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Study of prebiotic potential of *Terminalia chebula* and development of nasal synbiotic formulation

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ABSTRACT: The healthy population, including children and adults, naturally carries *Lactobacilli* in their nasal microflora, which probiotics can maintain. The potential benefits of probiotics and prebiotics to address nasal infections have not been thoroughly investigated. Hence, a novel reconstitutable synbiotic nasal spray dried powder (NSDP) has been proposed to protect and preserve the nasal microbiome. Several batches of NSDP were prepared by spray drying *Terminalia chebula* (*T. chebula*) with varying concentrations of carriers, including sorbitol, mannitol, and maltodextrin, to study the efficacy of the prebiotic potential of *T. chebula*. NSDP was evaluated for yield, LOD, micromeritic properties, size distribution by optical microscopy, laser diffraction, and surface topography by SEM. *L. casei* as a probiotic was added before spray drying to the optimized batch of NSDP. Probiotic-loaded NSDP was reconstituted and studied for pH, rheology, pump delivery, and viability enumeration before and after the spray drying and investigated for *in-vitro* efficacy by co-culture technique. Mannitol as a carrier showed parameters in a desirable range. The optimized mannitol batch yielded 37.3 %, with a moisture content of 4.6 ± 0.22 %, good flow properties, and an optimum particle size of 30.68± 4.1 µm. The pH of reconstituted optimized NSDP was about 6.2 to 6.3, with pump delivery 0.112 g/spray, and had shear thinning properties with significant results in viability and *in-vitro* efficacy by co-culture technique ($P < 0.0001$). The proposed NSDP formulation can be potentially used in restoring the nasal flora and can be further investigated for protective effects against nasal infections.

KEYWORDS: *Lactobacillus casei*; *Terminalia chebula*; Nasal spray dried powder; Probiotic; Prebiotic; Synbiotic.

1. INTRODUCTION

The nasal route is the most common pathway for entering viruses and bacteria, leading to many respiratory and local infections, including COPD, rhinitis, sinusitis, vestibulitis, Covid 19, and Mucor mycosis in the human body. After the Covid-19 pandemic, respiratory infection cases rose to millions, with more than 10 lakh deaths [1]. The dysbiosis of nasal flora is reported to play a significant role in acquiring these infections. Probiotics are one of the interesting therapeutic approaches in rebalancing the microflora and preventing infections. Despite being used predominantly by oral route for restoring GI flora, probiotics can confer health benefits and help in balancing microflora of other regions and showing promising potential for using such microorganisms as immune modulators [2]. Currently, the application of probiotics in respiratory health is being explored.

Probiotics are commonly used strains primarily belonging to the genera Bifidobacterium and *Lactobacillus* [3]. *Lactobacillus* species like *Lactobacillus casei* (*L. casei*) and *Lactobacillus plantarum* [4] have been proven effective in reducing the occurrence of nasal colonization with pathogenic bacteria [5].

The selection of a suitable probiotic for the nasal route depends on the ability of the probiotic to be tolerant to the local physiological conditions. Recent studies have demonstrated the effectiveness of *Lactobacillus casei* as a probiotic for the upper respiratory tract. It is reported to show preferred adherence to the epithelia of URT *in vivo*. Despite being an anaerobic strain, it is tolerant to nasal pH and higher oxygen levels. All these features make *L. casei* an ideal probiotic for URT [6].

Prebiotics refer to dietary fibers that are not readily soluble and are recognized for their ability to promote the growth of beneficial indigenous microorganisms, such as *lactobacilli*, thereby influencing the composition of the natural microflora and protecting against infections [7]. Established prebiotics include inulin,

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fructooligosaccharides (FOS), galactooligosaccharides (GOS), lactulose, and polydextrose, while emerging prebiotics include isomaltooligosaccharides (IMO), xylooligosaccharides (XOS), and lactitol. The inulin-derived (FOS) from chicory root, the arabinoxylooligosaccharides (AXOS) from wheat bran, and the xylooligosaccharides (XOS) have been shown to provide health benefits [8]. These prebiotics are now being explored for their health benefits in routes other than oral.

Terminalia chebula (*T. chebula*) is a constituent of the traditional medicine "Triphala" and can be used as a potential herbal prebiotic. *T. chebula* is reported to have anti-inflammatory, [9] antitumor, [10] antioxidant [11] anti-mutagenic activity, [12] can reduce blood sugar levels [13] and is useful in chronic sinusitis [14], asthma, [15] sore throat, [16] vomiting, hiccough, diarrhoea, dysentery, bleeding piles, ulcers, gout [17] heart [18] and bladder diseases [19]. *T. chebula* is also used in traditional medicine as 'Nasya' (nasal formulation) for treating sinusitis, cough, and allergic rhinitis [20].

The delivery of drugs through the nasal route has been used in ancient medicine. It can be an effective drug therapy requiring low doses and low oral bioavailability. Nasal administration is non-invasive, allows for precise dosing, and is easy to self-medicate. It provides a rapid onset of action, bypasses the gastrointestinal tract, and can effectively overcome the blood-brain barrier, resulting in high bioavailability. Additionally, the nasal route may be advantageous for actives that do not conform to the Lipinski rule [21]. The intranasal route of administration can be beneficial for developing new therapeutic drugs or improving the effectiveness of existing ones.

There has been a lack of exploration into using probiotics and prebiotics and formulating strategies targeting the upper respiratory tract through nasal administration. From previous research, Katarina Jokicevic et al. claimed that spray drying effectively maintains the viability and functionality of the recently isolated *L. casei* AMBR2 strain derived from the human upper respiratory tract (URT). It was found to be a better option for this specific strain when compared to traditional freeze-drying methods. In addition, powder formulation increases the stability of the formulation. In a preliminary study to test viability, safety, and effectiveness, spray drying showcased the potential and secure delivery of live *L. casei* AMBR2 cells via nasal administration in healthy volunteers [22].

Considering this, the present research aims to explore the prebiotic property of *T. chebula* and formulate a synbiotic formulation by combining it with the probiotic using spray drying. The formulated powder would be given by nasal route after reconstitution and would help maintain the nasal flora and prevent infections.

2. RESULTS AND DISCUSSION

2.1. Standardisation of *L. casei*

Gram staining and standardization of *L. casei* was performed against McFarland standard 4, and a linear relationship between OD and bacterial count (log cfu/ml) of *L. casei* was established as, y = 2926x + 7.9135. This equation was used for estimating the bacterial count from the OD values.

2.2. Characterization of *T. chebula* extract

The pharmacognostic evaluation of *T. chebula* values was within the acceptable range. With the optimized chromatographic conditions, the Rf value of standard gallic acid was found to be 0.65, as shown in Figure 1 (a). Overlay spectra of the peak of the same Rf value in the extract, as shown in Figure 1 (b), confirmed the presence of gallic acid in *T. chebula* extract.

2.3 Prebiotic efficacy of *T. chebula*

2.3.1. Growth kinetic of T. chebula

For the present work, *L. casei* was selected as a probiotic. The probiotic species administered nasally should be tolerant to the physiological conditions of the upper respiratory tract. The literature suggested good adherence and colonization capacity of *L. casei* in URT. Additionally, *L. casei* can tolerate the oxidative stress caused by aerobic conditions in the nasal passage. *In vitro* studies by (Ilke DE Boeck et al.) have confirmed the presence of a catalase gene dependent on manganese and haeme in *L. casei*, which is responsible for its oxygen tolerance [23].

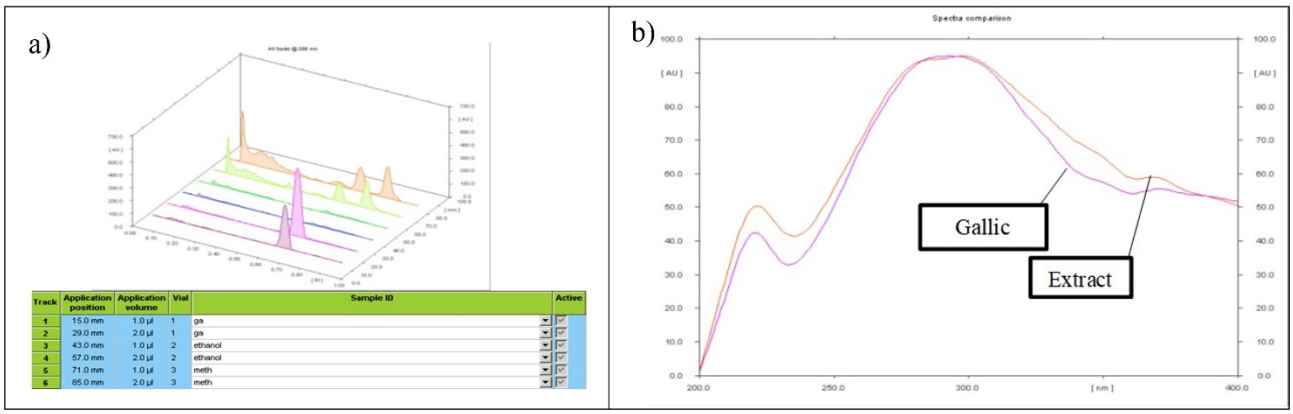

Figure 1. a) Densitogram obtained from High-Performance Thin-Layer Chromatography (HPTLC) analysis of Gallic Acid and extract. Gallic Acid exhibited a distinct peak at an Rf value of 0.65.; **b)** Overlay spectra of Gallic Acid and extract: A comparison of the UV-Vis spectra for Gallic Acid (purple) and the extract (red). The overlay highlights the distinctive absorption peaks, aiding in identifying and characterizing Gallic Acid within the extract.

T. chebula fruit extract is rich in hydrolyzable tannins and phenolic compounds, including gallic acid, chebulinic acid, ellagic acid, chebulagic acid, etc., attached to oligosaccharides or monosaccharides. The total carbohydrate content of *T. chebula* used as per literature is 36.3% with 16.4% crude fiber (Muhommad Tauseef Sultan et al.) [24]. *T. chebula* is an essential component of Triphala, an ayurvedic preparation used for treating GI ailments and improving GI functions. Considering these factors, the prebiotic potential of *T. chebula* was explored by investigating its effect on the growth of *L. casei*.

The sugar source of MRS was replaced with FOS and *T. chebula*, and the effect on *L. casei* was observed for 48h. *T. chebula* (5% w/v) showed significant positive effects on the growth of *L. casei*. There was a steady increase in the OD value of *L. casei* treated with *T. chebula*. The growth rate in the presence of *T. chebula* and FOS as standard was significantly higher ($p < 0.01$) compared with standard sugar sources in MRS. There was no significant difference in OD (p-value>0.1) of *T. chebula* and FOS.

The results of dry biomass further confirmed this against time. The value of μ max of *T. chebula* (0.1149 g. L^{-1} h^{-1}) and FOS (0.1396 g. L^{-1} h^{-1}) was higher compared to control (0.0923 g. L^{-1} h^{-1}). The td (doubling time) for FOS and *T. chebula* was found to be 4.9 hr and 6 hr, respectively. The results were plotted in the graph as shown in Figure 2(a) and 2(b).

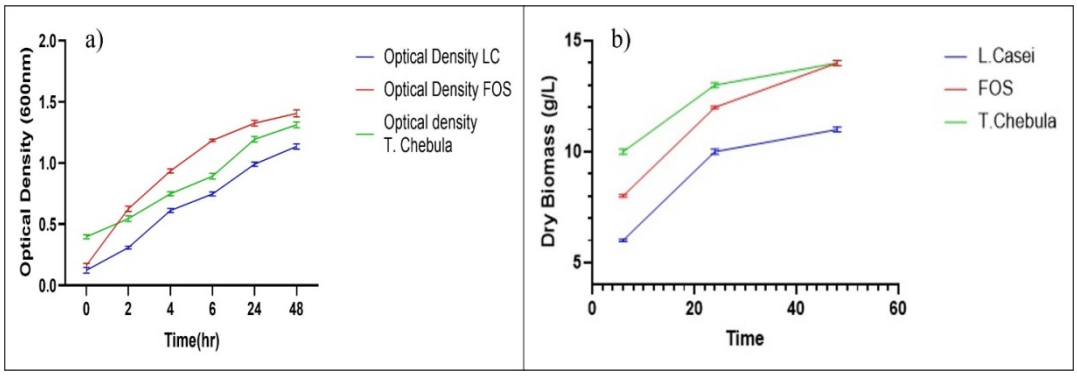

Figure 2. a) Effect of *T. chebula* on the growth of Lactobacilli casei (OD) over time; **b)** Effect of *T. chebula* on the growth of *L. casei* (dry biomass) over time.

*Error bars represent the standard deviation of three replicates.

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2.3.2. pH and % titratable acidity

L. casei can ferment sugar sources and produce various organic acids, including lactic acid is predominant. It utilizes 6-phosphogluconate/ phosphoketolose pathways to ferment sugar.

Table 1 indicates an increase in titratable acidity and a decrease in pH in all samples. The results were more pronounced at 24 h and 48h. FOS and *T. chebula* showed higher acid production compared to the control. *T. chebula* has more than 30% of carbohydrate content, the sugar source being mainly monosaccharides like glucose and fructose or oligosaccharides.

Table 1. pH and % titratable acidity of the blank, FOS, *T. chebula* samples.

Incubation time		pH	Titratable acidity (%)
6hr	Blank	6.28± 0.05	12.75 ± 0.4
	FOS	5.77 ± 0.06	12 ± 0.5
	T. chebula	6.24 ± 0.04	12.75 ± 0.3
24hr	Blank	6.27 ± 0.05	13.5 ± 0.3
	FOS	5.68 ± 0.04	14.75 ± 0.7
	T. chebula	6.16 ± 0.03	14.25 ± 0.5
48hr	Blank	6.25 ± 0.07	14.25 ± 0.6
	FOS	5.32 ± 0.04	15.75 ± 0.5
	T. chebula	6.01 ± 0.04	15 ± 0.3

The pH of the nasal cavity is usually in the range of 5.5 to 6.5, whereas it is higher (pH~7) in the nasopharyngeal region. *L. casei* was reported to be essential in maintaining this pH (Ilke De Boek et al.) [23]. The pH-lowering effect of *L. casei* was also confirmed, as mentioned in Table 1. After 48h, the pH of control was 6.25 ± 0.07, FOS was 5.32 ± 0.04, and with *T. chebula*, it was 6.01 ± 0.04. The pH lowering effect of FOS and *T. chebula* were almost similar (pH 0.009 and 0.005, respectively).

2.3.3. Antimicrobial activity

T. chebula contains polyphenol, an antimicrobial agent against many gram-positive pathogens, including *S. aureus*. The antimicrobial activity of *L. casei* supernatant was assessed against *S. aureus*, as mentioned in Table 2. All the samples demonstrated antibacterial activities at 24 and 48 h against *S. aureus*. *Lactobacillus* produces bacteriocin, which shows antibacterial activities against many pathogens, including *S. aureus* (Hanaa Khaleel Ibrahim et al.) [28]. *S. aureus* are gram-positive bacteria known to be sensitive to bacteriocin and showed significant zones against *S. aureus*. ($P>0.1$). The zone of inhibition of FOS and *T. chebula* were higher (P value 0.03 and 0.001) against *S. aureus* compared to the control. This was attributed to the highest growth of *L. casei* in the presence of prebiotics resulting in higher bacteriocin. The zone of inhibition of *T. chebula* was higher ($P <0.0001$) at 6h for *S. aureus*. In contrast, at 24h and 48h, there was no significant difference in FOS and *T. chebula* zones, as shown in Figure 3. The higher zone of inhibition for the *T. chebula* sample within the first 6h of the study could be attributed to the antibacterial effect of *T. chebula*.

Table 2. Antimicrobial activity of cell supernatant against *S. aureus*.

Sample	Zone of Inhibition at 6hr (cm)	Zone of Inhibition at 24hr (cm)	Zone of Inhibition at 48hr (cm)
L. casei	0.7 ± 0.5	0.8 ± 0.5	0.8 ± 0.5
FOS	0.9 ± 0.4	1.2 ± 0.3	1.3 ± 0.4
T. chebula	1.4 ± 0.5	1.5 ± 0.4	1.5 ± 0.4

All values are n =3 ±SD.

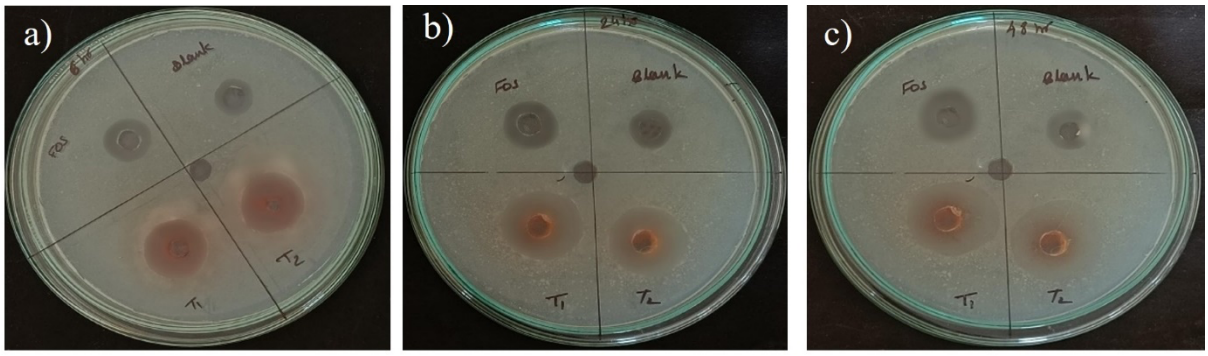

Figure 3. Antimicrobial Activity on *S. aureus*: **a)** 6 hours; **b)** 24 hours; **c)** 48 hours of exposure.

The figure showcases the time-dependent effects on the growth inhibition of *S. aureus*, providing its potential as an antimicrobial agent.

2.4. Formulation and Evaluation of NSDP

A spray-dried powder formulation of *L. casei* and *T. chebula* was prepared considering the stability of probiotics in powder form. Sugars like sorbitol, maltodextrin, and mannitol were screened in combination with *T. chebula* for spray drying of probiotics. Prebiotic sugars are reported to encapsulate the probiotic during spray drying and render thermal protection and stability. During spray drying, sugar molecules replaced the bacterial cell membrane, stabilized the bilayer membrane structure, and protected it from disruption. *L. casei* samples were harvested for spray drying during its early stationary phase (18 h culture). Various NSDP formulations (B1, B2, B3) were obtained using a combination of *T. chebula* and sorbitol, maltodextrin, or mannitol without probiotics. Batch B1 with sorbitol yielded only 12% yield with very high moisture content, whereas maltodextrin NSDP had the highest yield of 44.4%. However, batch (B2) was very hygroscopic, producing 6.18% of moisture. The moisture level in probiotic powder may affect the stability and shelf life. When probiotic was added to maltodextrin formulation (B6), the yield was drastically reduced to 8.5%. Formulation B3 with mannitol as the carrier showed 34.4% yield and 5.3% moisture content. Considering the yield and moisture content, mannitol was selected as a carrier, and the effect of the mannitol ratio with *T. chebula* on these parameters was further studied. When ratio of mannitol: *T. chebula* was varied from 1:1(B3), 2:1 (B4) and 3:1(B5) there was change in yield from 34.3%, 37.3% and 44.4%. Thus, as mannitol was increased with respect to *T. chebula*, the yield was also increased. The moisture content of B3 was 5.3%, B4 4.65%, and B5 3.4%. An increase in mannitol further reduced the moisture content. The flow properties of B3 were fair, whereas B4 and B5 demonstrated excellent flow behavior. The size of NSDP powder particles was measured by optical microscopy and laser diffractometry. The average size of B3 was 39.5µm, B4 30.6µm, and B5 with the lowest size of 8.7µm. The particle size of NSDP formulation played a critical role in its retention in the nasal cavity. The particles with size below 10µm do not retain in URT and are carried into the lungs, whereas sizes above 10µm are acceptable for nasal delivery. On the contrary, larger particles do not have longer retention in the nasal cavity and may irritate. The size of B4 and B5 was further measured by laser diffractometry. The size of B4 was found to be 30.65 ± 10.8 µm and for B5, 28.75 ± 11.65µm as shown in Figure 4. Thus, mannitol: *T. chebula* ratio of 2:1 was selected for spray drying of probiotic (B7). Mannitol with probiotics formed with a 26% yield with 5.1% moisture content, as mentioned in Table 3.

2.4.1. Total polyphenolic content

TPC of *T. chebula* and its NSDP formulation, B3, B4, B5, and B7, were evaluated. The formulation showed lower polyphenolic content than the extract, indicating loss during spray drying. The NSDP formulation showed TPC content in the range of 62.45±0.43% to 66.90±0.43%. Increasing mannitol preserved the TPC content, whereas the addition of probiotics reduced the TPC content to 58.81±1.04 %, as shown in Table 3.

2.4.2. Scanning electron microscopy

To study the morphological changes in *T. chebula* after spray drying, SEM study of *T. chebula* extract and NSDP formulation without probiotics was studied, as shown in Figure 5. The *T. chebula* sample showed asymmetric and irregular particles with particles in the range of 30 to 50µm. NSDP formulation was very spherical with a rough surface. The particles were observed in the range of 10-30µm.

Table 3. Evaluation of NSDP formulation

Parameter	Yield (%)	Particle Size		Bulk Density (g/cm³)	Tapped Density (g/cm³)	Carr's Index (%)	Hausner's Ratio	Angle of Repose (Degree)	Total Polyphenolic Content (%)
		Optical Microscopy (µm)	Laser Diffraction D_{90} (µm)						
B3	34.4	39.53± 5.1	-	0.232± 0.02	0.284± 0.005	19.22± 0.16	1.18± 0.03	30.89± 0.04	62.45± 0.43
B4	37.3	30.68± 4.88	30.65 ± 10.8 µm	0.283± 0.03	0.333± 0.002	11.5± 0.11	1.16± 0.02	27.64± 0.03	65.25± 0.42
B5	44.4	8.78± 2.31	28.75 ± 11.65µm	0.333± 0.03	0.376± 0.005	11± 0.10	1.12± 0.01	26.56± 0.03	66.90± 0.43

All values are n =3 ±SD.

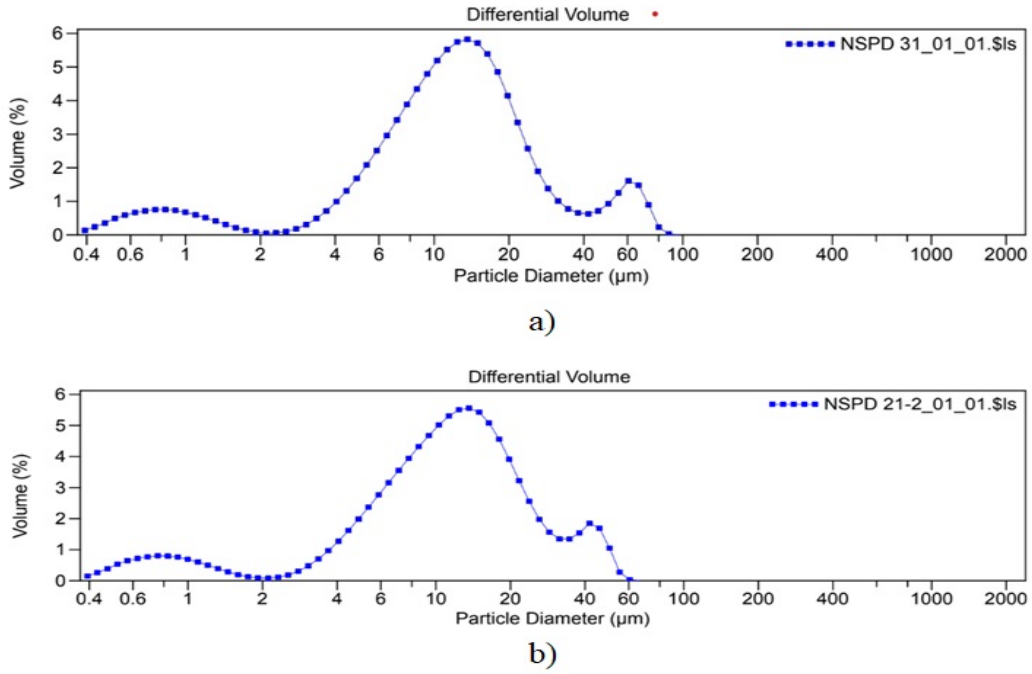

Figure 4. The particle size of **a)** Batch B4; **b)** Batch B5.

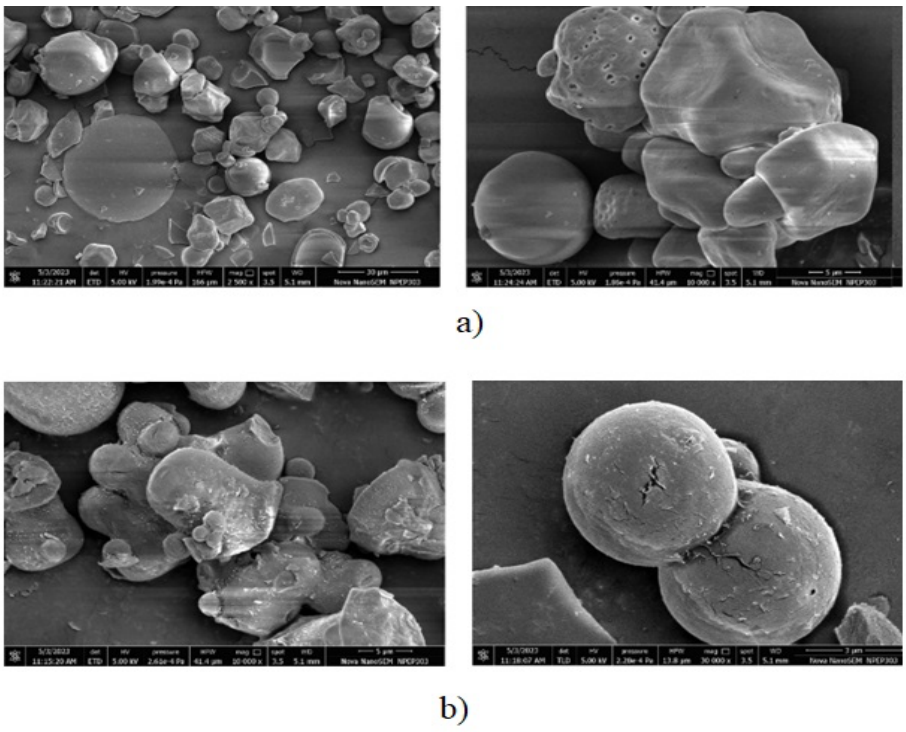

Figure 5. SEM of **a)** *Terminalia Chebula;* **b)** Nasal spray dried powder.

2.4.3. Differential scanning calorimetry

DSC study was performed to note the changes after spray drying, as shown in Figure 6. *T. chebula* sample indicated a broad endotherm at 86.6 °C. Mannitol indicated a sharp endotherm at 168.5 °C, representing its melting point. An endotherm at 160.0 °C was observed in soya lecithin samples, and an endotherm at 298.0 °C was observed for L. leucine, indicating its degradation. HPMC E5 showed a glass transition at 70.7 °C. NSDP formulation showed a single broad endotherm at 162.3 °C, indicating soya lecithin. The other endotherm was not evident in the DSC. These indicated complete dispersion of mannitol and *T. chebula* into each other during spray drying, causing the disappearance of their melting point.

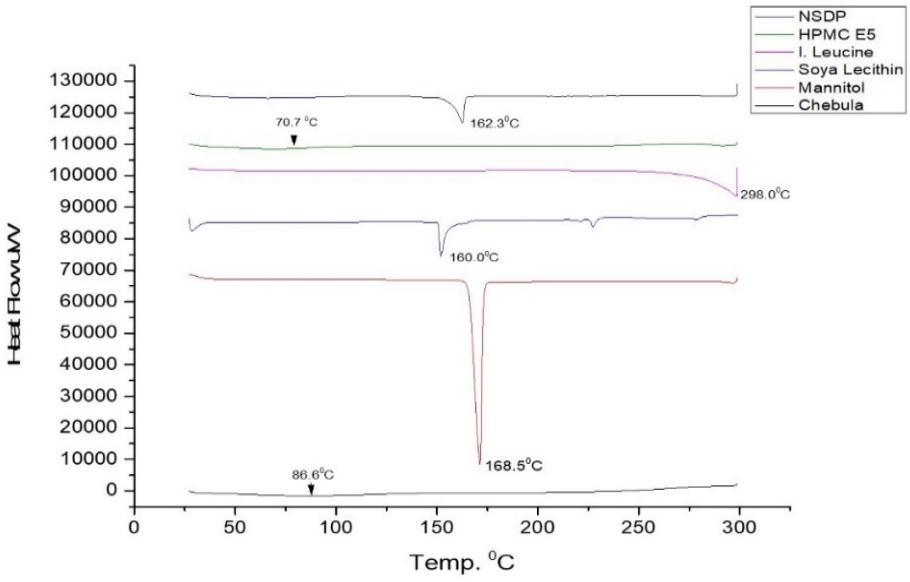

Figure 6. DSC of Formulation, *T. chebula*, and Excipients.

2.5. Evaluation of reconstituted NSDP

NSDP batch B7 was evaluated for pH, pump delivery, and rheology after reconstitution of 1.23 gm of powder with 20 ml of sterile water. The pH of the reconstituted solution was observed in a range of 6.2 to 6.3, which was suitable for the nasal cavity. Pump delivery from 20 consecutive pumps indicated that, on average, 0.112g of NSDP solution is sprayed with each pump. Viscosity was measured for the shear thinning system as different shear rates indicated NSDP formulation. At 0.5 rpm, viscosity was observed to be 142.1 cP which decreased to 1.57 cP at 100 rpm, indicating shear thinning.

2.5.1. Viability enumeration

Viability enumeration of NSDP formulation was done before and after spray drying to study the effect of the spray drying process on *L. casei* viability. There was no significant reduction in the viability of NSDP due to spray drying. The cell count of *L. casei* before spray drying was 8.16± 0.019 log cfu/ml, and after spray drying, it was reduced to 7.92 ± 0.004 log cfu/ml (less than 0.4 log reduction). The stability of probiotic suspension was also studied after 7 days stored at 4-8 °C and after spraying through a bottle since spraying probiotics in the form of droplets may cause harm to the cells. There was no significant change in viability after 7 days of storage at 7.88±0.004 log cfu/ml, and spraying through the bottle resulted in 7.81 log cfu/ml. This indicates the stability of formulation and the protective effect of *T. chebula* and mannitol on the probiotic, *L. casei*, as shown in Table 4 and Figure 7.

Table 4. Effect of spray drying and storage on the viability of *L. casei*.

Parameter	Bacterial count log cfu/ml
Before spray drying	8.16 ± 0.019
After spray drying	7.92 ± 0.004
After 7 days of storage at 4-8°C	7.88 ± 0.004
After spraying through a bottle	7.81± 0.019

All values are n =3 ±SD.

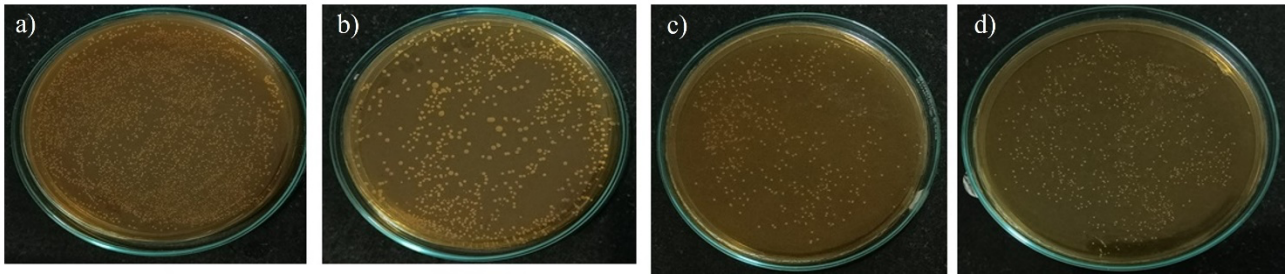

Figure 7. Colony count **a)** before spray drying **b)** after spray drying c) after 7 days storage at 4-8°C **d)** after spraying through the bottle.

2.5.2. In-vitro efficacy of NSDP by co-culture technique

A mixed culture technique was used to study the effect of synbiotic NSDP formulation on nasal pathogens, *S. aureus* and *C. albicans*. The formulation with probiotic and prebiotic was incubated alone in MRS media to observe the growth of *L. casei* and in the presence of *S. aureus* and *C. albicans* culture to study the mixed culture effect.

When the formulation was incubated in media without any pathogen, there was a sustained increase in *L. casei* at 24h (7.23± 0.02 log cfu/ml) and 48 h (7.44±0.02 log cfu/ml). The control of *S. aureus* and *C. albicans* also showed steady growth at 24h and 48h.

When the NSDP formulation was incubated in *S. aureus* culture, there was an increase in *L. casei* growth and a decrease in *S. aureus*. This shows *S. aureus* does not inhibit *L. casei*, whereas prebiotics supports the development of *L. casei*.

The microbial count of *S. aureus* is shown in Figure 8(a). at 0 times, was 6.81 ± 0.04 log cfu/ml, which was reduced to 6.67 log cfu/ml in 24h and 6.42 log cfu/ml ($p<0.0001$) 48 h. The decrease in *S. aureus* could be attributed to the antibacterial effect of *T. chebula* against pathogens. Also, antimicrobial bacteriocin and H2O2 secreted by lactobacilli further inhibit the growth of *S. aureus*.

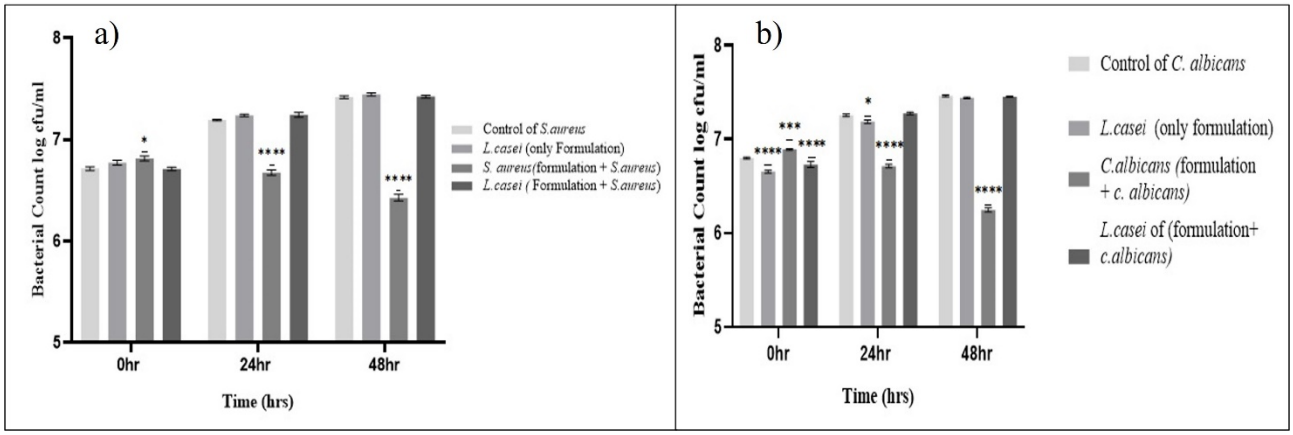

Figure 8. a) Effect of NSDP formulation on count of *L. casei* and *S. aureus*. **b)** Effect of NSDP formulation on *L. casei* and *C. albicans* count.

*error bar represents the standard deviation of three replicates.

Similar results were observed in *C. albicans*, as shown in Figure 8(b). *L. casei* concentration was increased with time without any inhibitory effect, whereas *C. albicans* count reduced to 6.71±0.03 log cfu/ml (p-value < 0.0001) and 6.24±0.03 log cfu/ml (p-value < 0.001) at 24h and 48h log cfu/ml compared to initial (6.89 logs cfu/ml). This is consistent with one of our previous studies of the effect of pectin and *L. casei* on *C. albicans*. Thus, the study demonstrated the effectiveness of synbiotic formulation's inhibitory effect on pathogen growth and stimulating *lactobacillus* count. Therefore, the developed formulation could be effectively used in protection against bacterial and fungal infections and would also help in restoring healthy nasal flora.

3. CONCLUSION

The spray drying technique successfully formulated a Nasal Spray Dried Powder (NSDP) containing probiotics and prebiotics. The prebiotic exhibited a positive impact on the probiotic, promoting their growth. By maintaining a carrier and drug ratio of 2:1, the formulation attained optimal results in terms of yield of 37.3 %, particle size by optical microscopy as 30.68 ± 4.88µm, by laser diffraction 30.65 ± 10.8µm, and moisture content of 5.1%. After reconstitution, the optimized batch resulted in a solution of 6.2 to 6.3 pH, and the sample's viscosity was found to be 142.1 cp at 0.5 rpm with shear thinning behavior. The viability of *L. casei* was retained even after spray drying, storage for 7 days at 4-8°C, and after spraying through a bottle. *In -vitro* study in the presence of *S. aureus* and *C. albicans* demonstrated an increase in lactobacilli count and a reduction in pathogen (*S. aureus* and *C. albicans*) over time. Conclusively, the developed formulation could be effectively used to restore the nasal flora and further tested against nasal injection in-vivo.

4. MATERIALS AND METHODS

4.1. Materials

Lactobacillus casei (NCIM -5303) were procured from the National chemical laboratory, Pune, India. Streptococcus aureus (ATCC -2592) and Candida albicans (ATCC 10231) were procured from Dr. D. Y. Patil Arts, Commerce, Science College, Pimpri, Pune, India. *T. chebula* was obtained as a gift sample from Mprex Healthcare Pvt. Ltd. Wakad, Pune. Sorbitol, mannitol, soya lecitin, *lactobacillus* MRS agar, MRS broth, McConkey agar and broth, chloramphenicol yeast glucose agar, and folin ciocalteu reagent were obtained from Himedia, Mumbai, India. Maltodextrin and HPMC E5 were obtained from Lobachemie, Mumbai, India. As a gift sample, fructooligosaccharides were obtained from Tata Chemicals Innovation Centre, Pune.

4.2. Methods

4.2.1. Standardization of L. casei

Gram staining of *L. casei* was performed to confirm the *lactobacillus* obtained from the National chemical laboratory. The standardization of *L. casei* was determined by culturing the bacteria for 24 hours and comparing its turbidity to a McFarland standard 4. The McFarland standard 4 was prepared by mixing 0.4ml of 1% $BaCl_2$ with 9.6ml of 1% H_2SO_4 resulting in a colony count of 1.2 x 109 cfu/ml. To establish a relationship between optical density and bacterial count, 1 ml of this cultured bacterium was added to 50 ml MRS Broth and incubated for 48 hours. The optical density and bacterial count (log cfu) were measured at 4, 6, 8, 10, 24, and 48 hours intervals. A graph was plotted with the optical density against the bacterial count, and an equation was derived from those data. This equation was used to estimate the colony counts in future studies [27].

4.2.2. Characterization of T. chebula

T. chebula was characterized for pharmacognostic properties like total ash value, acid-soluble ash value, and water-soluble ash value and assessed by folin ciocalteu colourimetric method for total polyphenolic content as shown in Table 5 [26].

Table 5. Characterization of *Terminalia chebula* extract.

Pharmacognostic Evaluation		
Parameter	**Standard Values**	**Sample values of drug sample**
Total Ash	NMT 5%	4.75%
Acid insoluble ash	NMT 5%	1%
Water soluble ash value	NLT 5%	3.5%
Total Polyphenolic Content	81.51 ± 0.3 %	

All values are n =3 ±SD.

To standardize *T. chebula* extract, a previously developed HPTLC method was performed using gallic acid. The extract (1µl) and standard (2µl) were loaded at 6mm band length on the pre-coated silica plate (10*10 cm TLC plate) using a Hamilton syringe and CAMAG LINOMAT instrument. The loaded sample was kept in a TLC twin trough developing chamber with a solvent system as chloroform: ethyl acetate: formic acid in the ratio (4:4:2). Plates were dried in hot air to evaporate the solvent from the plate. The plate was kept in a photo documentation chamber. The peak table, peak display, and peak densitogram were noted. The Rf value of extract and standard gallic acid were compared.

4.3. Prebiotic efficacy of T. chebula [27]

The kinetics of *L. casei* growth was estimated in reconstituted MRS broth containing tryptone, pectin, meat extract, potassium phosphate dibasic, sodium acetate, tri-ammonium citrate, magnesium sulphate and supplemented with *T. chebula*, and FOS. For the blank sample, *L. casei* culture in MRS broth was used. The cultivation of *L. casei* was done by adding 2% (v/v) inoculum in sterile reconstituted MRS broth (50 ml) in aseptic conditions. Subsequently, the flasks were incubated at 37± 2°C for 48 hours in an orbital shaking incubator (Remi electro tech, CIS 18, India). Samples were collected at intervals of 0, 2, 4, 6, 24, and 48 hours, and optical density (OD) was noted at 600nm. The culture containing *T. chebula* as prebiotic was centrifuged at 1000rpm in a cooling centrifuge (Remi electro tech, C-24 plus ZFCU-07420, India) to separate *T. chebula* from the bacterial samples.

4.3.1. Dry biomass determination

To monitor the process of cellular growth, a cooling centrifuge (Remi electro tech, C-24 plus ZFCU-07420, India) was used to separate the cultured bacterial cells by centrifugation of the sample at 1000 rpm for 30 minutes. The separated cell mass was dried in a hot air oven at 40°C (Remi electro tech, AI-7982, Mumbai, India) till constant weight to obtained as dry biomass. The plot of biomass data was made against time. The cell-free supernatant was further tested for further evaluation.

4.3.2. pH and determination of titratable acidity

A calibrated electronic digital pH meter (Elico, LI-120, Mumbai, India) was used to measure the pH.

To determine the % titratable acidity representing organic acid production by *L. casei*, the cell-free supernatant was titrated against 0.1 M NaOH.

$$\text{Titratable acidity} = \frac{n \times v \times m}{s \times 10}$$

Where "n" is the normality of base (0.1M NaOH); "v" is the volume of base (0.1M NaOH); "m" is the ratio of molecular weight of acid in the sample to the number of hydrogen ions in the acid molecules and "s" is the sample size in mL or g.

4.3.3. Antimicrobial Activity

The antimicrobial activity of the cell-free supernatant from the *L. casei* culture was evaluated against Streptococcus aureus (*S. aureus*) using agar well diffusion study. For *S. aureus* Tryptic Soy Agar was utilized as medium and sterilized for 20 minutes in an autoclave at 121°C and 15 psi pressure. The sterilized medium was added to sterile petri plates to solidify under aseptic conditions and placed in a hot air oven at 40°C for 30 minutes for moisture removal. *S. aureus* overnight cultures (0.1 ml each) were evenly distributed on the corresponding agar media and bored the wells of size 6 mm in diameter. The cell-free supernatant(100µl) was added to each well. The *L. casei* supernatant was added as a control, the FOS supernatant as a reference, and the *T. chebula* supernatant as a test sample. The samples were kept in a refrigerator for 30 mins to allow diffusion, plates were incubated at 37°C for 48 h, and zones of inhibition were measured.

4.4. Formulation of spray-dried powder

A laboratory scale spray dryer (Technosearch SDP-P-11, Mumbai, India) was used with an inlet temperature of 120 °C and outlet temperature of 60 °C, with a flow rate of 2ml/min. The nozzle diameter of 0.75µ was used, and the powder was harvested in the cyclone at an aspiration flow rate of 70 m3/hr for about 13 minutes with powder harvesting in a double cyclone separator. The NSDP formulation was optimized for the type of carrier (sorbitol, maltodextrin, mannitol) and carrier-to-prebiotic ratio (1:1, 2:1, 3:1), as shown in Table 1. Here, *T. chebula* is used as a prebiotic, and sorbitol, maltodextrin, and mannitol are used as a carrier for spray drying. Sorbitol and mannitol also enhanced the solubility of the prebiotic in the pre-spray solution. HPMC E5 was used to enhance the mucoadhesive property, and soya lecithin was used as a suspending agent.

Table 6. Composition of NSDP formulation.

Batches	*T. chebula* (gm)	Sorbitol (gm)	Maltodextrin (gm)	Mannitol (gm)	HPMC E5 (gm)	Soya Lecithin (gm)	L. Leucin (gm)	*L. casei* (cfu/ml)
B1	1	1	-	-	0.1	0.1	-	1.2×10^9
B2	1	-	2	-	0.05	0.1	0.157	-
B3	1	-	-	1	0.05	0.1	0.107	-
B4	1	-	-	2	0.05	0.1	0.157	-
B5	1	-	-	3	0.05	0.1	0.207	-
B6	1	-	2	-	0.05	0.1	0.157	1.2×10^9
B7	1	-	-	2	0.05	0.1	0.157	1.2×10^9

4.5. Evaluation parameter of NSDP

4.5.1. Powder Yield

The yield of NSDP was calculated by the ratio of the weight of spray-dried powder to the theoretical weight of solids before drying [22].

$$\% \text{ Yield} = \frac{\text{Weight of spray dried powder(g)}}{\text{Weight of solids before drying (g)}} \times 100$$

4.5.2. Loss on drying

Loss on drying (LOD) of the powder was calculated by drying 100mg powder samples till there was no change in the weight at 105°C [22]. Mass of powder before drying and after drying was recorded to find the LOD using an equation,

$$\text{LOD} = \frac{Initial\ weight\ of\ sample - Weight\ of\ sample\ after\ drying}{Initial\ weight\ of\ sample} \times 100$$

4.5.3. Micromeritic properties [28]

Micromeritic properties like Bulk and Tapped density, Carr's index, Hausner's ratio, and Angle of repose of nasal spray dried powder were evaluated as per the standard procedure mentioned in USP 2018.

4.5.4. Particle size [22]

Optical microscopy

NSDP formulations were studied for particle size by optical microscopy using calibrated eyepiece micrometer. Here the NSDP was sprayed on the slide, and the size of 100 particles was calculated under the microscope on the eyepiece scale, divided into 100 minor divisions. Each division is equivalent to 10µm. The arithmetic mean diameter of the particles was calculated.

Laser diffraction

Laser diffraction (Beckman Coulter LS Particle Size Analyzer, USA) was utilized to determine the particle size of the optimized NSDP batch. A cumulative undersize curve was constructed, allowing for the identification of granule sizes corresponding to the 10th, 50th, and 90th percentiles (D10, D50, and D90). The

calculation was performed within the range of 0.375 µm to 2000 µm. The outcomes were presented in terms of the arithmetic mean diameter.

4.5.5. Total Polyphenolic Content (TPC) [26]

The TPC of the sample was assessed by Folin-Ciocalteu colourimetric assay. NSDP sample was equivalent to 1mg/ml of *T. chebula* and was dissolved in 10ml of water: methanol (7:3) mixture, filtered with 0.45 µ membrane filter, and diluted to 200 µg/ml concentration. Sodium carbonate 4ml (7%) was added to this solution and left for 2-3 min. Further, 5ml of Folin Ciocalteu (10%) reagent was added and left in the dark for 30 min till dark blue colour appeared. The absorbance of the sample was measured at λmax of 735nm against blank. All the experiments were carried out in triplicate. A linear relationship between concentration and absorbance was developed using gallic acid as a standard in a 40 to 200 µg/ml concentration range.

4.5.6. Scanning Electron Microscopy (SEM)

SEM (FEI, Nova Nano 450, USA) was employed to analyze the morphology of the powder, enabling the examination of particle shape, surface roughness, cracks or voids, and other structural characteristics. SEM analysis was done of the batches without probiotics to prevent interference. The sample (5mg) was given a 20 nm platinum coating to enhance its electrical conductivity. Subsequently, the coated samples were randomly observed in the FESEM chamber using an acceleration voltage of 10.00 KV and a vacuum pressure of 1.30e-4 pa. Photomicrographs were captured at various magnifications.

4.5.7. Differential Scanning Calorimetry (DSC)

The study examined the thermal behaviour of the nasal spray-dried powder and contrasted it with raw materials, employing differential scanning calorimetry (HITACHI DSC7020, Japan). To perform the analysis, 1 mg of the sample was placed in aluminium DSC pan, which was crimp-sealed with pierced lids to maintain consistent pressure. The thermal properties of the samples were then investigated by subjecting them to a temperature range of 30°C to 300°C, with a heating rate of 10°C/min, using a blank aluminium pan as a reference.

4.6. Evaluation of reconstituted powder

The powder was added to 20ml of water for injection and evaluated for the following parameters:-

4.6.1. pH

A calibrated electronic digital pH meter (Elico, LI-120, Mumbai, India) was used to measure the pH.

4.6.2. Pump delivery

The nasal spray bottle was filled with the reconstituted sample, and the initial weight was recorded. Subsequently, the weight of the bottle was measured after each of the 20 consecutive sprays, and the pump delivery was assessed.

$$\text{Pump Delivery} = \frac{\text{Total weight of sample discharge}}{\text{no. of discharge}}$$

4.6.3. Rheology

A Brookfield viscometer (Brookfield RVDV II + PRO, USA) evaluated the viscosity of the reconstituted samples using a small sample adapter. The sample was placed in a sample chamber fitted with a flow jacket so that the 25°C temperature was maintained, with increasing rpm from 0.5 to 100 rpm.

4.6.4. Viability enumeration [22]

The viability of spray-dried powders was assessed by conducting ten-fold serial dilutions of the reconstituted powders on de Man Rogosa and Sharpe (MRS) agar plates. NSDP was mixed with 20 ml of distilled water and then serially diluted with MRS broth before plating. The MRS plates were incubated at 37°C for 48 hours, and the resulting bacterial colonies were counted. The formulation's viability was assessed before and after spray drying by spreading it on MRS agar plates. The viability of the formulation was also assessed after spraying it through the nasal bottle. The results were expressed in CFU/ml as mean value and ± standard deviation.

4.6.5. In-Vitro efficacy of NSDP using co-culture technique [27]

Minimal Essential Medium (MEM) (100µl) was prepared, which included yeast extract, Tween 80, tryptone, pectin, meat extract, potassium phosphate dibasic, sodium acetate, triammonium citrate, and magnesium sulphate. The media was then inoculated with 0.5 MacFarland standards of Streptococcus aureus and Candida albicans cultured overnight. Several flasks were prepared: flask 1 contained a blank, flask 2 contained the media and formulation, flask 3 contained the media, formulation, and *S. aureus*, flask 4 contained the media, formulation, and *C. albicans*, flask 5 was control containing media, and *S. aureus* and flask 6 was control containing media and *C. albicans*. The flasks were incubated for 48 hours. At 0, 24, and 48 hours of incubation, the sample (100µl) was removed from each flask and added to the sterilized broth to make serial dilutions (10-2, 10-4, 10-6). These dilutions (100 µl) were spread onto sterile agar plates, where they were subjected to a 24-hour incubation period at 37°C. MRS agar was used for the formulation containing *L. casei*, tryptic soy agar for *S. aureus*, chloramphenicol yeast glucose agar, and *C. albicans*. After 24 hours, the colonies were counted from each plate, and the colony count (cfu/ml) was calculated using the formula as follows,

$$\text{cfu/ml} = \frac{\text{No. of colonies} \times \text{Dilutionfactor}}{\text{Volume of culture plated in ml}}$$

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