

# Novel Fruit Juice Based Nutraceutical Formulation with Probiotic and Cholesterol Reduction Properties

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**Abstract-** This work encompasses the use of a sporulating probiotic culture, *Brevibacillus borstelensis*, to prepare a nutraceutical product with a mixed fruit juice as a carrier for the culture. Lyophilization of the formulation was carried out using different dehydrating agents, pectin, ascorbic acid, gelatin, gum Arabic and maltodextrin in specific permissible concentrations. Pectin gave the best results in terms of consistency and appearance of the nutraceutical powder, hence was used in the final formulation. The nutraceutical powder was then analysed for its shelf life, physical, chemical and nutritional profile. It was also tested for heavy metal contaminants, antibacterial activity and cholesterol reduction capability. Microbiological safety of the formulation was verified according to FSSAI manual. Encouraging results were obtained from the analyses. Lyophilization helped retain the nutrients of the fruits as well as improved the keeping quality of the formulation. The novel probiotic containing nutraceutical powder prepared could be helpful to lactose intolerant population. Its cholesterol reduction property could be beneficial to hypercholesterolaemic individuals.

**Keywords:** Nutraceutical, probiotic, *Brevibacillus borstelensis*, mixed fruit juice, cholesterol reduction

## I. INTRODUCTION

Food can be used as prophylactic and therapeutic agent. If it is natural and non-synthetic it has more advantages. The term Nutraceutical is a hybrid of nutrition and pharmaceutical and was introduced in 1989 by Stephen L. DeFelice. It can be defined as "Food, or parts of food, that provide medical or health benefits, including the prevention and treatment of disease" [1]. A lot of work is being carried out to prove the benefit of nutraceuticals like antioxidants, vitamins, amino acids, phytochemicals, and polyunsaturated fatty acids in foods which help to improve the immune system [2].

Fruits are perishable and available only seasonally. Food industries therefore process fruits to increase their shelf life. However, these processed fruits no longer retain the nutritive value of the original fruit. Hence nutraceutical products which claim to retain the nutritive value of these fruits must process them to minimize this loss.

Pomegranates have phytochemicals like polyphenols, tannins like punicalagins [3], protein, minerals like Ca, Mg, P, Fe, Na, K, Cu, S, Cl and vitamins like carotene, thiamine, riboflavin, nicotinic acid, vitamin C and pectin which act as

antioxidants and reduce blood pressure, and are helpful in preventing breast cancer [4]. Pineapples contain the active constituent bromelain which has antidiabetic, antioxidant, and anti-hyperlipidaemic properties [5]. Citrus fruits, rich in vitamins, healthy antioxidants and flavonoids are beneficial for prevention of cardiovascular disease [6].

Dehydration is one of the methods to make fruits available all year round and also increase their shelf life by reducing the  $a_w$  of fruits [7]. The methods include drying in hot air, osmotic dehydration, frying, drying in a microwave and freeze drying. Drying at high temperatures can destroy many of the nutrients in a fruit which are heat labile, so drying in hot air, frying and drying in a microwave were all avoided in the current work.

Fruit juices contain large amounts of simple sugars, such as glucose, fructose and sucrose, which make them very sticky during spray drying. Maltodextrins and gum Arabic are the most often used for fruit juices to improve texture and as drying agents [8].

Lyophilization or Freeze drying is the removal of water from a substance by sublimation. Freeze dried fruits maintain colours better than fruits dried by other methods, thus

indicating lesser loss of nutrients. Freeze drying allows the retention of ascorbic acid when conducted at low temperatures [9].

Probiotics can reduce cholesterol. It was observed that consumption of *Bifidobacterium* milk led to reduction in triglyceride, low-density lipid, and total cholesterol. [10] Another study showed a significant decrease in blood cholesterol when fermented milk containing *L. acidophilus* was consumed during controlled clinical trials. [11] A meta-analysis study review involving probiotics and serum lipids which tested strains of *Lactobacillus acidophilus*, *Bifidobacterium lactis*, *Lactobacillus plantarum* and *Lactobacillus helveticus*, found that they reduced total cholesterol by an average of 7.8 mg/dL and LDL cholesterol by 7.3 mg/dL [12].

Rest of the paper is organized as follows Section II includes Related work. Section III is Methodology which is further divided into subsections of Formulation and Analysis of the nutraceutical. Section IV contains Results and Discussion. Section V concludes the research work.

In the present study, a fruit juice-based nutraceutical powder was formulated with a probiotic *Brevibacillus borstelensis* (Gen Bank accession number MK156349) [13]. Being a non-dairy product, it can be used by lactose intolerant people. By testing the shelf life of the probiotic, studying the nutrition profile, antibacterial activity, chemical parameters, heavy metal contamination, cholesterol reduction ability and microbiological safety of the formulation, we will be arriving at a safe, novel nutraceutical having the benefits of natural fruit ingredients as well as the probiotic culture, which has a longer shelf life.

## II. RELATED WORK

Dairy products have been extensively used as carriers for probiotic cultures especially Lactic acid bacteria. However, there is an increased demand for non-dairy based probiotic supplements due to vegetarianism; cholesterol content of milk, lactose intolerance, milk allergy and other factors [14]. A number of studies have been conducted using fruit juices as a medium for probiotic growth and viability. [15-17] Food matrices like fruits and vegetables are being used as potential media as they contain beneficial nutrients and are an ideal carrier for probiotics. Several types of probiotic fruits and vegetable products have been developed and marketed, such as fruits and vegetables juices, dried fruits, fermented vegetables and deserts. Many probiotic strains, mainly species of *Lactobacillus* and *Bifidobacteria* have been used in the development of juice products, including pomegranate, orange, cashew apple, tomato, cabbage, beet and carrot juices [18]. Novel probiotics can be added to nutraceutical formulations, which have some beneficial effect on human health. [19]

## III. METHODOLOGY

### 1] Formulation of the nutraceutical

Fruits orange, sweet lime, pomegranate and pineapple were washed with water, blanched and used for preparing mixed fruit juice. It was then strained through sterile muslin cloth. Aseptic conditions were maintained during juice extraction, which was performed in a biosafety cabinet.

### Selection of the appropriate dehydrating agent:

To select the dehydrating agent only pineapple juice was lyophilized, since pineapple has the highest sugar content among the selected fruits and the agent which would give a good texture to it was also expected to do the same for the mixed fruit juice. It was dispensed in 5 separate flasks, each was mixed with a different dehydrating agent like pectin, ascorbic acid, gum Arabic, gelatin and maltodextrin. The probiotic *Brevibacillus borstelensis* was inoculated to a final density of  $10^{10}$  spores / ml. These were then lyophilized. The consistency and appearance of the juice powder obtained after lyophilization were compared and the one giving the best results was used for the final formulation.

### Formulation of the nutraceutical containing probiotic:

500ml of the mixed fruit juice was inoculated with 10 ml of spore suspension of *Brevibacillus borstelensis* to a final density of  $10^{10}$  spores / ml. Sterile pectin solution at a final concentration of 5% was mixed with the juice as the selected dehydrating agent. The flask was kept on a shaker maintained at 5°C for 2 hours. The sample was frozen at a temperature of -23°C for 12 hours. Lyophilization was carried out at 8-10mTorr pressure and -86°C temperature for 96 hours to obtain the juice in a powder form. The viability of probiotic culture was tested by spread plate technique before and after lyophilization.

### 2] Analysis of the Nutraceutical:

#### i. Shelf life of the probiotic:

Viable count of the probiotic culture in the nutraceutical preparation was carried out at monthly intervals for up to 6 months after lyophilization. Counts were expressed as cfu per gm of the powder.

#### ii. Antibacterial activity:

The Agar well diffusion method using Mueller Hinton agar plates was performed [20]. Test cultures used were *Bacillus cereus* NCIM 2109, *Bacillus subtilis* 6633, *Brevibacterium casei* MCC 2029, *Staphylococcus aureus*, MRSA, *Salmonella para typhi* B, *Salmonella abony* and *Escherichia coli* 500mg/ml, 250mg/ml, 125mg/ml and 62.5mg/ml concentrations of the nutraceutical powder were prepared using sterile saline. 50 µl of each was added to the wells. Zones of inhibition around the wells were measured and expressed in mms after incubation at 37°C for 24 hours.

### iii. Nutrition profile:

The nutraceutical sample was analysed for energy content (EL/SOP/509), total fat (EL/SOP/502), cholesterol (AOAC 976.26), total carbohydrates (IS 1656:2007, RA 2012), protein (EL/SOP/549), vitamin C (IS 538: 1970 RA 2010) and fatty acid profile (AOAC 996.06)

### iv. Chemical parameters:

Water activity (EL/SOP/ 530), Total soluble solids (TSS) using the refractometer, calibrated at 20°C. [21], Colour value - Konica Minolta, CM 3600d [22], tapped bulk density USP 23, bulk density USP 23 [https://www.usp.org]

### Heavy Metal contaminants:

The heavy metals like Arsenic, Lead, Mercury, Cadmium were estimated by EL/SOP/579.

**pH:** One gram of the sample was reconstituted in 100ml of neutralized distilled water and the pH was determined using pH meter.

**Titrateable acidity:** One gram of the sample was reconstituted in 100ml of neutralized distilled water. 0.3 ml of phenolphthalein indicator was added. The mixture was then titrated with 0.1 N NaOH till the end point was reached i.e. pH 8.2 [AOAC 17<sup>th</sup> ed, 2000]

% acid = [Vol of NaOH] x [N of NaOH] x [milliequivalent factor] x 100 / gram of sample

1 mL of 0.1 N NaOH equals Citric acid anhydrous - 0.0064 gm Tartaric acid - 0.0075 gm

% acid =  $\frac{[\text{ml of NaOH}] \times 0.1 \times 0.0064 \times 100}{1\text{gm}}$   
(Citric acid)

### v. Cholesterol reduction assay

An invitro assay was performed to test the ability of the nutraceutical as well as the prospective probiotic bacterium *Brevibacillus borstelensis* to reduce cholesterol using Zaks method [https://www.protocolpedia.com].

The test solutions were prepared using de Man, Rogosa, and Sharpe broth (MRS) broth with 0.3% bile salt and 100mcg/ml cholesterol, inoculated with the probiotic culture in one tube and the final nutraceutical product in another and incubated at 37°C for 24 hours. Reduction of cholesterol was estimated as per the above protocol.

### vi. Microbiological safety:

The nutraceutical was analysed for the presence of aerobic mesophiles, coliforms, pathogens like *Staphylococcus aureus*, *Salmonella species*, *Shigella species*, *Vibrio cholerae*, *Clostridium perfringens*, yeast and moulds. [23]

## IV. RESULT AND DISCUSSION

### [1]. Formulation of the nutraceutical

The weight of dry powder yield from 100 ml of the pineapple juice after lyophilization differed with the dehydrating agent used (Table 1).

Maltodextrin, Gum Arabic, Gelatin and Ascorbic acid gave sticky lumps, difficult to remove from the glass containers. In terms of consistency, appearance and hygroscopic property, the sample with pectin gave the best results.

Table 1: Comparison of the pineapple juice powder prepared using different dehydrating agents

Dehydrating agent	Weight of powder in g from 100 ml of juice	Colour	Average count of probiotic cfu/g
Maltodextrin	29.92	Brown	$5.8 \times 10^{10}$
Ascorbic acid	8.99	Yellow	$2.9 \times 10^{10}$
Gum Arabic	23.61	Light brown	$1.2 \times 10^{10}$
Pectin	16.04	Pale yellow	$3.03 \times 10^{10}$
Gelatin	10.63	Pale yellow	$4.8 \times 10^{10}$

A one log cycle reduction in viability of *Brevibacillus borstelensis* was observed upon lyophilization from the original count of  $1.08 \times 10^{11}$  cfu/ml before lyophilization. This indicates that the probiotic could tolerate the mixed fruit juice carrier as well as the lyophilization conditions well. In a similar study reported earlier, freeze-dried probiotic papaya juice powder did not show any log alteration in the number of probiotic organisms [24]. A probiotic beverage should possess a minimum viable count of  $10^6$  cfu/ml [25].

Pectin was used as the dehydrating agent to prepare the final nutraceutical product. Many manufacturers use pectin as a functional fiber. Benefits of pectin supplementation include reduction of cholesterol, balance of glucose levels, improvement in glycemic control for diabetics, reduction of blood pressure, support for cardiovascular health, cancer prevention as well as an overall improvement in gastrointestinal health. [26]

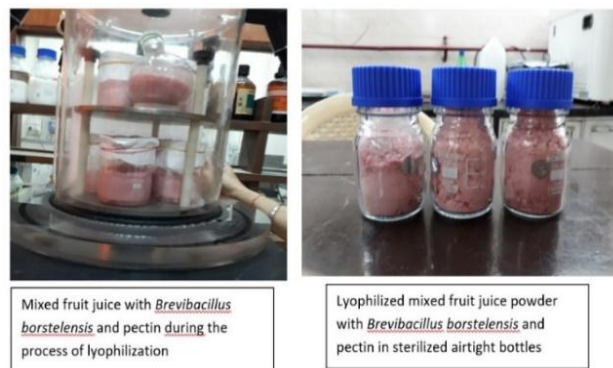
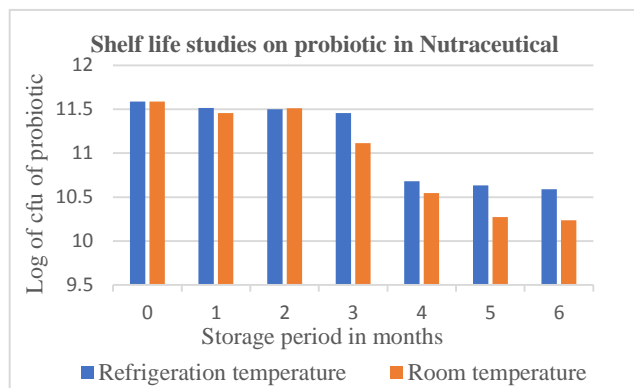


Figure 1: Lyophilized mixed fruit juice powder

## [2]. Analysis of the Nutraceutical:

### i. Shelf life studies:

The lyophilized probiotic in the nutraceutical preparation retained its viability till six months at refrigeration (4°C) and ambient temperature (30°C) as shown in Figure 2.



**Figure 2: Graph showing viability of the probiotic in the nutraceutical powder**

For up to two months, the viability was  $3.2 \times 10^{11}$  cfu / gm almost equal to the initial count which was  $3.8 \times 10^{11}$  / cfu / g irrespective of the storage temperature; which started declining subsequently till 6 months. Residual viability at the end of six months was  $3.9 \times 10^{10}$  cfu / g which was much greater than the recommended minimum numbers at the end of shelf life. Foods containing probiotics should maintain the number of bacteria till the end of shelf life mentioned on the label, since the minimum recommended number of probiotic cells at the time of consumption should be at least  $10^6$  cfu / g [27]. Since the product fulfils the requirement it can be safely stored between 4°C - 30°C for six months.

### ii. Antibacterial activity:

The nutraceutical was found to be more effective against Gram positive bacteria than Gram negative bacteria (Table 2). The diameter of zones of inhibition was directly proportional to the concentration of the nutraceutical powder. This may be due to higher cell number of the probiotic with increasing concentration of the nutraceutical. This shows that it can be used as a therapeutic agent against these pathogens during an infection.

Some of the fruit juices exhibit significant antibacterial effect due to their mineral content such as iron and other biologically active substances [28]. Medicinal importance of fruit juices has been increasing and fruits have become valuable sources to maintain human health due to their therapeutic value [29]. Pomegranate contains polyphenols, tannins, ellagic acid and anthocyanins like delphinidins, cyanidins which have antimicrobial properties [30].

Table 2: Antibacterial activity

Conc. Of Nutraceutical Test culture	Zone of inhibition in mm			
	62.5 µg/ml	125 µg/ml	250 µg/ml	500 µg/ml
<i>Bacillus cereus</i> NCIM 2109	11	12	14	20
<i>Bacillus subtilis</i> ATCC 6633	-	10	11	15
<i>Brevibacterium casei</i> MCC 2029	-	11	12	16
<i>Staphylococcus aureus</i>	-	-	09	14
Methicillin Resistant <i>S. aureus</i>	11	12	13	19
<i>Salmonella paratyphi</i> B	-	-	-	-
<i>Salmonella abony</i>	-	-	-	08
<i>Escherichia coli</i>	-	-	-	-

**Key:** - = zone of inhibition equal to or lesser than 7mm

### iii. Nutrition profile:

The nutraceutical sample was found to have an average energy value of 357.75Kcal / 100g, Total carbohydrates was 84.85g/100g, protein was 4.6g/100 g, Vitamin C was 130.83mg/100g, total fat and cholesterol were absent in the sample, the fatty acid profile showed less than 0.01% of saturated, monounsaturated and polyunsaturated and trans fatty acids.

The use of a nutraceutical-based treatment for hypercholesterolemia is increasing, and is considered to be an important preventive action to take when hypercholesterolemia is mild or moderate posing a low and/or moderate risk factor [31]. The prepared nutraceutical powder is a zero-cholesterol supplement, shows a good fatty acid profile and also proved to reduce cholesterol when tested.

The carbohydrate content of the powder is approximately 85% which may be the reason for the powder to give a high energy i.e. 356 Kcal/100gm. The protein content was 4.6 %, higher than fresh fruits, since the powder is a more concentrated form of the fruit solids due to dehydration. Moreover, there is no much loss of antioxidants as well as proteins due to process of freeze drying where high temperatures are not used. There was no reduction in nutritive value of the powder even after a month of storage.

### iv. Chemical parameters:

Listed in Table 3

#### Water activity $a_w$

$a_w$  of the nutraceutical powder was found to be 0.154.

Maintaining a low water activity controls microbial growth, extends shelf life and allows safe storage of some products without refrigeration [32]. Many powdered products can form cakes eventually if packaging is not proper. Hence, knowing the water activity can also help to decide the type of packaging required for a product to increase its shelf life.

Table 3: Chemical parameters

Parameter	Result
Water activity	0.154 at 29.7°C
Total soluble solids	12%
Colour	L=71.99, a= 12.18, b= 7.35
Bulk density	0.28 g/ml
Tapped density	0.38 g/ml
Carr index	26.4
Heavy metal contaminants As, Pb, Hg, Cd	< 0.1mg/kg
pH	3.23
Titrateable acidity	0.512% (citric acid)

### The total soluble solids (TSS)

TSS in the nutraceutical powder was determined using refractometer and was found to be 12%. TSS is expressed as degrees Brix. The brix estimates all soluble solids like sucrose, fructose, vitamins, minerals, amino acids, proteins, hormones, etc. in the nutraceutical. Researchers have reported TSS in pineapple, apple, plum, orange and mango fruit as 9, 12, 16, 10 and 20-degree Brix respectively [33]. Maintenance or slight increase in TSS content of the juice during storage is desirable for safeguarding good juice quality [34].

### Colour value

When a colour is expressed in CIELAB,  $L^*$  defines lightness,  $a^*$  denotes the red/green value and  $b^*$  the yellow/blue value. Since  $L^*$  scale value of the powder was 71.99, it indicates the colour is light,  $a^*$  scale value was 12 and this positive number indicates the sample is red and not green,  $b^*$  scale value was 7.35, indicating that the sample is yellow and not blue. All three values are required to completely describe the colour of the powder. If the colour of the product prepared from fruits and vegetables are preserved even after the processing it indicates that the nutrients are also preserved. Since the nutraceutical powder was prepared by Lyophilization and not subjected to heat treatment after extraction, it may have preserved most if not all the nutrients.

### Tapped Bulk density and Bulk density

The Hausner ratio is correlated to the flowability of a powder. It is calculated by the formula:  $H = \text{Tapped density} / \text{Bulk density}$ . A Hausner ratio greater than 1.25 is said to indicate poor flowability. The Tapped bulk density of the nutraceutical powder was found to be 0.38g/ml. Its bulk density was 0.28 g/ml. The Hausner ratio of the powder  $H = 0.38 / 0.28 = 1.36$ , which is higher than 1.25, thus indicating poor flowability.

The Carr index is also said to measure flowability. Its formula is  $C = 100 \times (1 - \text{Bulk density} / \text{Tapped density})$ . A Carr index greater than 25 is said to have poor flowability. [35] The Carr index of the powder  $C = 100 \times (1 - 0.28/0.38) = 26.4$ , which is greater than 25, confirming poor flowability. This may be due to its hygroscopic nature. The lyophilized nutraceutical powder needs to be stored in an airtight sterile container to maintain its flowability.

### Metal contaminants:

Metals such as arsenic, lead, cadmium, mercury and others are found in certain foods. At high levels, these metals can be toxic, but eliminating them entirely from our food supply is not always possible because these metals have polluted our air, water and soil.

According to American National standard, 2008 and European Union Pharmacopoeia, 2010 the fixed cut off limits for lead in food is 10ppm, Arsenic is 5ppm, Cadmium it is 0.3ppm. In Europe, the European Commission discussed the need for setting maximum levels of Pb, Cd, and Hg in food supplements to amend the Commission Regulation (EC) No. 1881/2006, because monitoring studies found high levels of these metals in certain food supplements. The following limits were then set: 3.0mg/kg for Pb; 1.0mg/kg for Cd and 0.10mg/kg for Hg [36].

The nutraceutical powder prepared from mixed fruit juice and probiotic had less than 0.1mg/kg of all four heavy metals tested, Hg, Cd, Pb and As. All are within acceptable limits. The powder thus does not pose a potential health hazard in terms of heavy metal toxicity.

### pH and Titrateable acidity:

**pH** of the reconstituted nutraceutical powder measured with the help of pH meter was found to be 3.23. The presence of organic acids like citric acid, lactic acid, malic acid and tartaric acids in fruit juices lowers the pH of these juices. This acts as a natural preservative for the juice. This will also give the tart flavour to the reconstituted powder, a favourable organoleptic property of the nutraceutical preparation.

The **titrateable acidity** of fruits is used, along with sugar content, as an indicator of maturity, generally the higher the maturity, the lower the acid content. The acidity of a fruit juice avoids alteration of its organoleptic nature and fermentation processes.

Acidity was calculated in terms of citric acid since this is the predominant organic acid in citrus fruits, and in pineapple and pomegranate.

*Acidity = 0.512% (w/w) of nutraceutical powder*

### v. Cholesterol reduction assay:

The probiotic culture *Brevibacillus borstelensis* was able to reduce cholesterol when incubated with it for 24 hours to the



extent of 34% and the nutraceutical containing the probiotic culture reduced cholesterol by 25%. [Table 4]

Table 4: Reduction of cholesterol by the probiotic and nutraceutical formulation

Test sample	Residual Concentration of cholesterol in µg/ ml	Reduction in cholesterol in supernatant µg/ ml
Control	99.02	---
<i>Brevibacillus borstelensis</i>	65	34.02
Nutraceutical	73.82	25.2

The nutraceutical powder prepared by lyophilization contains spores (dormant forms) of *Brevibacillus borstelensis*, which after inoculation into the Rogosa broth after a time lag would germinate into vegetative cells of the culture. These vegetative cells are metabolically active and begin to metabolize cholesterol. Hence during the 24-hour incubation, the young culture of *Brevibacillus borstelensis* could reduce more cholesterol than the nutraceutical sample.

The bile salt hydrolase activity of probiotics has been associated with their cholesterol lowering properties [37]. One of the probable mechanisms used by this bacterium to reduce cholesterol appears to be deconjugation of bile salts, since it was also found to be positive for bile salt hydrolase activity. The manifestation of cardiovascular disease can be reduced in individuals suffering from hypercholesterolemia, if total and in particular LDL cholesterol can be controlled with use of the right probiotic supplements. This method could be effective in dealing with coronary heart disease [38].

#### vi. Microbiological safety

The nutraceutical was devoid of aerobic mesophiles except the probiotic *Brevibacillus borstelensis*. Neither coliforms, nor yeasts or moulds were detected in the sample. Spoilage causing organisms were ruled out from the preparation. Pathogens like *Staphylococcus aureus*, *Salmonella species*, *Shigella species*, *Vibrio cholerae*, *Clostridium perfringens* were absent when tested. The sample therefore proved to be safe for consumption.

## V. CONCLUSION

The mixed fruit juice-based nutraceutical powder containing the novel probiotic culture, *Brevibacillus borstelensis* was good in terms of nutritional profile, antibacterial activity and cholesterol reducing ability. It proved to be a suitable carrier for the probiotic culture, and was safe for consumption by lactose intolerant population. Lyophilization helped to improve the shelf stability of the product, apart from retaining nutrients and improving the viability of the

probiotic. It was also found to be safe for consumption as per FSSAI microbiological safety tests.

Future studies will aim at including multiple probiotic cultures each exhibiting unique health benefits in the formulation and improving the consistency of the powder in terms of its hygroscopic nature and converting it to a free-flowing powder for its optimal storage and use.

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