Original Research Article

Anti-microbial susceptibility pattern of spores used in *Bacillus clausii* suspension: an in vitro study

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Received: 18 February 2020

Accepted: 21 March 2020

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ABSTRACT

Background: *Bacillus* species have been used as probiotics as they have high stability to gastrointestinal conditions and impart health benefit on the host. Primarily used in their spore form the diversity of *Bacillus* species being used and their applications are remarkable. Here, we present the results of the antimicrobial susceptibility testing of the *B. clausii* spore suspension (Benegut®).

Methods: *Bacillus clausii* spore suspension (Benegut®), used in oral bacteriotherapy was tested for the susceptibility to therapeutically useful antibiotics. Twelve commercially prepared, paper antibiotic discs of different drugs having fixed concentrations were used. The antimicrobial susceptibility test was performed by the disc diffusion technique, using soybean casein digestive agar (SCDA) as media and the results were categorized as susceptible, intermediate, or resistant; using the criteria published by the Clinical and Laboratory Standards Institute.

Results: *B. clausii* was found to be resistant to broad-spectrum antibiotic chloramphenicol, antimycobacterial rifampicin, beta-lactamase inhibitor amoxi-clav, first-generation antibiotic cefaloridine, penicillin ampicillin, and tetracycline. *B. clausii* was resistant to both the aminoglycoside antibiotics (streptomycin and kanamycin) studied. Off first-generation fluoroquinolones studied, *B. clausii* was resistant to ciprofloxacin but partially sensitive to norfloxacin, ofloxacin and to macrolide azithromycin.

Conclusions: Antimicrobial resistance is an important feature of probiotics. The results of our study indicate that the antimicrobial and immunomodulatory activity *B. clausii* spore suspension (Benegut®) can profess beneficial effects and can be a useful source of improving the intestinal imbalance of microbial flora and is safe for human consumption.

Keywords: *B. clausii*, Probiotics, Resistance, Susceptibility

INTRODUCTION

Probiotics are live microorganisms which when administered in adequate quantities confer health benefits to the host.¹ Microorganisms have applications in every area of human life and have been explored for human benefits in the food and pharmaceutical industry.² In pharmaceutical industries various antibiotics and vaccines are produced from fungi, viruses and bacteria.² Food and beverage industries also use microbes such as *Lactobacillus*, *Saccharomyces cerevisiae* and *Propionibacterium sharmanii* for manufacturing

processed foods and drinks.³ The potential benefits of probiotics include improved nutrition and growth and prevention of various gastrointestinal disorders.⁴ Probiotics can also be used as an adjunct to antibiotic therapy, as the microorganisms present in them are resistant to antibiotics and therefore prevent the intestinal microbial flora imbalance.^{5,6}

However, the use of probiotics for health benefits is giving rise to concerns regarding resistance transfer from the probiotic microorganisms to the disease-causing pathogenic bacteria.⁵⁻⁷ The antimicrobial resistance to

antibiotics can be classified as intrinsic which is non-transferrable and acquired resistance which is transferrable.⁷ The resistance transfer, acquired in nature, can take place either by a mutation in a gene or by the acquisition of foreign DNA through horizontal gene transfer which includes transduction, transformation, and conjugation.⁸

The most common group of bacteria used as probiotics belongs to the group of lactic acid bacteria, which involves *Lactobacillus* and *Enterococcus*.⁹ Due to horizontal gene transfer, concerns are still raised, particularly in lactic acid bacteria strains that carry mobile genetic elements such as plasmids.¹⁰ *Enterococcus*, on the other hand, has member strains that are opportunistic pathogens and are sometimes the etiologic agents of some human nosocomial infections, such as bacteremia and infective endocarditis.⁹ Therefore, bacterial spore formers, mostly of the genus *Bacillus* which is a gram-positive species constitute a major probiotic product in use today.⁵ Primarily used in their spore form, these products have been shown to prevent gastrointestinal disorders, also the diversity of *Bacillus* species being used and their applications are astonishing.⁵ It has been suggested that the spore-bearing bacilli have some potential advantages over other non-spore formers such as *Lactobacillus* spp.⁶ *Bacillus* spores are heat-stable, capable of surviving the low pH of the gastric barrier, additionally products made on them can be stored at room temperature without any deleterious effect on viability.⁶

Benegut® is a *Bacillus clausii* spore suspension, used in the treatment of alterations in the intestinal microbial flora. In this article, we present the results of the antimicrobial susceptibility testing of the *B. clausii* spore suspension (Benegut®) using Soybean Casein Digestive Agar (SCDA).

METHODS

The antimicrobial susceptibility test was performed by the disc diffusion technique to assess the susceptibility of the strains of *B. clausii* against the antibiotics.

Table 1: Composition of soybean casein digestive agar.

Chemicals	%
Tryptone	1.5
Peptone	0.5
NaCl	0.5
Agar	2
pH - 8.0±0.2	

The advantages of the disk method are simplicity of testing not requiring any special equipment, flexibility in the selection of disks for testing and the results that can be easily interpreted by the clinicians. The media used for

the antimicrobial susceptibility testing of *B. clausii* was SCDA. The composition of this media is mentioned in Table 1. One hundred mL of enriched broth of soybean casein digestive medium (SCDM) was prepared (Table 2) and one vial of *B. clausii* spore suspension (Benegut®) was inoculated on it. After that 500µL of this prepared media was spread on the SCDA in aseptic conditions and the broth was incubated at 37°C±2°C for 18-24 hrs.

Table 2: Composition of soybean casein digestive medium.

Chemicals	%
Tryptone	1.7
Soya peptone	0.3
Sodium Chloride	0.5
Dextrose	0.25
Dipotassium hydrogen phosphate	0.25
pH - 7.3±0.2	

Table 3: Antibiotic discs and their concentration.

Antibiotic name	Disc concentration	Disc source
Streptomycin	10 mcg	HiMedia
Chloramphenicol	10 mcg	HiMedia
Tetracycline	30 mcg	HiMedia
Rifampicin	30 mcg	HiMedia
Azithromycin	15 mcg	HiMedia
Amoxycylav	30 mcg	HiMedia
Cefaloridine	10 mcg	HiMedia
Ciprofloxacin	5 mcg	HiMedia
Norfloxacin	10 mcg	HiMedia
Ofloxacin	2 mcg	HiMedia
Ampicillin	10 mcg	HiMedia
Kanamycin	30 mcg	HiMedia

Table 3 contains the concentration and source of the antibiotic discs used. Twelve commercially prepared, paper antibiotic discs of different drugs having fixed concentrations were placed in the middle of the culture inoculated agar plates. These agar plates were incubated at 37±2°C for 24 to 48 hours and observed. The zones of growth inhibition around each antibiotic disks were measured to the nearest millimeter. The diameter of the zone gives the measure of the susceptibility of the isolate and the amount of drug diffused through the agar medium. The zone diameters of each drug are interpreted using the criteria published by the Clinical and Laboratory Standards Institute (CLSI, formerly the National Committee for Clinical Laboratory Standards or NCCLS) or those included in the US Food and Drug Administration (FDA)-approved product inserts for the disks. Positive, medium and LAFU control were also incubated at 37°C for 24-48 hrs. Growth observed in the plate was regarded as positive and inhibition of zone around antibiotic disc was regarded as negative.

RESULTS

The antimicrobial susceptibility of *B. clausii* spore suspension (Benegut®) to antibiotics was accessed by the disc diffusion technique and the results were categorized as susceptible, intermediate or resistant. A bacterial strain is said to be susceptible to a given antibiotic when it is inhibited in vitro by the antibiotic in therapeutic dose range; intermediate if it is inhibited by a concentration that is associated with an uncertain therapeutic effect and resistant when it is inhibited by a concentration that is associated with a high likelihood of therapeutic failure.¹¹ *B. clausii* was found to be resistant to broad-spectrum antibiotic chloramphenicol, antimycobacterial rifampicin, beta-lactamase inhibitor amoxiclav, first-generation antibiotic cefaloridine, penicillin ampicillin, and tetracycline. *B. clausii* was resistant to both the aminoglycoside antibiotics (streptomycin and kanamycin) studied. Off first-generation fluoroquinolones studied, *B. clausii* was resistant to ciprofloxacin but partially sensitive to norfloxacin, ofloxacin and to macrolide azithromycin. The test results are summarized in Table 4. Positive and medium control was also maintained and growth was observed in positive control and not in medium control.

Table 4: Results of the antibiotic susceptibility test.

Name of the Antibiotic	Benegut®	
	Resistance	Sensitivity
Streptomycin	✓	✗
Chloramphenicol	✓	✗
Tetracycline	✓	✗
Rifampicin	✓	✗
Azithromycin	Partially resistance	Partially sensitive
Amoxiclav	✓	✗
Cefaloridine	✓	✗
Ciprofloxacin	✓	✗
Norfloxacin	Partially resistance	Partially sensitive
Ofloxacin	Partially resistance	Partially sensitive
Ampicillin	✓	✗
Kanamycin	✓	✗
Positive control	Growth was observed	
Medium control	No growth was observed	
LAFU control	No growth was observed	

✓=Resistant

✗=Zone of inhibition more than 1.0 cm

Partially resistant= Zone of inhibition less than 1.0 cm

DISCUSSION

Commercially available probiotics contain microorganisms with antibiotic resistance, due to which they are used in association with antibiotic therapies in order to prevent microbial flora imbalance.¹² Therefore, it

is necessary to access the pattern and stability of resistance spectra in probiotics, in order to better manage the advantage of antibiotic-resistant probiotics during antibiotic therapy.⁶ Antibiotic resistance is a common phenomenon in gram-positive bacteria.¹³ It is accomplished by genes acquired either horizontally through plasmids, or foreign DNA recombination, or mutations at different chromosomal loci in the bacterial genome.¹³

Members of the genus *Bacillus* are aerobic or facultative aerobic, endospore-forming and rod-shaped gram-positive bacteria, which inhabit a wide range of habitats, mostly soil, and sediments.¹² These bacteria do not belong to the commensal microbiota of the gastrointestinal tract, but some strains of the genus are included in food supplements and used in human nutrition as probiotics, notably *B. clausii*.¹²

The *B. clausii* probiotic strains are resistant to clinically important antibiotics, including macrolides and aminoglycosides.^{14,15} Resistance to aminoglycosides is reported to be due to the synthesis of an aminoglycoside-inactivating enzyme encoded by an aadD2 chromosomal gene, and to macrolides due to the presence of an erm(34) gene.¹⁴ The resistance of *B. clausii* to rifampicin is reported to be due to a chromosomal mutation.⁷ A study by Galopin et al, showed that the resistance to chloramphenicol in probiotic containing *B. clausii* was due to the production of CAT encoded by the catBcl gene.¹⁶ There are domains found in *B. clausii* which confer resistance to different drugs, such as streptomycin (Pfam) and tetracycline.¹³ Moreover, all the *Bacillus* probiotics contain different classes of beta-lactamases rendering them resistant to penicillins such as ampicillin and amoxiclav.¹³ These findings are consistent with the fact that the genes found in *B. clausii* have not been detected in pathogenic bacteria despite the long term use.⁷

The present study evaluated the antibiotic susceptibility profile of *B. clausii* spore suspension (Benegut®). The antimicrobial susceptibility test of *B. clausii* spore suspension (Benegut®) was performed against 12 antibiotics using the disc diffusion technique. In this study, we found that the Benegut® was resistant to streptomycin, chloramphenicol, tetracycline, rifampicin, amoxiclav, cefaloridine, ciprofloxacin, ampicillin, and kanamycin; partially resistant to azithromycin, norfloxacin, ofloxacin. In the case of positive and medium controls, growth was observed in positive control and not in medium control.

A couple of studies have reported similar susceptibility of *B. clausii* to antibiotics as reported in our study. A study conducted by Gueimonde et al, accessing the antibiotic resistance patterns of the *Lactobacillus* and *Bacillus* strains, also showed that the strains of *Bacillus* had genes resistant to macrolides, tetracycline, aminoglycosides, chloramphenicol, and beta-lactamases.¹² Another study testing *Enterogermina* (containing four strains of *Bacillus*

species [O/C, N/R, T, and SIN]) found that different strains had varied susceptibility towards different antibiotics. O/C strain was found to be resistant to chloramphenicol, N/R was resistant to rifampicin, T showed resistance towards tetracycline and SIN showed resistance to streptomycin.¹⁷

Gueimonde et al. examined five commercial preparations of *Bacillus* strains by disc diffusion technique.¹² The authors reported that *B. subtilis* had a basal level of resistance to streptomycin. Among the five probiotics examined, Bactisubtil was resistant to both chloramphenicol and tetracycline, Domuvar, which is closely related to Enterogermina was found to be resistant to chloramphenicol.¹² According to another study testing probiotics containing *Bacillus* strains, all the strains of *Bacillus* were resistant to streptomycin but susceptible to chloramphenicol, kanamycin, and tetracycline.¹⁸ The *B. clausii* strain (ATCC 9799) was reported to be resistant to cephalosporins in the study by Hong et al, and Girlich et al, and sensitive in a study by Abbrescia et al.^{5,6,19} In this study out of three fluoroquinolones (ciprofloxacin, norfloxacin, and ofloxacin) tested, *B. clausii* probiotic strains were reported to be resistant to ciprofloxacin but partially resistant to norfloxacin and ofloxacin. However, in a study by Abbrescia et al., *B.* strains were reported to be sensitive to fluoroquinolones (ciprofloxacin, levofloxacin, norfloxacin, and moxifloxacin).⁶

In the era of antibiotics, probiotic use is increasingly employed in association with antibiotics therapy for preserving the intestinal microbial flora, owing to antimicrobial resistance.⁶ Antimicrobial resistance is a desirable feature in probiotics. Although, microbes used as probiotics are not exempted from the natural processes governing antibiotic resistance.²⁰

Therefore, it is imperative to screen microbes effectively for antibiotic resistance genes before using them as probiotics.²⁰ Extensive research has been conducted on the antimicrobial resistance patterns of probiotic strains and it was found that probiotic strains possess both acquired and intrinsic resistance.^{9,12,20}

B. clausii, probiotic in the spore form appears to have an important role to play management of acute diarrhea and recurrent respiratory infections in children, with a good safety profile.^{21,22} A systematic review and a meta-analysis of randomized controlled trials of *B. clausii* in acute childhood diarrhea by Ianiro et al, showed that the mean duration of diarrhea was reduced by 9.12 hours with *B. clausii* treatment, as compared to controls ($p=0.015$).²¹ The authors also reported that the administration of *B. clausii* preparations significantly reduced the duration of hospitalization by a mean of 0.85 days compared to controls ($p = 0.017$).²¹ This is important considering that in low-income countries, children under three years old experience on average three episodes of diarrhea every year.^{21,23}

Marseglia et al, performed a pilot study to assess the efficacy and the safety of *B. clausii* in the prevention of recurrent respiratory infections in children.²² Results showed that *B. clausii* treatment was significantly effective ($p=0.037$) in reducing the duration of respiratory infections in children with recurrent disease. Moreover, the duration of respiratory infections episodes during the follow-up period was also significantly shortened ($p=0.049$).²²

The use of probiotics in the treatment and prevention of disease, particularly gastrointestinal disease, has yielded many successful results.²⁴ As regard to *B. clausii*, its professed beneficial effect has been credited to antimicrobial and immunomodulatory activity.²⁵ *Bacillus* probiotics have an overall excellent health-promoting record, especially in preventing and curing of diarrhea, gingivitis, *H. pylori* infection and maintaining homeostasis of the intestine.²⁶ *B. clausii* survives transit in the gut and maintains a considerable intestinal titre for up to 12 days after a single oral administration.²⁵ *B. clausii* strains show different ability to survive and persist, suggesting a strain dependent adaptation to this environment.²⁵ Analysis of the whole genome sequence of *B. clausii* has revealed that the antibiotic resistance genes are present in chromosomal DNA which is intrinsic and not transferable.²⁷ Toxin genes were also found to be absent. These results suggest consumption of *B. clausii* is safe for humans.²⁷

In conclusion, resistant traits found in *B. clausii* spore suspension (Benegut®) in our study were similar to those reported from other commercial preparations of *Bacillus* strains. Benegut® can be a useful source of improving the intestinal imbalance of microbial flora; and effective treatment of diarrhea and other intestinal malfunctions.

ACKNOWLEDGEMENTS

Authors would like to thank MIS team at medONE Pharma Solutions, Gurugram, Delhi NCR, for assistance in the preparation of this manuscript.

Funding: No funding sources

Conflict of interest: None declared

Ethical approval: The study was approved by the Institutional Ethics Committee

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Cite this article as: Srinivas A. Anti-microbial susceptibility pattern of spores used in *Bacillus clausii* suspension: an in vitro study. Int J Contemp Pediatr 2020;7:980-4.