined daily doses (DDDs) were used, with 54.9% from watch category antibiotics. Azithromycin was the most consumed antibiotic (640 million DDDs, 12.6%), followed by Cefixime, and the most consumed FDC was Amoxicillin-Clavulanic acid 500/125 mg (Koya et al., 2019). In rural India, informal healthcare providers significa ntly contribute to antibiotic availability, with a survey in West Bengal showing that 85% stock tablets, 74% syrups/ suspensions, and 18% injections. The most commonly stocked ATC class is beta-lactam antibacterials, particularly penicillins (78%), including Amoxicillin and Ampicill in, followed by other beta-lactam antibacterials (57%), like Cephalosporins. The frequently stocked oral antibiotics include Amoxicillin, Cefixime, Azithromycin, and Ciprofloxacin (Gautham et al., 2021).

Antibiotic treatments can cause unfavorable side effects, such as antibiotic-associated diarrhea, primarily due to disruptions in the intestinal microbiota and damage to intestinal barriers. These treatments alter the production of mucin, cytokines, and antimicrobial peptides, weakening the intestinal epithelial barrier. While broad-spectrum antibiotics are commonly used, their effects on the microbiome vary based on their mode of action, spectrum, delivery route, duration, and the host's attributes (Neuman et al., 2020; Duan et al., 2022). Short-term consequences of antibiotic use include antibiotic-associated diarrhea, *Clostridium difficile* associated diarrhea, and *Helicobacter pylori* infections, lasting from weeks to months. Long-term effects can persist for up to two years and include changes in gut microbiota, as well as the development of obesity, allergies, and asthma. The parallel intake of probiotic bacteria might reduce these events. The high sensitivity of the bacterial cells to the antibiotic molecule completely prevents a stable colonization in the intestine, thus ensuring only non-significant and transient effects. In this regard, the use of antibiotic-resistant beneficial bacteria could represent a worthy strategy (Jernberg et al., 2007; Zimmermann et al., 2019).

Bacillus is a genus of spore-forming bacteria found in the air, water, food, soil, and the human gut (Elshaghabee et al., 2017). The commercial *Bacillus* probiotic strains in use are *B. cereus, B. clausii, B. coagulans, B. licheniformis, B.mesentericus, B. polyfermenticus, B. pumilus*, and *B. subtilis*. These strains have antimicrobial, anticancer, antioxidant, and vitamin production properties (Lee et al., 2019).

Spore-bearing bacilli offer advantages over non-spore formers by undergoing a complex developmental process that allows them to survive harsh conditions, such as lack of water, nutrients, extreme temperatures, pH changes, UV radiation, and chemicals (Cutting & Ricca, 2014). When conditions improve, these spores germinate into vegetative cells that can grow and reproduce. *Bacillus* spores are metabolically inactive, tolerate bile salts, survive the acidic gastrointestinal environment, and are more stable than vegetative bacteria during the processing and storage of probiotic formulations (Elshaghabee et al., 2017; Hong et al., 2005; Sanders et al., 2003).

Bacillus clausii is a Gram-positive, aerobic, endospore-forming, facultative alkaliphilic rod bacterium used as a human probiotic (Senesi et al., 2001). Its ability to form spores grants it tolerance to heat, acid, and salt, ensuring safe passage through the gastrointestinal tract without cell loss.

Bacillus clausii, a resilient probiotic strain, shows promise in combating antibiotic resistance and is often used to mitigate gastrointestinal side effects of antibiotic treatment (Plomer et al., 2020; Nista et al., 2004). It aids gut immune homeostasis and has antimicrobial and immunomodulatory properties, making it effective for treating and preventing intestinal bacterial disorders, especially diarrhoea, as supported by clinical trials and a systematic review (Wong-Chew et al., 2022). To exert a measurable beneficial effect, probiotics need to survive the hostile environment of the gastrointestinal tract and have the ability to multiply and colonize the intestine (Ciffo, 1984). In the clinical context, strains that do not display this tolerance are unlikely to be viable and/or colonize the gastrointestinal tract and will therefore have reduced or no efficacy (Kolaček et al., 2017). *Bacillus* spores can withstand the acidic environment of stomach. It possess remarkable gastric resistance, enabling them to survive stomach acidity and reach the intestine tract and germinate successfully (Ciffo et al., 1987). Previous studies have shown that the spore-forming lactic acid bacteria exhibit significant acid and bile tolerance making them promising candidates for probiotic use (Hyronimus et al., 2000).

A spore mixture of four bacterial strains of *B. clausii*, known as O/C, SIN, N/R and T, characterized by an extended pattern of resistance to many antibiotics, are marketed in Italy as an OTC medicinal supplement (Abbrescia et al., 2019). In the current study, we evaluated the antibiotic sensitivity of our formulation, which contains a mixture of four *Bacillus clausii* strains (ACC0075, ACC0076, ACC0077, and ACC0079), against various classes of antibiotics. The antibiotics selected for this study are those that are most widely prescribed in India. The detailed analysis on broad spectrum of resistance and susceptibility patterns on these antibiotics is crucial for understanding their potential application. In addition, Antibiotic resistance is a growing global concern because it threatens the effectiveness of medications that have long been essential in treating bacterial infections lead to excessive or inappropriate use of antibiotics. Therefore, the results of this study will be valuable in supporting the idea that consuming antibiotic-resistant probiotics can help mitigate the effects of antibiotic therapy. To provide health benefits, the strains must be able to withstand various gastrointestinal conditions over extended periods. Therefore, this study also demonstrates the potential of the four *Bacillus clausii* strains (ACC0075, ACC0076, ACC0077, and ACC0079) in surviving these conditions, the strains were tested for their ability to endure the harsh environment of the gastrointestinal tract, ensuring they can reach the intestine and successfully colonize.

Materials and Methods

Bacterial Strains and Culture Conditions

Bacillus clausii strain ACC0075, ACC0076, ACC0077 and ACC0079 and a formulation containing mixture of 4 strains of *Bacillus clausii* (ACC0075, ACC0076, ACC0077 and ACC0079) was used for study. All 4 Strains were routinely cultured in Nutrient broth at 37°C in shaking at 180-200 rpm for 14±2 hours. For viable count determination, diluted cultures were pour plated with nutrient agar and were incubated at 37°C up to 48 hours.

Antibiotic Susceptibility Testing

Antibiotic susceptibility testing were performed using "Clinical and Laboratory Standards Institute, 33rd edition, M100 Performance standard for antimicrobial susceptibility testing" (CLSI, 2023). The present experiment provided data on the antibiotic susceptibility profile of each *Bacillus clausii strain* (ACC0075, ACC0076, ACC0077 and ACC0079) and formulation containing these four *Bacillus clausii* strains.

Vegetative Cell Preparation for Gut Tolerance Study

1mL of 0.4 Billion CFU/mL commercial formulation containing mixture of 4 strains of *Bacillus clausii* (ACC0075, ACC0076, ACC0077 and ACC0079) was grown in Nutrient broth at 37°C in shaking at 180-200 rpm for 14±2 hours.

Spore Mixture Preparation for Gut Tolerance Study

0.4 Billion CFU/mL commercial formulation containing mixture of 4 strains of *Bacillus clausii* (ACC0075, ACC0076, ACC0077 and ACC0079) was used for the study.

pH Tolerance Study

To perform study with vegetative cells, 1 ml of cell suspension was aliquoted into 10 Eppendorf tubes, and the cells were pelleted by centrifugation at 8000 RPM for 30 minutes. Supernatant was discarded. The cell pellets were resuspended in 1 mL of different sterile saline solutions (0.9% sodium chloride) with pH values ranging from 1 to 10 and incubated at 37°C for 2 hours. Samples were taken immediately after suspending in different pH conditions, marking the 0-hour time point. Subsequent samples were collected at 1 hour and 2 hours post-incubation at 37°C. The samples were serially diluted and pour-plated to assess the viable cell count per mL at each time point. The percent survival was then calculated.

To perform study with spores, 1 ml of formulation spore suspension was aliquoted into 10 Eppendorf tubes, and the spore were pelleted by centrifugation at 8000 RPM for 30 minutes. Supernatant was discarded. The spore pellets were resuspended in 1 mL of different sterile saline solutions (0.9% sodium chloride) with pH values ranging from 1 to 10 and incubated at 37°C for 2 hours. Samples were taken immediately after suspending in different pH conditions, marking the 0-hour time point. Subsequent samples were collected at 1 hour and 2 hours post-incubation at 37°C. The samples were serially diluted and pour-plated to assess the viable cell count per mL at each time point. The percent survival was then calculated.

Stimulated Gastric Juice Tolerance study

Simulated gastric juice was prepared by dissolving glucose (3.5 g/L), NaCl (2.05 g/L), KH_2PO_4 (0.60 g/L), $CaCl_2$ (0.11 g/L), and KCl (0.37 g/L) in water, then adjusting the pH to 2.0 with 1M HCl. The solution was autoclaved at 121°C for 15 minutes, and pepsin was added at a concentration of 13.3 mg/L (Corcoran et al., 2005).

To conduct the study with vegetative cells, 5 ml of cell suspension was centrifuged at 8000 RPM for 30 minutes. The supernatant was discarded, and the cell pellet was resuspended in 5 mL of gastric juice and incubated at 37 °C. Samples were taken after one hour and two hours of cell incubation in simulated gastric juice. The samples were serially diluted and pour-plated onto nutrient agar to determine viable cell counts at each time point. Percent survival was then calculated.

To perform study with spores, 5 ml of spore suspension was centrifuged at 8000 RPM for 30 minutes. The supernatant was discarded, and the spore pellet was resuspended in 5 mL of gastric juice and incubated at 37 °C. Samples were taken after one hour and two hours of incubation in simulated gastric juice. The samples were serially diluted and pour-plated onto nutrient agar to determine viable cell counts at each time point. Percent survival was then calculated.

Bile Tolerance Study

Bile Tolerance of Vegetative Cells

A 1% inoculum of vegetative cells was added to nutrient broth containing bile at concentrations ranging from 0.5% to 1%, with the pH adjusted to 8. The mixture was incubated at 37°C with shaking at 180-200 rpm for up to 24 hours. Percent survival was assessed by plating the samples onto nutrient agar plates and incubating at 37°C for 24-48 hours.

Germination of Formulated Spore under Bile Condition

A 1% of spore suspension was added to nutrient broth containing bile at concentrations ranging from 0.5% to 1%, with the pH adjusted to 8. The mixture was incubated at 37°C with shaking at 180-200 rpm for up to 24 hours. The germination percentage was assessed through microscopic observation.

Results

Antibiotic Sensitivity Test

The *B. clausii* probiotic strains are resistant to clinically important antibiotics, including macrolides and aminoglycosides (Bozdogan et al., 2003; Mazza et al., 1992). B. clausii strains exhibits resistance to macrolide, lincosamide and streptogramin B antibiotics (MLSB). Pattern of resistance defines an MLSB phenotype generally due to the presence of an erm gene encoding a ribosomal methylase (Bozdogan et al., 2004). The strain also harbor specific antibiotic-defense mechanisms, such as an aminoglycoside resistance gene (aadD2), a chloramphenicol acetyltransferase gene, cat(Bcl) or a β-lactamase (Bozdogan et al., 2004; Girlich et al., 2007; Galopin et al., 2009).

The four strains(ACC0075, ACC0076, ACC0077 and ACC0079) exhibited diverse susceptibility patterns across the tested antibiotics, indicating individual differences in their resistance profile. The resistance of each strain and formulation was estimated by measuring the MIC of antibiotics most representative of each class and the results are tabulated in Table 1. *Bacillus clausii* ACC0076 strain displayed higher MIC values for antibiotics, Azithromycin, Cefixime, Cefpodoxime, Rifampicin and Rifaximin, suggesting the greater resistance compared to the others. *Bacillus clausii* ACC0077 strain demonstrated lower MIC values for most antibiotics, suggesting greater susceptibility compared to other strains. This was observed for Cephalexin, Doxycycline, Levofloxacin, Ofloxacin, Rifampicin and Rifaximin.

Rifaximin is an antibiotic with broad-spectrum activity against Gram positive and Gram negative aerobic and anaerobic bacteria, effective for the treatment of travelers' diarrhea and other gastrointestinal infective conditions such as Clostridium difficile colitis. Rifaximin can down-regulate the inflammatory response triggered by the gut microbes by inhibiting the activation of the nuclear factor (NF)-κB via the pregnane X receptor (PXR) and by reducing the expression of the pro-inflammatory cytokines interleukin (IL)-1B and tumor necrosis factor-alpha (TNFα) (Scarpignato & Pelosini, 2006; Scarpignato & Pelosini, 2005; Marchese et al., 2000). Our study showed ACC0075 and formulation mixture of 4 strains of *Bacillus clausii* (ACC0075, ACC0076, ACC0077 and ACC0079) exhibited Rifampicin resistance. This resistance profile can open up a new therapeutic strategies of combination of Rifaximin and *Bacillus clausii* in treatment of travelers' diarrhoea.

The strains of *Bacillus clausii* (ACC0075, ACC0076, ACC0077 and ACC0079), as well as the formulation containing these strains, demonstrated resistance to spectrum of antibiotics, including Amoxicillin, Azithromycin, Chloramphenicol, Cefixime, Cefpodoxime, Novobiocin, Penicillin, Rifampicin, Rifaximin and Streptomycin. Recently, the presence of cfr-like genes in several *Bacillus* species has been reported. Cfr genes encode ribosome methyltransferases providing resistance to several classes of antibiotics including Phenicols, Oxazolidinone, Lincosamides, Pleuromutilins, and Streptogramin A (Dai et al., 2010). Conversely, they exhibited sensitivity to six antibiotics: Cephalexin, Doxycycline and Levofloxacin. Notably, both individual strains and the composite formulation displayed a moderate susceptibility to Minocycline, Ofloxacin and Tetracycline.

Table 1: Minimum inhibitory concentration (MIC) for *Bacillus clausii*.

S.No.	Antibiotics	Antibiotic class	MIC (µg/mL)				
			Bacillus clausii (ACC0075)	*Bacillus clausii* (ACC0076)	*Bacillus clausii* (ACC0077)	*Bacillus clausii* (ACC0079	Formulation
1	Amoxicillin	Beta Lactams	16	256	4	2	64
2	Azithromycin	Macrolide	> 256	> 256	> 256	> 256	> 256
3	Chloramphenicol	Chloramphenicol	64	32	64	64	64
4	Cefixime	Cephalosporins	> 256	> 256	64	> 256	> 256
5	Cefpodoxime	Cephalosporins	> 256	> 256	> 256	> 256	> 256
6	Cephalexin	Cephalosporins	0.5	4	2	2	1
7	Doxycycline	Tetracycline	8	0.125	2	4	4
8	Levofloxacin	Fluoroquniolones	16	2	2	2	2
9	Minocycline	Tetracycline	16	0.125	4	16	4
10	Novobiocin	Aminocoumarin	128	64	64	32	64
11	Ofloxacin	Quinolones	64	8	2	2	8
12	Penicillin	Beta Lactams	16	256	16	2	32
13	Rifampicin	Rifamycin	256	0.008	0.016	0.008	128
14	Rifaximin	Rifamycin	256	0.008	0.016	0.008	128
15	Streptomycin	Aminoglycoside	64	256	64	32	256
16	Tetracycline	Tetracycline	2	4	16	2	16

Based on this study, *Bacillus clausii strains* (ACC0075, ACC0076, ACC0077 and ACC0079) can survive and exert its probiotic effects even in the presence of antibiotics, making it a valuable option for maintaining gut health during antibiotic therapy.

Gut Tolerance Study

The digestive tract, starting from the mouth and ending at the anus, experiences variations in pH from acidic to alkaline. Acidic pH is one of the most challenging hostile conditions that can be encountered by probiotic strains in low-pH foods, during gastric transit and following exposure to fatty acids in the small intestine (Cotter & Hill, 2003). Probiotics will be exposed to gastric juice when they enter the stomach. Gastric juice, which is highly acidic due to hydrochloric acid, presents a challenging environment for probiotic bacteria. The stomach pH will be in the range of 1-3, this acidic environment activates the secreted pepsin and causes denaturation of food. The physical and chemical digestion process will take 15min-4hours in stomach (Sensoy, 2021). Studies has shown that *Bacillus clausii* vegetative cells exhibited a board pH tolerance survival rate up to 41%-99.7% from pH 2 to 12 (Chelliah et al., 2024). When our formulated spore suspension and formulated vegetative cells suspension was subjected to different pH range up to 1 for 2 hours, the cultures were found to survive with the survival rate of 45.7% for vegetative cells and 89.2% for spore suspension at the extreme acidic pH. The study also evaluated the ability of *Bacillus clausii* strains to endure the acidic environment of gastric juice with activated pepsin mimicking the stomach. The formulated spore suspensions exhibited better survival rate up to 95.2% for 2hrs when compared to vegetative cells in gastric juice. The data indicates that while vegetative cells are sensitive to low pH, spore suspensions are more resilient, highlighting the advantage of spore-forming probiotics in acidic environments like the stomach.

The pH in the duodenum rises to around 6 to 7 or even slightly higher, creating a netrual pH environment. Next in small intestine, pH remains neutral to slightly alkaline (around 7 to 8), which is conducive for the action of enzymes that further digest food and for the absorption of nutrients. Food typically takes about 3 to 5 hours to pass through the duodenum, jejunum, and ileum (Sensoy, 2021). Probiotic to get colonize in the gut should first by pass this varying pH exposure. Probiotics thrive in the alkaline environment of the small intestine, which aids food breakdown and nutrient availability for probiotics. The survival rate of spores remained higher, surpassing 95%, emphasizing the benefit of consuming the probiotic in spore form. In contrast, the survival rate of vegetative cells was 73% under extreme alkaline conditions. A decline in survival was observed as the pH increased from 7 to 10, suggesting potential stress or unfavorable conditions at these higher alkalinities. Data suggest that the *Bacillus clausii* strains can maintain their metabolic activity and cellular functions at alkaline condition in small intestine helping them to survive and colonize the gut. This pH tolerance is crucial, as the ability of probiotic strains to endure such environment is essential for their effectiveness in promoting gut health.

Bile secretion in the duodenum is a crucial part of the digestive process, particularly in the emulsification and absorption of fats. Bile is another challenge faced by probiotic bacteria in the upper parts of the small intestine, and they must possess specific tolerance mechanisms to counter this stress and successfully colonize the gut. In earlier reports, strains with increased bile salt tolerance has been obtained by selection toward other stress conditions such as acidic pH (Sanchez et al., 2012). The study examined the impact of bile salts on the viability cells in formulated vegetative cells. Culture was exposed to 0.5-1% bile salts for a period of 24 hours. Result showed that the survival rate of vegetative cells tends to decline as bile concentration increases, especially notable at 1.0%. To withstand the stress produced by Bile, the microorganism tolerance requires different defense mechanisms including the presence of efflux pumps, bile salt hydrolase (BSH) enzyme, the intrinsic capacity of cells to maintain intracellular homeostasis and modifications in the architecture and composition of the cell membrane. In fact, it was strongly suggested that BSH could play an important role in the colonization and survival of bacteria in the gut (Bustos et al., 2018). Germination rate of *Bacillus clausii* spore was examined. Data indicates that *Bacillus clausii* formulated spore suspension can germinate in presence of different concentration of bile ranging from 0.5 % to 1.0 % there by indicating survival and colonization. The resistance of bacterial spore to bile is due to the combination of tough, multilayered structure, dehydration, specific biochemical defenses (like efflux pumps and bile salt hydrolase), and their ability to withstand harsh conditions typically found in the intestines (Bustos et al., 2018; Driks, 2002; Henriques & Moran, 2007). This enables them to survive the bile-rich environment of the gastrointestinal tract and potentially colonize or infect the host.

Table 2: pH tolerance pattern for vegetative cells and spore.

S.No.	pH Range	Vegetative cells % survival	Spore % survival
1	1	45.7	89.2
2	2	85.5	92.1
3	3	87.5	94.5
4	4	90.8	94.9
5	5	91.0	95.7
6	6	92.0	98.1
7	7	94.5	97.0
8	8	96.6	98.4
9	9	93.1	95.7
10	10	88.8	95.5

The table presents a clear pattern of survival rates of vegetative cells and spores across different pH levels where the spore suspension is most stable indicating it is more resilient than vegetative cells, especially at lower pH levels, demonstrating their advantage in harsh conditions.

Table 3: Gastric tolerance.

S.No.	Simulated gastric juice exposure with 13.3 mg/L of pepsin	Vegetative cells % survival	Spore % survival
1	Control (0 hour)	100.0	100.0
2	1 hour	92.1	98.3
3	2 hrs.	81.0	95.2

The formulation shows a relatively high survival rate even after 2 hours of exposure, suggesting that it is quite resistant to degradation under the conditions simulated by the gastric juice with pepsin. Survival of spores tends to be better compared to vegetative cells. The small decrease in survival rate for both spore and vegetative cells indicates that there is minimal impact, highlighting the formulation's robustness in a simulated gastric environment.

Table 4: Bile tolerance pattern for vegetative cell suspension.

S.No.	Bile Concentration	Vegetative cells % survival
1	Control (No Bile)	100
2	0.5%	82.6
3	0.6%	82.9
4	0.7%	84.0
5	0.8%	82.2
6	0.9%	82.6
7	1.0%	80.1

The survival rate of vegetative cells decreased with increasing bile concentration.

Table 5: Germination of formulated spore under bile condition

S.No.	Bile concentration	Microscopic observation	% of Germination
1	Control (No Bile)	Vegetative cells. No spores	100
2	0.5%	Vegetative cells. No spores	100
3	0.6%	Vegetative cells. No spores	100
4	0.7%	Vegetative cells. No spores	100
5	0.8%	Vegetative cells. No spores	100
6	0.9%	Vegetative cells. No spores	100
7	1.0%	Vegetative cells. No spores	100

Discussion

Gut microbiota play a crucial role in maintaining overall health, influencing a wide range of physiological processes including digestion, immune function and metabolism. Imbalances in gut microbiota, known as dysbiosis are associated with various diseases, including infections, inflammatory conditions, metabolic disorders and potentially mental health issues (Young, 2012). Previous study investigated the variety of aerobic actinobacteria, particularly focused on the genus *Bacillus*, found in human faecal samples. Researchers collected 124 isolates from the faeces of ten healthy adult donors, identifying a significant presence of *Bacillus* species through 16S rRNA gene sequence analysis (Hoyles et al., 2012). *Bacillus* species stand out as promising probiotic qualities due to their stability and beneficial properties like antimicrobial, anticancer and antioxidant properties. Various clinical trials, including randomized controlled trails have shown that B. clausii can help manage conditions such as acute diarrhea, antibiotic- associated diarrhea and irritable bowel syndrome (Lee et al., 2019). Generally regarded as safe for consumption, with no significant adverse effects reported in the reviewed studies (Acosta-Rodríguez-Buen et al., 2022).

The study introduces a *Bacillus clausii* strains ACC0075, ACC0076, ACC0077 and ACC0079, which possess distinct antibiotic resistant properties and are capable of withstanding the harsh conditions of gastrointestinal environment. The study presents a detailed analysis of the antibiotic resistance profiles of four *Bacillus clausii* strains. The antibiotics used in this study are amongst the most commonly prescribed nationwide. The use of probiotics that are resistant to commonly prescribed antibiotics can offer several potential benefits, particularly in the context of managing or preventing infections and maintaining gut health. The key findings indicate a broad spectrum of antibiotic resistance and susceptibility patterns among these strains, which is crucial for understanding their potential application in therapeutic contexts, especially during antibiotic therapy. Consuming antibiotics-resistant probiotics in individuals receiving antibiotic therapy may help support gut health, reduce side effects, prevent pathogenic overgrowth, and potentially reduce the overall need for further antibiotic treatments. This approach could be particularly valuable in a time when antibiotic resistance is a growing concern globally.

The *Bacillus clausii* strains exhibit resistance to macrolides, lincosamides, and streptogramins (MLSB phenotype), typically associated with the presence of the erm gene. This resistance is a common defence mechanism in bacteria and indicates that these strains can survive in environments with these antibiotics, which might be relevant for their persistence in the gut. ACC0076 strain shows higher minimum inhibitory concentration (MIC) values for Azithromycin, Cefixime, Cefpodoxime, Rifampicin, and Rifaximin, suggesting a greater resistance to these antibiotics. Higher resistance might indicate a more robust survival capability in the presence of these antibiotics. ACC0077 strain demonstrates lower MIC values for several antibiotics, including Cephalexin, Doxycycline, Levofloxacin, Ofloxacin, Rifampicin, and Rifaximin. This suggests greater susceptibility compared to other strains. The strains, along with the mixed formulation, show resistance to a wide range of antibiotics, including Amoxicillin, Azithromycin, Chloramphenicol, Cefixime, Cefpodoxime, Novobiocin, Penicillin, Rifampicin, Rifaximin, and Streptomycin. The presence of cfr-like genes, which provide resistance to multiple classes of antibiotics, underscores the complex resistance mechanisms in these strains. The observed resistance of mixed strain formulation to Rifaximin can be used in combination with *Bacillus clausii* formulation can be a strategic approach, particularly in treating gastrointestinal infections, preventing antibiotic-associated side effects, and preserving gut microbiota during antibiotic therapy. This combination leverages the targeted antibacterial effects of rifamycins while supporting gut health and microbiota balance with the resilient, antibiotic-resistant probiotic *Bacillus clausii*. This approach can be particularly valuable in managing conditions like traveler's diarrhea, IBS, or hepatic encephalopathy, and in minimizing the adverse effects of antibiotic treatment on the gut (Scarpignato & Pelosini, 2006). The strains exhibit sensitivity to Cephalexin, Doxycycline, and Levofloxacin. Moderate susceptibility is observed to Minocycline, Ofloxacin, and Tetracycline. The study's findings highlight the critical importance of comprehending the antibiotic resistance profiles of *Bacillus clausii* strains to optimize their clinical applications. Overall, the study offers a thorough understanding of these profiles, which is vital for the safe and effective use of *Bacillus clausii* in medical and therapeutic settings. The results underscore the necessity for ongoing research and thoughtful consideration of resistance mechanisms when developing probiotic and therapeutic interventions.

The study demonstrates that *Bacillus clausii* strains exhibit significant resilience across various challenging conditions, including extreme pH levels, gastric acidity, and bile salt concentrations. The strains' ability to survive and maintain viability under these conditions is essential for their effectiveness as probiotics. Specifically, the formulation's tolerance to both acidic and alkaline environments, coupled with high survival rates in simulated gastric conditions, supports the potential of these strains to confer health benefits through their gastrointestinal transit. The superior bile tolerance of *Bacillus clausii* strains further emphasizes its potential for effective gut colonization and probiotic activity. Overall, these findings underscore the robust nature of *Bacillus clausii* strains and their potential utility in promoting gut health in diverse condition.

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