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A Comparative Evaluation of Bifilac Clausi Vs Enterogermina and Other Selected Brands for Probiosimilarity

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Abstract
Purpose: *To compare the qualitative and quantitative parameters of various Bacillus clausii brands that are available in the Indian market with the International reference product, Enterogermina.*
Study Centre: *JSS College of Pharmacy, Ooty*
Study Period: *06th Nov 2022 to 30th Dec 2022*
Methods & Materials: *Various parameters like total spore count, number of strains isolated (based on their sensitivity/ resistance pattern to antibiotics), pH & Transmittance, antibiotic resistance test, microbial purity, DNA sequencing and species identification were tested with various Bacillus Clausii brands that are available in Indian market and were compared with the International reference product, Enterogermina.*
Results: *Only Bifilac Clausi (Bacillus clausii - TIL 19T, TIL 21C, TIL 28S, TIL 30R) a product of Tablets (India) Limited passed all the qualitative and quantitative parameters in terms of assay, same number of strains (4 strains), taxonomical similarity, pH and suspension clarity, as well as resistance/sensitivity pattern to commonly used antibiotics.*
Conclusion: *Based on the results of the testing of various Bacillus clausii formulations, we conclude that only Bifilac Clausi (Bacillus clausii - TIL 19T, TIL 21C, TIL 28S, TIL 30R) showed a similarity to the International reference product, Enterogermina. Hence Bifilac Clausi was found to be probiosimilar to Enterogermina and is a viable alternative to Enterogermina to provide similar probiotic benefits.*
Keywords: *[Probiosimilar, Bacillus clausii formulation, Indian market, Qualitative & Quantitative parameters, 16S RNA sequence]*

Introduction

Probiotics are defined as **"**live microorganisms which, when administered in adequate amounts, confer a beneficial effect on host health**"** (Hill et al., 2014). In the recent past, probiotics has been used in various therapy areas to correct the dysbiosis in gastrointestinal issues, addressing antibiotic associated side effects, immune system

modulation, dysbiosis in genitourinary tract, lactose intolerance and used in variety of clinical conditions to restore the microflora in humans.

The last 10 years have been a game changer for probiotic market as novel and newer probiotics preparations have been launched across the globe and both patients and medical fraternity has started to realise importance of probiotics in an array of health conditions. Different types of probiotic strains are known to act in specific systems of human bodies and have gained importance in clinical applications in respective therapy areas.

Commercially available probiotic strains are manufactured, cultured and undergo various levels of standardization and each strain has been assigned a specific deposit number. All probiotic strains have been given an International Depository Authority (IDA) like the ATCC, DSM or CNCM which is specific for a particular strain and a specific health value is clearly attributable to the strain specified for a specific deposit number. It is generally believed that that a specific strain with a defined code number offers unique health benefits. Similar to biomolecules, the idea of generic probiotics and the creation of formulations that are similar to those of a patent-expired product can provide a workable solution to increase probiotic access for people in developing nations. The methods for demonstrating the clinical equivalency of biosimilars to generic biomolecules have advanced significantly. Similar need exists in the case of probiotics too. Though the guidelines like The European Society for Paediatric Gastroenterology Hepatology and Nutrition (ESPGHAN) and World Gastroenterology Organization (WGO) recommend probiotic strains for various clinical disorders, an effective method for assessing the biosimilarities between probiotics is missing.

Studies have shown that the safety and effectiveness of probiotics cannot be generalized because each strain is unique. Differences in strain-specific efficacy were first reported in 2010 with the help of genomic analysis that characterized bacterial and fungal strains in detail. International probiotics guidelines and experts in probiotics recommend to mention the strain numbers or designations of probiotics in the studies. However, these norms are yet to be consistently applied.

A robust approach to evaluate the bio-similarities between marketed probiotics still needs to be developed. 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 at JSS College of Pharmacy, Ooty.

Methodology

A total of 6 different brands of *Bacillus Clausii* were tested and compared with the International reference product *Bacillus Clausii*, Enterogermina. The details of the formulations are listed in the table 1 below.

Table 1: Characteristics of products containing Bacillus Clausii

Sl. No.	Manufacturer	Brand Name	Batch No.	Mfg. Date
1	Tablets (India) Limited	Bifilac Clausi	AHLA2I1	05/2022
2	Sanofi S.p.A.	Enterogermina	21286	07/2022
3	Unique biotech ltd	Tufpro	ZEZ0042	07/2022
4	Unique biotech ltd	Novogermina	NTF22012UH	08/2022
5	Unique biotech ltd	Gutgermina	GMSUQ2221	06/2022
6	Unique biotech ltd	Entroflora	UB01822	06/2022
7	Virchow Biotech Pvt Ltd	Entromax	A6EEU085	03/2021

Evaluation of Quantitative & Qualitative Parameters

1. **Estimation of total spore quantity in the oral suspension**

The total spores in oral suspension samples were enumerated as described by Ghelardi et al. with relevant modifications[10]. The samples were serially diluted in 0.1% peptone and seeded (100 µl per plate) on MHA plates for enumeration of B. clausii strains. Plating was performed in triplicate and the plates were incubated at 37°C for 24 h.

2. **Simple staining and spore staining:**

A sample smear was prepared using a sterile technique and then 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. The washed slide was examined under a microscope. The sample was observed for the presence of bright green spores and brownish-red to pink vegetative cell morphology

3. **Detection of microbial purity of spores:**

The detection of some of the most commonly found microbial contaminants 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 moulds count from Bifilac Clausi, Enterogermina, Entromax, Novogermina, Gutgermina, Entroflora and Tufpro oral suspension were carried out using the Indian Pharmacopoeia, 2018 procedure.

4. **Strain isolation based on antibiotic sensitivity/resistance pattern.**

Ghelardi et al. described a method for isolating antibiotic resistance strains from oral suspension samples[13]. For the selection of B. clausii strains, samples were serially diluted in 0.1% peptone and seeded (100 µl per plate) on MHA plates containing antibiotics. Plating was done in triplicate and incubated for 24 hours at 37-degree C.

5. **Taxonomical identification by 16S r RNA sequencing**

The isolates after molecular characterization was sequenced by 16S rRNA using 27F (5’ AGAGTTTGATCCTGGCTCAG-3’) and 1492R (5’- TACGGYTACCTTGTTACGACTT-3’) primers. Gene sequences of 16S rRNA were used to construct the phylogenetic tree for determination of nearest bacterial species by UPGMA method using MEGA 7.0.21 software [11].

Genomic DNA extraction procedure: Purified microbial colonies were cultured in LB broth for 24 hours in a shaker at the optimum temperature. DNA was isolated from gram-negative or gram-positive bacteria by centrifuging a 1ml cell suspension at 8000g for 2 minutes to pellet cells. The cells were washed twice with 400µl STE Buffer (100 mM NaCl, 10 mM Tris/HCL, 1 mM EDTA, pH 8.0) after the supernatant was removed. The cells were then centrifuged at 8000g for 2 minutes at 10°C, resuspended pellets in 200 µl TE Buffer (10 mM TRIS/HCL, 1 mM EDTA, pH 8.0). 20 µl lysozyme (20 mg/ml) was added and incubated for 30 minutes at 37 C. This tube was then filled with 100 µl Tris-saturated phenol (pH 8.0), and the vortex mixing was carried out for the 90s to lyse cells. Following that, the samples were centrifuged at 13000g for 5 minutes at 4°C to separate the aqueous phase from the organic phase. A total of 160 µl of upper aqueous phase was transferred to a clean 1.5 ml tube. To make 200 µl, 40 µl TE buffer was added and combined with 100 µl chloroform before centrifuging for 5 minutes at 13000 g at 4°C. The lysate was purified by chloroform extraction until no white interface was visible. The upper aqueous phase from 150 µl was transferred to a clean 1.5 ml tube. 1 ml of 100% ethanol was added and

centrifuged at 13000 g for 5 minutes at 4°C. After discarding the supernatant, the pellet was rinsed with 1 ml of 70% ethanol (centrifuged for 2 minutes at 13000 g at 4°C). After air drying, the pellet was dissolved in 40 µl of TE buffer/nuclease-free water. Purified DNA was then utilised immediately in the following experiment or stored at -20°C. Electrophoresis in a 0.7% agarose gel in 1 TAE buffer was used to resolve the purified genomic DNA products. After that, the gels were pre-stained with 10 mg/ml ethidium bromide.

PCR Amplification: A PCR was performed in a total volume of 20 µl containing10 µl master mixture, 1 µl of µM each of 8F (5′ AGAGTTTGATCCTGGCTCAG3′) and 1942R (5′ GGTTACCTTGTTACGACTT3′) and 40 ng template DNA. PCR conditions were as follows: denaturation at 95°C for 5 min; 30 cycles of 94°C for 1 min, primer specific annealing temperature at 53.8°C for 45 sec and extension at 72°C for 1 min, and a final extension at 72°C for 5 min. The PCR products were resolved by electrophoresis in a 1% agarose gel in 1 × TAE buffer. The gels were pre-stained with 10 mg/ml ethidium bromide.

DNA Sequencing and species identification: Amplified PCR product was purified using Qiaquick PCR purification kit (QIAGEN, USA). Then sequencing reactions were carried out in both directions using same forward and reverse primers used for amplification with BigDye Version 3.1 kit (Applied Bio-systems) on an ABI-PRISM 3730 DNA Sequencer (Applied Bio-systems). And ambiguous sequences from the base called sequences were corrected with Chromas (Version 2.01) and the sequences were assembled with Bio-Edit (Version 7.0.9.0). Later the search for sequence homolog of potential isolate for species identification was made using the BLASTn program (NCBI) and the nucleotide sequence was verified using GenBank.

6. Antibiotic susceptibility test

The antibiotic susceptibility test was performed using Mueller–Hinton agar 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 (15µg), Cefotaxime (30 µg), Ofloxacin (5 µg) and Ciprofloxacin (5 µg) (Himedia, India) and incubated at 37 °C for 24 h. All tests followed the testing and quality assurance practices outlined by the European Committee on Antimicrobial Susceptibility Testing (Eucast) (http://www.eucast.org) [12]

7. pH and Transmittance

The pH of the products were 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 was measured directly from the sample using the UV-visible spectrophotometer (Make: Shimadzu; Model: UV-1601).

Results

1. Estimation of total spores' quantity of oral suspension sample

Bifilac Clausi, Tufpro and Enterogermina matched the count provided on their respective labels when the actual spore count was compared to the counts claimed on the label. (Table 2)

Table 2: Total spore counts of tested products

Manufacturer	Brand Name	Batch No.	Mfg. Date	Claim (CFU)	Observed Result (CFU)
Tablets (India) Ltd	Bifilac Clausi	AHLA2I1	05/2022	2.0 Billion	2.3 Billion
Sanofi S.p.A.	Enterogermina	21286	07/2022	2.0 Billion	2.1 Billion
Unique biotech Ltd	Tufpro	ZEZ0042	07/2022	2.0 Billion	2.0 Billion
Unique biotech Ltd	Novogermina	NTF22012UH	08/2022	2.0 Billion	1.8 Billion*
Unique biotech Ltd	Gutgermina	GMSUQ2221	06/2022	2.0 Billion	1.9 Billion*
Unique biotech Ltd	Entroflora	UB01822	06/2022	2.0 Billion	1.9 Billion*
Virchow Biotech	Entromax	A6EEU085	03/2021	2.0 Billion	0.5 Billion*

*Not meeting the label claim

2. Simple staining and spore staining:

By examining the spores under a microscope, it became apparent that Bifilac Clausi and Enterogermina alone contained spores, while the other examined commercial brands contained a mixture of spores, debris, or vegetative cells. The maximum density of high-quality spores was found in Enterogermina and Bifilac Clausi, according to the microscopic analysis of spore staining (figure 1 & 2). Entromax has the lowest spore density.

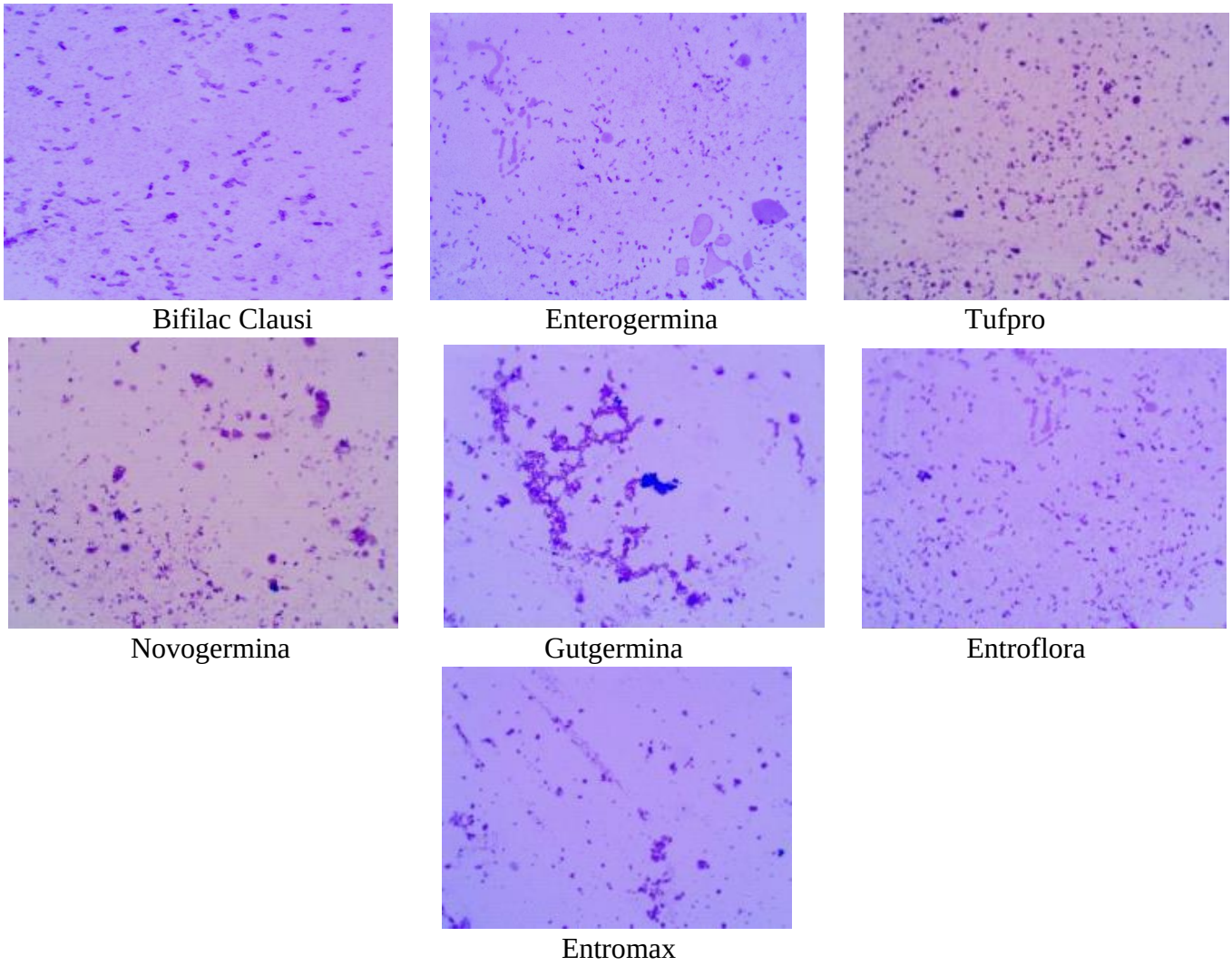

Fig 1 : Simple Staining of Probiotic brands

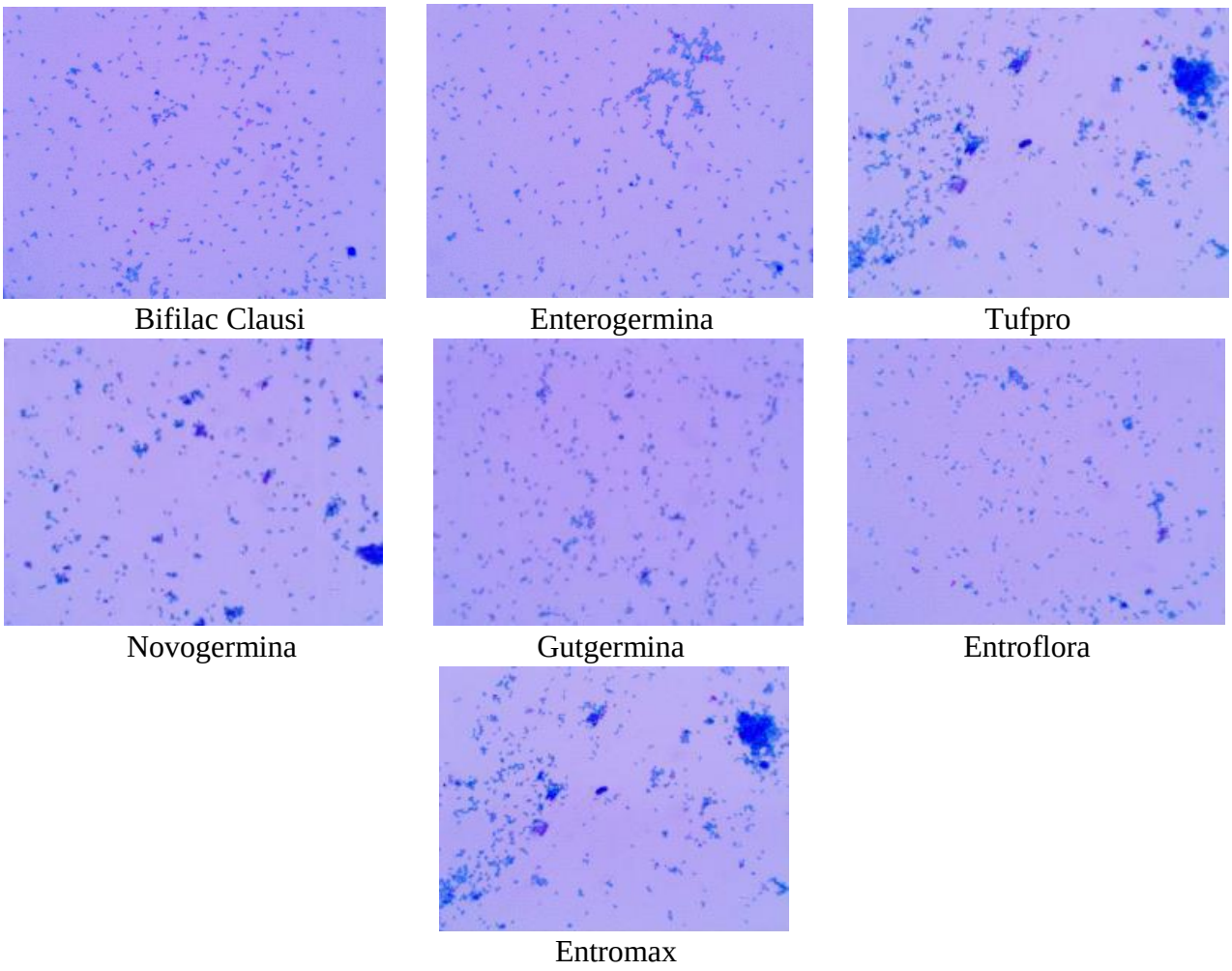

Fig 2 : Spore Staining of Probiotic brands

3. Microbial purity of strains:

None of the tested products contained any harmful bacteria, such as *Escherichia coli, Pseudomonas aeruginosa, Salmonella enterica, Shigella boydii, Candida albicans, Staphylococcus aureus,* or Total Yeast and Mould.

4. Isolation of strains

Four strains were isolated from Bifilac Clausi and Enterogermina oral suspension based on 16S rRNA sequencing whereas the other tested products had only one strain isolated (Table 3).

Table 3: Test isolates by 16S rRNA gene sequencing

Sl. No.	Manufacturer	Brand Name	Number of strains isolated
1	Tablets(India)Limited	Bifilac Clausi	4
2	Sanofi S.p.A.	Enterogermina	4
3	Unique biotech ltd	Tufpro	1
		Novogermina	1
		Gutgermina	1
		Entroflora	1
4	Virchow Biotech Pvt Ltd	Entromax	1

5. Characterisation of strains

Based on 16S rRNA sequencing, only Bifilac Clausi had four strains similar to that in the international reference product (Table 4). The strains isolated from the other marketed products did not match the identity of the ones in Enterogermina. Therefore, only Bifilac Clausi contained probiotic strains taxonomically similar to Enterogermina, as confirmed by 16S rRNA sequencing.

Table 4: Characterisation of strains

Product Name	Name of the strains identified	Number of strains identified	Number of Probiosimilar strains
Bifilac Clausi	Bifilac Clausi I	4	4
	Bifilac Clausi 2		
	Bifilac Clausi 3		
	Bifilac Clausi 4		
Enterogermina	Enterogermina I	4	4
	Enterogermina 2		
	Enterogermina 3		
	Enterogermina 4		
Tufpro	Tufpro	1	None
Novogermina	Novogermina	1	None
Gutgermina	Gutgermina	1	None
Entroflora	Entroflora	1	None
Entromax	Entromax	1	None

6. Antibiotic resistance pattern

Both Bifilac Clausi and Enterogermina were resistant to all the ten antibiotics tested. (Table 5). The other products, including Tufpro, Novogermina, Gutgermina, Entroflora, and Entromax, showed sensitivity to all the antibiotics tested except for Cefotaxime and Azithromycin.

Table 5 : Antibiotic resistance pattern of Probiotic brands

Product name	Antibiotics									
	C	RIF	TE	HLS	AMC	CTX	AZM	CFM	OF	CIP
Bifilac Clausi	R	R	R	R	R	R	R	R	R	R
Enterogermina	R	R	R	R	R	R	R	R	R	R
Tufpro	S	S	S	S	S	R	R	S	S	S
Novogermina	S	S	S	S	S	R	R	S	S	S
Gutgermina	S	S	S	S	S	R	R	S	S	S
Entroflora	S	S	S	S	S	R	R	S	S	S
Entromax	S	S	S	S	S	R	R	S	S	S

Note: R- Resistance, S – Sensitive, C – Chloramphenicol, RIF – Rifampicin, TE - Tetracycline, HLS- Streptomycin, AMC – Amoxyclav, CFM – Cefixime, AZM – Azithromycin, CTX – Cefotaxime, OF – Ofloxacin, CIP – Ciprofloxacin.

7. pH and Transmittance

The pH of the products were 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). 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 6).

Table 6: pH & Transmittance of the tested

Manufacturer	Brand Name	pH	Absorbance (ABS)	Transmittance (%)
Tablets (India) Ltd	Bifilac Clausi	6.90	0.896	12.5 %
Sanofi S.p.A.	Enterogermina	6.98	0.871	13.6 %
Unique Biotech Ltd	Tufpro	7.18	1.152	6.2 %
	Novogermina	7.10	1.238	5.5 %
	Gutgermina	7.15	1.208	5.1 %
	Entroflora	7.20	1.180	6.0 %
Virchow Biotech Pvt. Ltd	Entromax	7.20	0.047	89.5%

Discussion

This study outlines a comprehensive methodology that uses tried-and-true methods to evaluate probiosimilarities between commercially available probiotics. When comparing seven different *Bacillus clausii* probiotic products that are marketed in India to the international reference brand Enterogermina, we used a two-pronged strategy that included microbiological assessment and genetic analysis.

Characterization in both qualitative and quantitative terms was a major component of the microbiological analysis. The anticipated viable spore count during the course of the product's shelf life was listed on all labels of marketed probiotic products. The quantity of viable spores is closely linked to the probiotics quality. Reduced count of viable probiotics could deliver sub therapeutic effect and impede claimed health benefit.

The World Health Organisation (WHO) and the Food and Agriculture Organisation (FAO) advise including the number of viable cells in a probiotic formulation as a crucial quality characteristic of the final product[17]. With this characteristic in mind, the quantitative analysis of the number of spore counts showed that, among the tested products, only Bifilac Clausi, Tufpro and Enterogermina had estimated spore counts that matched with the reported spore counts on their respective labels (Table 2). The spore count in the remaining marketed products were much lower than what was stated on the label. Similar discrepancies between the label of the marketed product and the laboratory estimate for the *Bacillus clausii* spore count were noted by Patrone et al. Out of 7 tested commercial probiotic products made and marketed in India and Pakistan, Patrone et al. found a comparable discrepancy between the label of the product and the laboratory estimate of the *Bacillus clausii* spore count.[18] They discovered that only Enterogermina and Ospor, two of the five brands, actually contained B. clausii spores as claimed on the label.

We used the spore staining method to compare the quality of the spores in the tested products with that in the reference product. Only Bifilac Clausi had the highest matching purity of spores. Other products had significant amounts of debris or vegetative cells. Reduced efficacy is most likely caused by the administration of probiotic products that do not meet the necessary quality standards. We also evaluated the materials analytically and chemometrically. Only Bifilac Clausi, showed pH and transmittance that were almost identical to the

international reference standard, Enterogermina (Table 6). The lowest transmittance of 13–15% reveals the best spore counts in Enterogermina and Bifilac Clausi. The spore count was lowest in Entromax.

Since probiotics are created to not impede the effectiveness of antibiotics, their antibiotic susceptibility pattern is essential in clinical settings. Bifilac Clausi and Enterogermina, were resistant to most commonly used popular antibiotic.[19]

A probiotic product's quality and effectiveness greatly depends on the number of strains it contains. The probiotic strain identification is important in determining the species beneficial effects in the genomic setting. There were four strains of Bacillus Clausii in Enterogermina oral suspension. Only Bifilac Clausi among the other commercial *Bacillus clausii* products had isolates of four strains (Table 4). All other commercially available brands that were evaluated, in contrast, had just one strain of Bacillus clausii.

Thus only Bifilac Clausi was identified as being probiosimilar to the international reference standard, Enterogermina, out of the many marketed brands of *Bacillus clausii* that were evaluated for the seven qualitative and quantitative parameters.

Conclusion

Based on the results of the testing of *Bacillus clausii* formulations, it can be concluded that only Bifilac Clausi showed a similarity to the International reference product, Enterogermina. Bifilac Clausi (*Bacillus clausii* - TIL 19T, TIL 21C, TIL 28S, TIL 30R) was found to be probiosimilar to Enterogermina in terms of quantitative assay (CFU count), having the same number of strains (4 strains), taxonomical similarity, pH and suspension clarity, as well as resistance pattern to commonly used antibiotics. Therefore, it can be inferred that Bifilac Clausi is a viable alternative to Enterogermina and can be used to provide similar probiotic benefits.

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Disclosure: Nil

The author reports no conflicts of interest in this work.

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