Indian Journal of Applied Microbiology
ISSN (Online): 2454-289X, ISSN (Print): 2249-8400

https://doi.org/10.46798/ijam.2023.v25i02.4
Volume 25 Number 2
July - September 2023, pp. 47-59

Probiosimilarity Study: A Comprehensive Approach to Compare the Attributes of Marketed Probiotics Containing *Bacillus clausii*

Mathan Natarajan[1*], Ragul Kessavane[2]

[1]General Manager (Research & Development),
[2]Executive Scientist (Research & Development), Allianz Biosciences Pvt Ltd,
R.S. No.55/1, 2 & 3, Whirlpool Road, Thiruvandarkoil, Puducherry- 605102, India.

Abstract

Background: Probiotics are widely accepted functional foods for general well-being and a healthy lifestyle. A robust approach to evaluate the bio-similarities between marketed probiotics still needs to be developed. **Aim:** We aimed to define an approach that uses time-tested methods to evaluate pro-biosimilarity between different marketed probiotics. **Methods:** A total of five different *Bacillus clausii* formulations were compared with the international reference standard Enterogermina. The spore quantity and microbial purity of the samples were estimated. The antibiotic resistance strains in oral suspension samples were isolated and subjected to molecular characterization by 16S rRNA sequencing. The antibiotic susceptibility test was performed using commercially available antibiotics. The pH and transmittance of the samples were measured. **Results:** Bifilac Clausi had the highest, while Entromax had the lowest density of quality spores. None of the products had contaminant pathogenic microorganisms. Based on 16S rRNA sequencing, four strains from Bifilac Clausi and Enterogermina and one strain from other samples were identified. The identity of the strains from Bifilac Clausi matched those of Enterogermina. Bifilac Clausi and Enterogermina were resistant to the ten antibiotics tested. **Conclusions:** Among the different Indian brands of *Bacillus clausii*, only Bifilac Clausi was found to be a pro-biosimilar to the international reference standard, Enterogermina.

Keywords: Probiotics, Biosimilarity, *Bacillus clausii*, Enterogermina, antibiotic resistance

*Author for Correspondence. E-mail: nm@abpl.co.in

Introduction

Probiotics are widely accepted for the general well-being and healthy lifestyle of people across the globe. Probiotics find wide applications, such as maintaining gut microbiota balance and gut membrane integrity to resolve several gastrointestinal issues, counteracting antibiotic side effects, maintaining homeostasis of the immune system, amelioration of symptoms of lactose intolerance and reducing the risk of various diseases [1-3].

Over the last few decades, the probiotics market has evolved to introduce several new pharmaceutical preparations of novel probiotic strains in the form of sachets, capsules and numerous other formulations tailored to address specific clinical disorders and health needs. Most of the probiotic strains introduced in the market are unique cultures that are assigned specific deposit numbers. Such strains are subjected to standardisation and large-scale commercial scale of production. Although patenting the probiotics per se has been a challenge, the code number provided by the International Depository Authority (IDA) like the American type culture collection (ATCC), German collection of microorganisms (DSM) or Collection Nationale de Cultures de Microorganisms (CNCM) is specific for a particular strain. It is broadly accepted that a specific strain with a defined code number renders unique health values. Just like the biomolecules, the concept of generic probiotics and the development of formulations similar to that of a patent-expired product can offer a practical solution to enhance access to probiotics for people in underdeveloped countries and to enhance the reach of certain valuable probiotics to a large section of the population in need [4]. There has been a significant development in the methodologies to prove the chemical and clinical equivalence of biosimilars of generic biomolecules. At the same time, such a need exists in the field of probiotics too. Although there are guidelines by associations such as the American Gastroenterological Association (AGA) and the World Gastroenterology Organization (WGO) [5,6] to define the quality standards of probiotics, a robust approach to evaluate the biosimilarities between probiotics that comprise live microbes is lacking. Several points must be considered to establish the probiosimilarities between the commercialized probiotics.

A systematic meta-analysis strongly suggests that specific strains contribute to the efficacy of the probiotic products [7].However, the unique characterization of the various marketed probiotics remains challenging due to the distinguishing features attributable to the mode and extent of action, strain-specific delivery of clinical efficacy, differences in the in-house processes and final product quality[8]. A detailed genomic approach based analysis in 2010 demonstrated the differences in strain-specific efficacy of diverse strains of bacterial or fungal origin[9]. The International probiotic guidelines recommend using strain designations when reporting clinical trials to facilitate documentation of strain-specific efficacy. This recommendation has yet to be uniformly followed [10,11].The antibiotic resistance patterns of different strains could also change due to their adaptability and complex interaction with the exposed surroundings [12].

Considering this, we define an approach that uses time-tested methods to evaluate probiosimilarity between different marketed probiotics in this study. As a proof of concept, we tested the probiosimilarities of *Bacillus clausii* formulations marketed in India with an international reference standard of *Bacillus clausii*, Enterogermina.

Methodology

Five different *Bacillus clausii* formulations were compared with the international reference standard of *Bacillus clausii*, Enterogermina at IIT-Madras, India in December 2021. The details of the formulations are listed in Table 1.

Table 1: Characteristics of Products

Sl. No.	Product Name	Manufacturer	Batch No.	Mfg. Date
1	Enterogermina	Sanofi S.p.A.	01234	11/2020
2	Bifilac Clausi	Tablets (India) Limited	AHLAISI	03/2021
3	Tufpro	Unique biotech ltd	ZEY0017	04/2021
4	Novogermina	Unique biotech ltd	NTF21008UH	05/2021
5	Progermina	Genetek Life sciences Pvt. Ltd	GBC21016	04/2021
6	Entromax	Virchow Biotech Pvt Ltd	A6EEU085	03/2021

1. Estimation of total spore quantity of oral suspension sample

The total spore in oral suspension was enumerated according to a previously described method by Ghelardi et al. with slight modifications [13]. To enumerate *B. clausii* strains, the samples were serially diluted in 0.1% peptone, seeded (100 µl per plate) on plates, and incubated at 37^{o} C for 24 h. The experiment was performed in triplicates.

2. Simple and spore staining

A sample smear was prepared under sterile conditions, air-dried and heat-fixed. The blotting paper was saturated with 0.5% malachite green stain solution and steamed for 5 minutes. The slide was washed and counterstained with 0.5% safranin for 30 seconds and examined under a microscope. The sample was observed for the presence of bright green spores and brownish-red to pink vegetative cell morphology.

3. Microbial purity of spore detection

The detection of some of the most commonly found microbial contaminants in pharmaceutical preparation or those organisms of public health importance was carried out using colony morphology and differential biochemical tests. *Escherichia coli, Pseudomonas aeruginosa, Salmonella enterica, Shigella boydii, Candida albicans, Staphylococcus aureus*, total yeast and

mould count from Bifilac Clausi, Enterogermina, Entromax, Novogermina, Progermina and Tufpro products were carried out according to the procedure in Indian Pharmacopeia, 2018 [14].

4. Isolation of strains based on their sensitivity/resistance pattern to antibiotics

The antibiotic resistance strains in oral suspension were isolated using the method described by Ghelardi et al. [13]. Samples were serially diluted in 0.1% peptone and seeded (100 µl per plate) on Mueller—Hinton agar (MHA) plates containing antibiotics and incubated at 37^{o}C for 24 h. The experiment was performed in triplicates.

5. Taxonomical identification (16S rRNA sequencing)

Molecular characterization of the selected isolates was performed using 16S rRNA sequencing.

Primers used:

27F (5'-AGAGTTTGATCCTGGCTCAG-3') and

1492R (5'-TACGGYTACCTTGTTACGACTT-3').

To identify the closest match of bacterial species, gene sequences of 16S rRNA were used to construct the phylogenetic tree using the UPGMA method (MEGA 7.0.21 software) [15].

Genomic DNA extraction procedure

Genomic DNA was extracted using the standard molecular biology protocol. Isolated and purified bacterial colonies were grown in LB broth for 24 hours. Cells from 1ml of cell suspension were pelleted at 8000g for 2 min, washed twice with 400 µl STE Buffer (100 mM NaCl, 10 mM Tris/HCL, 1 mM EDTA, pH 8.0), centrifuged at 8000g for 2 min at 10°C and resuspended in 200 µl TE Buffer (10 mM TRIS /HCl, 1 mM EDTA, pH 8.0). For cell lysis, 20 µl lysozyme (20mg/ml) was added and incubated at 37˚C for 30 minutes, followed by the addition of 100µl Tris–saturated phenol (pH 8.0) and vortex mixed for 90seconds.The aqueous was separated from the organic phase upon centrifugation at 13000g for 5 min at 4°C. 160 µl upper aqueous phase with 40 µl TE buffer subjected to multiple runs of 100 µl chloroform extraction for 5 min at 13000 g at 4°C. Ethanol extraction with 1ml of absolute ethanol added to 150 µl upper aqueous was performed with high-speed centrifugation at 13000g at 4°C for 5 min. The pellet was washed with 1 ml of 70% ethanol (13000g at 4°C for 2 min), air-dried and suspended in 40 µl of TE buffer or nuclease-free water and stored at -20°C. The purified genomic DNA products were resolved by electrophoresis in a 0.7% agarose gel in 1x TAE buffer, stained with 10mg/ml of ethidium bromide.

PCR Amplification

A PCR was performed in a total volume of 20 µl containing 10 µl master mixture, 40 ng of template DNA and 1 ml of 1µM each of the following primers:

8F (5′AGAGTTTGATCCTGGCTCAG3′) and 1942R (5′GGTTACCTTGTTACGACTT3′)

PCR conditions were as follows:

Denaturation 95°C

for 5 min, 30 cycles

94°C for 1 min

Primer-specific annealing temperature at 53.8°C for 45 sec

extension at 72°C for 1 min

final extension at 72°C for 5 min.

The reaction contents were run on 1% agarose gel in 1x TAE buffer containing10mg/ml, and the resolved products were visualized under UV.

DNA sequencing and species identification

The amplified PCR product was purified using a Qiaquick PCR purification kit (QIAGEN, USA). Sequencing in both directions was performed using the PCR primers with BigDye Version 3.1 kit (Applied Biosystems). The experiment was performed using ABI-PRISM 3730 DNA sequencer (Applied Biosystems), and any ambiguous sequences from the base called sequences were corrected with Chromas (Version 2.01). The sequences were assembled with bio-Edit (Version 7.0.9.0), an identity search was done using the BLASTn program (NCBI) and the final nucleotide sequence was submitted to GenBank for reference.

6. Antibiotic susceptibility test

The antibiotic susceptibility test was performed using plates overlaid with 0.1 ml of selected isolates against commercially available antibiotic discs viz., Chloramphenicol (50 µg), Rifampicin (30 µg), Tetracycline (30 µg), Streptomycin (300 µg), Amoxyclav (30 µg), Cefixime (5 µg), Azithromycin (15 µg), Cephotaxime (30 µg), Ofloxacin (5 µg) and Ciprofloxacin (5 µg) (HiMedia, India). They were incubated at 37^{o}C for 24 h. All tests followed the testing and quality assurance practices outlined by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) [16].

Verification of Rifampicin Resistance

Rifampicin (100 mg) was dissolved and made up to 10 ml in formamide to obtain a final stock concentration of10 mg/ml. 1 ml of the solution is diluted to 10 ml with sterile purified water (1 mg/ml). 1 ml of solution was taken in a Petri dish and made up to 20 ml with MHA cooled to 45°C. A concentration of 50 µg/ml of Rifampicin was obtained. The medium was left to solidify. 0.5 ml of the sample was spread on a petri dish and incubated at 37°C for 24 to 48 hours.

Verification of Tetracycline Resistance:

Tetracycline hydrochloride (10.8 mg), corresponding to 10mg of the bases, was dissolved in sterile, purified water and made up to 10 ml with the same solvent (1mg/ml). One ml of the above solution was made up to 20 ml with MHA in a petri dish and cooled to 45°C to achieve a final concentration of 50 µg/ml of Tetracycline. Once the medium solidified, 0.5 ml of the sample was spread on a petri dish and incubated at 37°C for 48 to 72 hours.

Verification of Chloramphenicol Resistance:

Chloramphenicol (1g) was dissolved in ethanol-water and made up to 10 ml to obtain a 100 mg/ml concentration. It was diluted 1:1000 times to achieve a concentration of 1 mg/ml. To obtain a media preparation containing 50 µg/ml of chloramphenicol, 0.1 ml of this diluted solution was made up to 20 ml with MHA and allowed to cool to 45°C on a petri dish. 0.5 ml of the test sample was spread on the dish and incubated at 37°C for 24 to 48 hours.

Verification of Streptomycin Resistance:

A 10 ml stock of Streptomycin (10 mg) was made with sterile water to 10 mg/ml. One ml of this solution was made up to 20 ml with MHA and allowed to cool to 45°C and solidify on a petri dish to obtain a final concentration of 500 µg/ml of Streptomycin. 0.5 ml of a test sample was spread on the petri dish and incubated at 37°C for 24 to 48 hours.

7. pH and Transmittance

The pH of the products was measured directly from the sample using the pH meter (Make: Digisun Electronics, Model: 7007). Transmittance provides the measure of turbidity in the suspension. The Optical density and Transmittance of the products were measured directly from the sample using the UV-visible spectrophotometer (Make: Shimadzu; Model: UV-1601).

Results

1. Estimation of total spore quantity of oral suspension sample

Comparing the actual spore count to the numbers claimed on the label, Bifilac Clausi and Enterogermina matched the count indicated in their respective label (Table 2).

Table 2: Total spore count of oral suspension sample.

Sl. No.	Product Name	Manufacturer	Batch No.	Mfg. Date	Claim (CFU)	Observed Result (CFU)
1	Bifilac Clausi	Tablets (India) Limited	AHLAISI	03/2021	2.0 Billion	2.3 Billion
2	Enterogermina	Sanofi S.p.A.	01234	11/2020	2.0 Billion	2.1 Billion

Sl. No.	Product Name	Manufacturer	Batch No.	Mfg. Date	Claim (CFU)	Observed Result (CFU)
3	Tufpro	Unique biotech ltd	ZEY0017	04/2021	2.0 Billion	1.8 Billion
4	Novogermina	Unique biotech ltd	NTF21008UH	05/2021	2.0 Billion	1.7 Billion
5	Progermina	Genetek Life sciences, Pvt. Ltd,	GBC21016	04/2021	2.0 Billion	1.8 Billion
6	Entromax	Virchow Biotech Pvt Ltd	A6EEU085	03/2021	2.0 Billion	0.1 Billion

2. Simple and Spore staining

Evaluating the quality of the spores by microscopic examination indicated that Bifilac Clausi and Enterogermina contained only spores, and other marketed brands that were tested contained a mix of spores, debris, or vegetative cells (figure 1). The microscopic examination of spore staining (figure 2) demonstrated that Enterogermina and Bifilac Clausi had the highest density with quality spores. Entromax had the least density of spores. The other samples had other debris and vegetative forms.

Figure 1: Simple staining of a) Bifilac Clausi b) Enterogermina c) Tufpro d) Progermina e) Novogermina f) Entromax

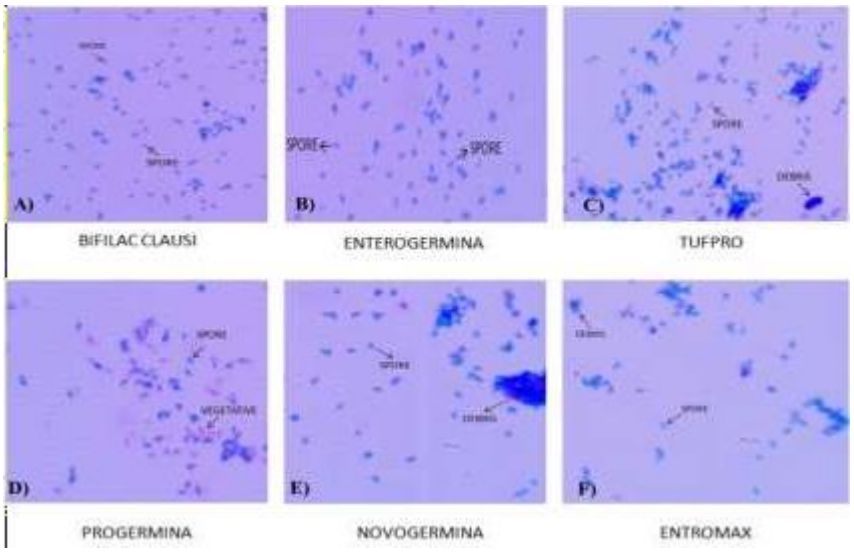
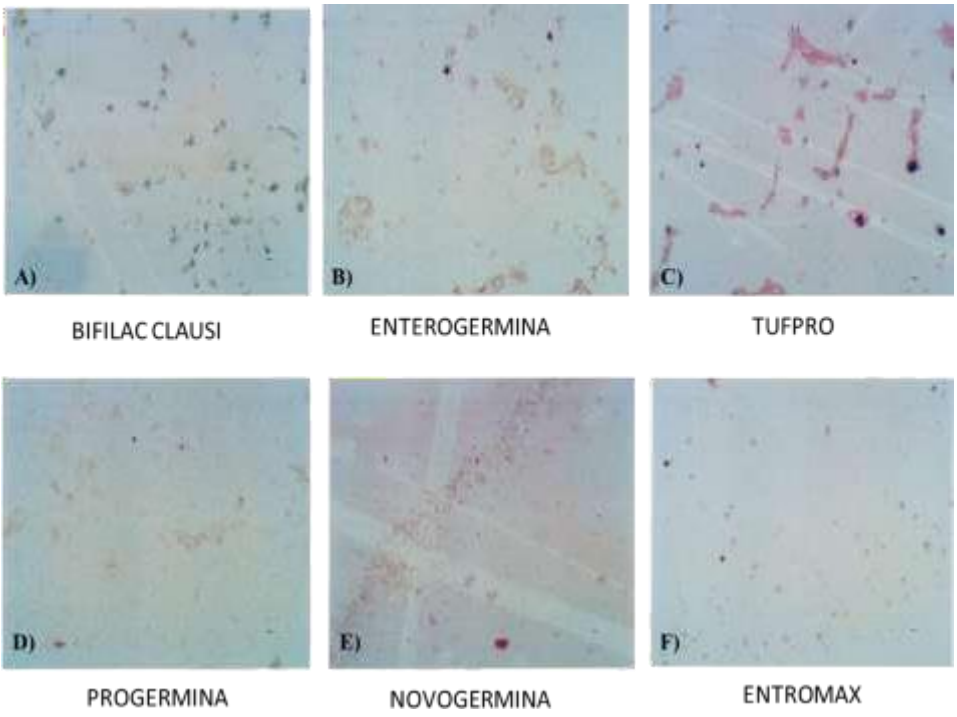


Fig 2: Spore staining a) Bifilac Clausi b) Enterogermina c) Tufpro d) Progermina e) Novogermina f). Entromax



3. Microbial purity of strains:

None of the products had pathogenic microorganisms (Escherichia coli, Pseudomonas aeruginosa, Salmonella enterica, Shigella boydii, Candida albicans, Staphylococcus aureus and Total Yeast and mould).

4. Isolation of strains

Based on the 16S rRNA sequencing, the total number of strains isolated from Bifilac Clausi and Enterogermina oral suspension was four, and the other tested products had only one strain isolated (Table 3).

Table 3: Test isolates by 16S rRNA gene sequencing

S. No.	Product Name	Name of the strains identified	No: of strains identified	No: of Probio similar strains
1	Bifilac Clausi	Bifilac Clausi 1	4	4
		Bifilac Clausi 2		
		Bifilac Clausi 3		
		Bifilac Clausi 4		
2	Enterogermina	Enterogermina 1	4	4
		Enterogermina 2		
		Enterogermina 3		
		Enterogermina 4		
3	Tufpro	Tufpro	1	None
4	Novogermina	Novogermina	1	None
5	Progermina	Progermina	1	None
6	Entromax	Entromax	1	None

5. Characterisation of strains

Based on 16S rRNA sequencing, only Bifilac Clausi had four strains similar to that in the international reference product (Table 3). The other strains isolated from the other samples did not match the identity of the ones in Enterogermina. Therefore, only Bifilac Clausi contained probiotic strains taxonomically similar to Enterogermina, as shown by 16S DNA sequencing.

6. Antibiotic resistance pattern

Bifilac Clausi and Enterogermina were resistant to all the ten antibiotics tested. (Table 4).

Table 4: Antibiotic resistance/sensitivity patterns of isolates

Antibiotics	Bifilac Clausi	Enterogermina	Tufpro	Novogermina	Progermina	Entromax
Chloramphenicol	R	R	S	S	S	S
Rifampicin	R	R	S	S	S	S
Tetracycline	R	R	S	S	S	S
Streptomycin	R	R	S	S	S	S
Amoxyclav	R	R	S	S	S	S
Cefixime	R	R	R	R	S	R
Azithromycin	R	R	R	R	R	R
Cephotaxime	R	R	S	S	S	S
Ofloxacin	R	R	S	S	S	S
Ciprofloxacin	R	R	S	S	S	S

*S: Sensitive; R: resistance

7. pH and Transmittance

Analysis of the physical properties revealed that among the samples, only Bifilac Clausi had almost the same pH and transmittance as the international reference standard, Enterogermina (Table 5).

Table 5: pH and transmittance **of products**

Sl. No.	Product Name	pH	Absorbance	Transmittance
1	Bifilac Clausi	6.95	0.858	13.9%
2	Enterogermina	6.91	0.835	14.6%
3	Tufpro	7.30	1.228	5.9%
4	Novogermina	7.02	1.437	3.7%
5	Progermina	7.78	1.567	2.7%
6	Entromax	7.20	0.047	89.5%

Discussion

This study defines a comprehensive approach using time-tested methods to evaluate the probiosimilarities between different marketed probiotics. We employed a two-folded strategy comprising microbiological evaluation and genomic analysis to demarcate the features of

probiosimilarities of five different marketed probiotics products of *Bacillus clausii* in India compared to the international reference brand, Enterogermina.

The microbiological examination mainly included qualitative and quantitative characterisation. All labels of a marketed probiotic product list the expected viable spore count during the product's shelf-life. The actual viable spore count is directly proportional to the quality of the probiotics. A lower count of viable microorganisms could impede a practical health benefit. Food and Agriculture Organization (FAO) and the World Health Organization (WHO) recommend listing the number of viable cells in a probiotic formulation as an essential qualitative attribute of the product.[17] This is an essential aspect of probiotics tailored to benefit the consumer. Focusing on this attribute, the quantitative evaluation of the number of spore counts revealed that among the tested products, the estimated spore count of only Bifilac Clausi and Enterogermina matched the indicated spore count in their respective labels (Table 2). The rest of the formulations had a significantly lesser spore count than what was mentioned on the label. Patrone et al. reported a similar mismatch between the label of the marketed product and the laboratory estimate for the *Bacillus clausii* spore count for five commercial probiotic products manufactured and marketed in India and Pakistan.[18] They found that only two of the five brands, Enterogermina and Ospor, contained *B. clausii* spores, as indicated by the label.

We next considered the quality of the spores in the tested products against the reference product by spore staining technique. We found that only Bifilac Clausi and Enterogermina samples contained the utmost matched purity of the spores. Other marketed products contained debris or vegetative cells in high quantities. The administration of probiotic products that do not comply with the required quality most likely leads to reduced efficacy.

We also performed analytical and chemometric assessments of the samples. Among the samples, we found that only Bifilac Clausi had almost the same pH and transmittance as the international reference standard, Enterogermina (Table 5). The results of the analysis of the physical properties corroborate with the quality estimation of the samples. Enterogermina and Bifilac Clausi have the optimal spore count, as indicated by the lowest transmittance of 13-15%. Entromax had the least spore count.

The antibiotic susceptibility pattern of probiotics is paramount in clinical settings as probiotics are designed to not interfere with the working of antibiotics. Probiotics can contribute to intestinal microbial balance, especially when co-administered with antibiotic therapy. They could help restore the balance of the beneficial microbial flora of the digestive tract. [19] Probiotic formulations containing *Bacillus clausii* strains are recommended to be used along with various antibiotics. Only Bifilac Clausi and Enterogermina are resistant to all the tested commonly used antibiotics. [20] This indicates that Bifilac Clausi has been designed to retain the antibiotic trait (Table 5). Concerns have been raised regarding the transfer of antibiotic resistance of probiotics to pathogenic organisms through horizontal gene transfer. This depends on the localisation of the resistance gene elements along the chromosome. Based on the 16S rRNA genomic characterization of the products, our study findings did not indicate any mutagenic strains or

transfer or acquisition of new antibiotic resistance genes in Bifilac Clausi or Enterogermina (Table 3).

In the genomic context, the strain identity of probiotics is highly relevant and vital to reap the positive benefits of the species. Enterogermina oral suspension had four strains. Among the other marketed products of *Bacillus clausii*, we could isolate four strains only from Bifilac Clausi (Table 3). In contrast, all other marketed products tested had only a single strain of *Bacillus clausii*. Needless to say, the number of strains in a probiotic product is a crucial aspect of its quality and efficacy.

Thus, our comprehensive approach evaluates the relevant and essential attributes of probiotics that relate to their promising potential. It successfully engages microbiological and physical techniques in spore characterization and the quality of the products. Additionally, the independent genomic approach was instrumental in strain characterization. Using this multimodal approach, we could successfully compare and identify the Indian brands of *Bacillus clausii* to display the potential probiotics characteristics that closely match the attributes of the international reference brand, Enterogermina. Among the various marketed brands of *Bacillus clausii* tested for the seven qualitative and quantitative parameters, only Bifilac Clausi was found to be probiosimilar to the international reference standard, Enterogermina.

Data Sharing: 16S rRNA sequencing report can be provided upon request.

Acknowledgements: Nil

Declaration of interest statement: Nil

References

1. Amara, A.A. and Shibl, A., 2015, ‒Role of probiotics in health improvement, infection control and disease treatment and management‖, Saudi Pharm J.,23(2), pp.107-14.
2. Kechagia, M., Basoulis, D., Konstantopoulou, S., Dimitriadi, D., Gyftopoulou, K., Skarmoutsou, N., et al.,2013, ‒Health benefits of probiotics: a review‖, ISRN Nutr.,pp.481651.
3. Nagpal, R., Kumar, A., Kumar, M., Behare, P.V., Jain, S. and Yadav, H.,2012, ‒Probiotics, their health benefits and applications for developing healthier foods: a review‖, FEMS Microbiol Lett., 334(1), pp.1-15.
4. Kort, R., Westerik, N., Mariela Serrano, L., Douillard, F.P., Gottstein, W., Mukisa, I.M., et al. 2015,‒A novel consortium of Lactobacillus rhamnosus and Streptococcus thermophilus for increased access to functional fermented foods‖, Microb Cell Fact.,14(1), pp.195.
5. Merenstein, D. and Salminen, S., 2017, ‒Probiotics and prebiotics‖, World Gastroenterology Organisation Global Guidelines.
6. Su, G.L., Ko, C.W., Bercik, P., Falck-Ytter, Y., Sultan, S., Weizman, A.V., et al. 2020, ‒AGA clinical practice guidelines on the role of probiotics in the management of gastrointestinal disorders‖,Gastroenterology, 159(2), pp.697-705.

7. McFarland, L.V., Evans, C.T., Goldstein EJC.,2018. ‒Strain-specificity and disease-specificity of probiotic efficacy: A systematic review and meta-analysis‖, Front Med (Lausanne)5, pp.124.

8. Szajewska, H., Canani, R.B., Guarino, A., Hojsak, I., Indrio, F., Kolacek, S., et al. 2016, ‒Probiotics for the prevention of antibiotic-associated diarrhea in children‖, J Pediatr Gastroenterol Nutr.,62(3), pp. 495-506.

9. Khatri, I., Tomar, R., Ganesan, K., Prasad, G.S. and Subramanian, S.,2017, ‒Complete genome sequence and comparative genomics of the probiotic yeast Saccharomyces boulardii‖, Sci Rep., 7(1), pp. 371.

10. Cruchet, S., Furnes, R., Maruy, A., Hebel, E., Palacios, J., Medina, F., et al. 2015, ‒The use of probiotics in pediatric gastroenterology: a review of the literature and recommendations by Latin-American experts‖,Paediatr Drugs., 17(3), pp.199-216.

11. Hill, C., Guarner, F., Reid, G., Gibson, G.R., Merenstein, D.J., Pot, B., et al. 2014, ‖Expert consensus document. The International Scientific Association for Probiotics and Prebiotics consensus statement on the scope and appropriate use of the term probiotic‖, Nat Rev Gastroenterol Hepatol., 11(8), pp.506-14.

12. Imperial, I.C.V.J. and Ilana, J.A.,2016, ‒Addressing the antibiotic resistance problem with probiotics: reducing the risk of its double-edged sword effect‖, Front Microbiol.,7, pp.1983.

13. Ghelardi, E., Celandroni, F., Salvetti, S., Gueye, S.A.,Lupetti, A. and Senesi, S.,2015, ‒Survival and persistence of Bacillus clausii in the human gastrointestinal tract following oral administration as spore-based probiotic formulation‖ J ApplMicrobiol., 119(2), pp.552-9.

14. Thota, P., Thota, A., Medhi, B., Sidhu, S., Kumar, P., Selvan, V.K., et al. 2018, ‒Drug safety alerts of pharmacovigilance programme of India: A scope for targeted spontaneous reporting in India‖,Perspect Clin Res., 9(1), pp.51-5.

15. Kumar, S., Stecher, G. and Tamura, K., 2016,‒MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets‖, Mol BiolEvol., 33(7), pp.1870-4.

16. European Food Safety Authority (EFSA) EFSA. ‒Technical guidance-Update of the criteria used in the assessment of bacterial resistance to antibiotics of human or veterinary importance‖. EFSA J. 2008, 6, pp.732.

17. Sharma, S., Arora, M. and Baldi, A.,2013, ‒Probiotics in India: current status and future prospects‖, Pharm Aspire., 1, pp.1-12.

18. Patrone, V., Molinari, P. and Morelli, L.,2016, ‒Microbiological and molecular characterization of commercially available probiotics containing Bacillus clausii from India and Pakistan‖, Int J Food Microbiol., 237, pp.92-7.

19. Gueimonde, M., Sánchez, B., G de Los Reyes-Gavilán, C. andMargolles, A.,2013, ‒Antibiotic resistance in probiotic bacteria‖, Front Microbiol., 4, pp.202.

20. Ouwehand, A.C., Forssten, S., Hibberd, A.A., Lyra, A. and Stahl, B. 2016, ‒Probiotic approach to prevent antibiotic resistance‖, Ann Med., 48(4), pp.246-55.