

Evaluation of Prebiotic Score of Edible Mushroom Extract

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Abstract- Nowadays for our health benefits, prebiotics and probiotics are used in yogurts and fermented food which are largely available in the market. Prebiotics are non-digestible dietary fibers and oligosaccharides that stimulate the growth of bifidobacteria and lactic acid bacteria in the gastrointestinal tract. Industries are significantly increasing the production of prebiotics and probiotics supplemented foods due to the increment in numbers of health-conscious consumers. The objective of this work is to study the prebiotic score of edible mushrooms (*Pleurotus sajor-caju*, *P. Florida* and *Lentinus edodes*) in comparison with commercial prebiotics. The prebiotic score was calculated by comparing the bacterial growth with respect to the growth of enteric pathogen at 0 h and 24 h in the presence and absence of prebiotics. Among the three mushroom extracts studied, *P. sajor-caju* was found to produce the good prebiotic score with *L. acidophilus*.

Keywords- *Prebiotics, Mushrooms, Prebiotic score, Lactobacillus, Enteric Pathogen*

INTRODUCTION-

It has been well proved that humans are superorganisms and harbor millions and millions of microorganisms in their body. The gastrointestinal tract is considered to be heavily populated by microorganisms than other parts of the human body and is termed as the biggest immune organ. Recent research results are quoting that the microorganisms present in the gut are mainly involved in developing our immunity and they are in constant communication with the brain. These are the living organism (bacteria and yeast) which when administered in adequate amounts confer a health benefit on the host (Fuller, 1989). Probiotics strain are very sensitive in nature that depends upon the environmental condition such as heat, moisture, oxygen, acid, etc. Probiotics bacteria are helpful to maintain the balance of microflora in the intestine. The genus *Lactobacillus* and *Bifidobacterium* are considered to be the main probiotic bacteria. Probiotics are also found in the dietary supplements. Probiotics have the capability to endure the antibacterial mechanisms that operate in the gut (Gilliland and Speck, 1977).

Polysaccharides and some oligosaccharides are termed as prebiotics due to their nature of supporting the growth of microorganisms in the gut. The concept of prebiotics defines as "non-digestible food ingredients that beneficially affect host health by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon" (Gibson & Roberfroid, 1995). A prebiotic is a fermentable fiber, that is to say, no digestible carbohydrates affected to get fermentation by colonic bacteria. All the

fibers do not have prebiotics effect. Since all the fiber is not fermentable. Prebiotics are added to foods which do not contain fiber such as yogurt or fermented milk. The addition of fibers increases the effectiveness of lactic fermentations because prebiotics favor's the growth of lactic acid bacteria (Kaplan & Hutkins, 2000; Schrezenmeier & de Vrese, 2001). Prebiotics such as oligosaccharides and inulin have become a great interest as a functional food ingredient because it is able to manipulate the composition of colonic microbiota in human gut by inhibition of exogenous pathogens (Rycroft et al., 2001), thus improving the host health (Roberfroid, 2000 and 2002). Wang (2009) suggested that the effect of prebiotics was actually indirect because it is the changes in the gastrointestinal microbiota compositions (bifidobacteria, lactobacilli, as well as the histolyticum subgroup; Bacteroides and clostridia) that give rise to the prebiotic effect. Bifidobacteria and lactobacilli are the beneficial bacteria that serve as prebiotics target (Macfarlane et al., 2008). A positive effect of prebiotic reflects the significant increase in numbers of bifidobacteria and lactobacilli while retarding the development of histolyticum subgroup (Palframan et al., 2003).

MUSHROOMS AS PREBIOTIC SOURCES

Other than its medicinal properties, consumption of edible mushrooms also leads to a significant health improvement. This is because they are low in calories, sodium, fat, and cholesterol, while containing the high percentage of protein, carbohydrate, fiber, vitamins, and minerals. These nutritional properties make mushrooms a very good dietary food, which can contribute to the formulation of a well-balanced diet (Manzi et al., 2001). Mushrooms seem to be a potential candidate for prebiotics as it contains carbohydrates like chitin, hemicellulose, α and β -glucans, mannans, xylans, and galactans. There are great advantages of incorporating the mushrooms extracts in food as its polysaccharides were reported to exhibit immunomodulation properties, and anticancer activities (Wasser, 2002). The medicinal properties of mushrooms have been confirmed through intensive research conducted worldwide. Nutritional and therapeutic properties of mushrooms growing throughout the world were studied and reviewed by several scientists (Chang, 1996; Manzi et al., 2001; Sanmee et al., 2003; Agrahar-Murugkar and Subbulakshmi, 2005; Barros et al., 2008; Kalac, 2009; Roy et al., 2009). Synytsya et al. (2009) studied the prebiotic effects of glucans isolated from fruit bodies of *Pleurotus ostreatus* and *P. eryngii* using nine probiotic strains of *Lactobacillus*, *Bifidobacterium* and *Enterococcus* and

reported that these glucans can be applied to the synbiotic construction of selected probiotic strains. Prebiotic values of mushrooms have been thoroughly reviewed by Aida et al. (2009).

PREBIOTIC MOLECULES IN MUSHROOMS-

Most of mushrooms polysaccharides present as linear and branched glucans with different types of glycosidic linkages such as (1/3), (1/6)- β -glucans and (1/3)- α -glucans. Even though mushroom polysaccharides are of different chemical composition, most of them belonging to the group of β -glucans (Wasser, 2002). Digestive enzymes secreted by the pancreas or brush border of vertebrates, and of mammals, in particular, are unable to hydrolyze β -glucosidic bonds. This makes them resistant to acid hydrolysis in the stomach and remains non-digestible by human digestive enzymes (Van Loo, 2006). The non-digestible property of mushroom carbohydrate enables it to be considered as a potential source of prebiotic, as it meets part of prebiotic's definition (Gibson et al., 2004). Even though scientific experiments have proven a significant prebiotic effect of *Pleurotus ostreatus* and *P. eryngii* on selective microorganisms (Synytsya et al., 2009), an intensive and more thorough research need to be conducted to evaluate the prebiotic characters of other edible mushrooms.

PREBIOTIC ACTIVITY SCORE AND PREBIOTIC INDEX-

Prebiotic Score or Prebiotic Activity Score is used to evaluate the effectiveness of prebiotics in terms of supporting the growth of probiotics or pathogens. Huebner et al. (2007) reported the following equation to determine the prebiotic activity score which is a quantitative score for the activity within the food sample. A prebiotic score can be calculated using growth of

probiotic bacterial cells counted in the presence and absence of prebiotics in comparison to the growth of enteric pathogenic bacterial cells in the presence and absence of prebiotics. The growth of the probiotic and pathogenic bacterial cells is determined by spread plate or pour plate method using MRS agar and selective agar, respectively. Number of bacterial cells in terms of colony forming units (CFU) per one mL of the diluted sample was calculated and the log of CFU per mL was used to calculate the prebiotic activity score. Growth determined by optical density values can also be utilized to calculate the prebiotic activity score. Higher prebiotic score denotes that the prebiotic molecules support the growth of probiotic bacteria and does not support the growth of pathogenic bacteria in the presence of prebiotic molecules. Lower prebiotic score denotes that the prebiotic molecules support the growth of pathogenic bacteria and does not support the growth of probiotic bacteria in the presence of prebiotic molecules. Another terminology Measure of the Prebiotic Effect (MPE) denotes the analysis that considers the number of dominant bacterial groups and end products of fermentation such as short-chain fatty acids (SCFA) and substrate assimilation. MPE was first described by Jelena Veluvic in association with Glenn R Gibson and Robert Rastall. The prebiotic index or PI was

introduced to compare the prebiotic efficiency of different dietary oligosaccharides. Prebiotic Index equation considers bifidobacteria (Bif), Bacteroides (Bac), Lactobacilli (Lac) and Clostridia (Clos) to finally arrive in the Prebiotic Index value. Therefore, the aim of this work was to investigate to the prebiotic score of the two commercial prebiotics (inulin and raffinose) and three edible mushroom extracts (*Pleurotus sajor-caju*, *P. florida*, and *Lentinus edodes*). Hereafter this result may be analyzing the consolidation of probiotics and prebiotics of the three edible mushrooms and that could be added into the dairy foods.

MATERIALS AND METHODS-

1.COLLECTION OF BACTERIA

Lactobacillus acidophilus NCIM 2660 was purchased from National Collection of Industrial Microorganisms (NCIM), Pune, India. *Lactobacillus paracasei* KACC 12361 (Korean Agriculture Culture Collection (KACC), Myongji University, Yongin, Republic of Korea). *Lactobacillus* species were cultured using MRS agar (Himedia / Difco (BD), Mumbai, India). The enteric pathogens used were *Salmonella typhimurium* MTCC 3224, *Enterobacter cloacae* NCIM 2164 and *Escherichia coli* (a clinical isolate). Enteric pathogens were cultured in TSB (Trypticase soya broth).

2.COMMERCIAL PREBIOTICS

The commercial prebiotics D (+) Raffinose pentahydrate and Inulin-S from chicory, and a non-prebiotic substrate D-(+)-Glucose anhydrous were purchased from Himedia, Mumbai, India.

3.COLLECTION OF EDIBLE MUSHROOMS

Edible mushrooms like *Pleurotus sajor-caju* and *P. Florida* were collected from Mr. Basanta, Udyan Fresh, Chandrasekhar Pur, Bhubaneswar, Odisha. Shiitake (*Lentinula edodes*) mushroom was obtained from Prof.Satyawathi Sharma, Centre for Rural Development and Technology, IIT Delhi, Delhi, India.

4.PREPARATION OF CARBOHYDRATE RICH MUSHROOM EXTRACT

Collected mushrooms were washed with distilled water to remove dirt and extraneous matters. After drying in the oven at 70°C for 48 hours it was ground to fine powder. First cold extraction was done with cold distilled water for 3 hours. After drying it was washed twice with 80% ether followed by drying and protein precipitation with Sevag reagent (chloroform: butanol in ratio 4:1). Then after air drying, it was dissolved in deionized water (pH adjusted to 7) and kept in a hot water bath at 50°C. The slurry was kept on continuous stirring for 10 hours followed by centrifugation at 5000 rpm for 20 min. The supernatant was collected and 3 parts absolute ethanol was added to it followed by air drying and re-suspension in deionized water. The extract was then lyophilized for 4 hours to concentrate it and then stored in an airtight container until use.

5. PREBIOTIC ACTIVITY SCORE

Prebiotic activity in this study shows the ability of the test substrate to support and sustain the growth of beneficial organisms (probiotics) and not those of pathogenic ones. This activity is also related to the level of growth of the two types of organisms in presence of glucose (non-prebiotic substrate). Therefore, the prebiotic molecule has a better prebiotic activity score when their metabolism is comparable to that of glucose and is more preferred by probiotic bacteria rather than pathogenic bacteria. To keep the initial cell count uniform, McFarland Standard 2.0 (McFarland Latex Standards from Hardy Diagnostics (2014-12-10), measured at the UCSF DeRisi Lab) was selected. All the bacterial which were grown overnight were diluted until the OD 600nm value of 0.242. Diluted bacterial cultures were inoculated at 1% (v/v) level into a respective tube containing MRS broth supplemented with either 1% (w/v) of prebiotic (mushroom extract powder) or glucose. The culture was incubated at 37°C in ambient atmosphere for 24 hours. Growth in the broth was measured before and after incubation by reading the optical density values at 600 nm. Cell load or the number of bacterial cells before and after incubation was enumerated by plating (spread plate method) appropriate dilutions of MRS broth on MRS agar and counting the colonies after 24 hours' incubation of MRS agar plate. In addition, overnight cultures of different enteric pathogens were added at 1% (v/v) to respective tubes containing TS broth containing 1% (w/v) prebiotics (mushroom extract powder) or glucose. Optical density values and cell load of enteric pathogens were measured as mentioned previously for probiotic bacteria by using TSI agar plates. After 24 hours of incubation, the cell numbers were counted and colony forming units (CFU) per one mL of the diluted broth sample was calculated by using the following formula.

CFU/mL = Number of colonies counted / (dilution factor x volume of sample plated for analysis)

Prebiotic activity score was calculated by using both optical density and log CFU/mL values using the following equation:

$$\text{Prebiotic activity score} = \frac{[(\text{PBP24h}-\text{PBP0h})/(\text{PBG24h}-\text{PBG0h})] - [(\text{EPP24h}-\text{EPP0h})/(\text{EPG24h}-\text{EPG0h})]}{1}$$

Where,

PBP24h = Log CFU ml⁻¹ probiotic growing on the prebiotic at 24 h

PBP0h = Log CFU ml⁻¹ probiotic growing on the prebiotic at 0 h

PBG24h = Log CFU ml⁻¹ probiotic growing on glucose at 24 h

PBG0h = Log CFU ml⁻¹ probiotic growing on glucose at 0 h

EPP24h = Log CFU ml⁻¹ enteric pathogen growing on the prebiotic at 24 h

EPP0h = Log CFU ml⁻¹ enteric pathogen growing on the prebiotic at 0 h
EPG24h = Log CFU ml⁻¹ enteric pathogen growing on glucose at 24 h
EPG0h = Log CFU ml⁻¹ enteric pathogen growing on glucose at 0 h
STATISTICAL ANALYSIS

Results of the experiments are presented as a mean ± standard deviation. Mean and the Standard deviation was calculated using MS office Excel version 10 program. One-way analysis of variance (ANOVA) by Duncan's new multiple range tests was used to compare the mean values and was done with the SPSS (IBM) package version 19.0. Differences were considered to be significant at p<0.05.

RESULTS AND DISCUSSION

Mushrooms were reported to be the source of prebiotics in many studies. The present study was carried out to evaluate the prebiotic activity score of inulin, raffinose, and three edible mushrooms (*P. sajor-caju*, *P. Florida*, *L. edodes*) extracts using *L. acidophilus* NCIM 2660 and *L. paracasei* KACC 12361 as probiotic cultures and *S. typhimurium* MTCC 3224, *E. cloacae* NCIM 2164 and *E. coli* (a clinical isolate) as enteric pathogens. Prebiotic activity score was calculated using the equation mentioned in section 1.

Table-1 shows the increase in cell density after 24 hours of incubation of probiotic and pathogenic bacteria in the presence and absence of glucose, inulin, and raffinose. Inulin and raffinose were found to improve the growth of both of the probiotic bacteria studied. However, inulin was found to also support the growth of all the pathogenic bacteria studied. Raffinose was found not to support the growth of *E. coli* and *E. cloacae* but not *S. typhimurium*.

Table 1: Effect of glucose, inulin, and raffinose on the growth of bacteria (Log10CFU/mL).

Prebiotics	Log10 values of CFU/mL				
	LP	LA	ST	E.Coli	EC
Control	2.55±0.42 ^b	3.85±0.62 ^a	2.80±0.45 ^b	4.10±0.57 ^a	2.70±0.51 ^a
Glucose	3.38±0.62 ^{ab}	3.75±0.82 ^a	3.91±0.55 ^{ab}	4.05±0.52 ^a	1.75±0.31 ^a
Inulin	4.07±0.86 ^a	4.25±0.76 ^a	4.30±0.82 ^a	4.30±0.71 ^a	2.30±0.48 ^a
Raffinose	3.73±0.27 ^a	4.39±0.51 ^a	2.94±0.48 ^b	4.07±0.55 ^a	1.67±0.71 ^a

Table-2 shows that the prebiotic scores calculated for inulin and raffinose. Inulin supports the growth of *L. acidophilus* than the growth of *S. typhimurium* and *E. coli*. Inulin also supports the growth of the pathogen, *E. cloacae*. When compared to the control (without prebiotics), the prebiotic score of inulin on the growth of *L. acidophilus* over *S. typhimurium* is very less. However, the growth improvement shown by inulin on *L. acidophilus* over *E. coli* is higher than the control and is statistically significant (P<0.05). Contrary to *L. acidophilus*, the prebiotic scores calculated for inulin on *L. plantarum* are higher than the

control and statistically significant ($P < 0.05$) except for *L. plantarum* with *S. typhimurium* (statistically not significant, $P < 0.05$).

Table 2: Prebiotic activity score (calculated from CFU) of bacterial strains grown on inulin and raffinose.

	LA S.T.	LA E. coli	LA E.C	LP S.T.	LP E. coli	LP E.C.
Control	0.31±0.09 ^a	0.02±0.02 ^c	-0.51±0.05 ^c	0.04±0.01 ^b	-0.26±0.07 ^b	-0.79±0.07 ^c
Inulin	0.04±0.01 ^b	0.07±0.01 ^b	-0.02±0.00 ^b	0.11±0.07 ^b	0.14±0.03 ^a	0.05±0.02 ^b
Raffinose	0.42±0.06 ^a	0.17±0.02 ^a	0.22±0.02 ^a	0.35±0.09 ^a	0.10±0.02 ^a	0.15±0.05 ^a

Compared to inulin, the prebiotic score calculated for raffinose shows that it has statistically significant ($P < 0.05$) growth improvement characteristics on *L. acidophilus* and *L. plantarum* than the pathogens tested except for prebiotic score calculated for *L. acidophilus* with *S. typhimurium* (statistically insignificant, $P < 0.05$). The prebiotic scores calculated in the presence of raffinose for all the treatments tested are higher than the control (without prebiotics) which clearly shows the selective growth enhancement properties of raffinose.

Among the three pathogens tested, inulin shows the good prebiotic effect against *E. coli* when tested with both *L. acidophilus* and *L. plantarum*. Similarly, raffinose was showing a good prebiotic effect against *S. typhimurium* when tested with both *L. acidophilus* and *L. plantarum*.

Table 3: Effect of mushroom extracts on the growth of bacteria

Prebiotics	L.acidophilus KACC 12361	E.coli	E.Cloacae NCIM 2164
	Log10CFU/mL		
Control	5.00±0.50 ^a	4.12±0.82 ^a	4.06±0.21 ^c
Glucose	4.14±0.62 ^a	3.53±0.75 ^a	5.84±0.44 ^a
L.edodes	3.99±0.76 ^a	4.26±0.54 ^a	5.50±0.23 ^{ab}
P.Sajor.Caju	4.56±0.45 ^a	3.15±0.23 ^a	4.77±0.67 ^{bc}
P.Florida	4.68±0.82 ^a	4.00±0.71 ^a	6.14±0.51 ^a

Table-3 shows the effect of mushroom extracts on the growth of *L. acidophilus*, *E. coli*, and *E. cloacae* for 24 hours. All the three mushroom extracts showed no effect on the growth of *L.*

acidophilus and *E. coli* when compared to control.

Table 4: Prebiotic activity score calculated using log CFU values

Bacterial culture	Control	L.edodes	P.sajor-caju	P.florida
<i>L.acidophilus</i> NCIM 2660	0.04±0.01 ^b	-0.24±0.05 ^c	0.20±0.03 ^a	0.001±0.00 ^b
<i>E.Coli</i> NCIM 2660	0.51±0.04 ^a	0.023±0.00 ^c	0.28±0.02 ^b	0.08±0.03 ^c

Table-4 shows the prebiotic scores calculated for the carbohydrate-rich extract of three mushrooms, *L. edodes*, *P. sajor-caju*, and *P. florida* using *L. acidophilus* and two pathogens, *E. coli* and *E. cloacae*. Among the three mushroom extracts tested, *P. sajor-caju* shows higher prebiotic effects than the other two mushrooms. The growth improvement shown by *P. sajor-caju* extract on *L. acidophilus* is higher than control and statistically significant ($P < 0.05$) when it was calculated with *E. coli*. However, *P. sajor-caju*'s growth improvement effect was lesser ($P < 0.05$) than control for *L. acidophilus* when it was calculated with *E. cloacae*.

The prebiotic activity of mushroom extract is due to the presence of prebiotic components like beta-glucan. Aida et al. (2009) reviewed the potential of mushroom as prebiotics. Following carbohydrate constituents were isolated from various types of mushrooms: beta-glucan (*Pleurotus ostreatus*, *P. eryngii*, *P. tuberregium*, and *Ganoderma lucidum*), Krestin (mycelial biomass of *Trametes versicolor*), Chitin (*Boletus spp.*, *Agaricus spp.*), Lentinan (fruiting bodies of *Lentinus edodes*), Schizophyllan (*Schizophyllum commune*), (1→3)-alpha-d-glucan and beta-(1→3)-linked glucans (spores of *G. lucidum*). Even though the mushrooms polysaccharides are of different chemical composition, most of them belonging to the group of beta-glucans (Wasser, 2002). In *Pleurotus spp.* it ranges from 2.2 to 5.3 mg/g of dry matter, while in *Lentinus edodes*, it was reported to be around 2 mg/g of dry matter.

The non-digestible property of mushroom carbohydrate enables it to be considered as a potential source of prebiotic, as it meets part of prebiotic's definition. Synytsya et al. (2008) reported that the mushroom extract of *P. ostreatus* and *P. eryngii* were able to stimulate the growth of probiotics, *Lactobacillus spp.*, *Bifidobacterium sp.*, and *Enterococcus faecium*. They observed a maximum growth rate, maximum biomass concentration, and final acid production. Chou et al. (2013) reported that polysaccharides from the bases and stipes of *L. edodes*, *P. eryngii*, and *Flammulina velutipes* can enhance the survival rate of *L. acidophilus* and *L. casei* and *B. longum subsp. longum* during cold storage.

Many research studies also have indicated that polysaccharides from *Pleurotus spp.* (Synytsya et al., 2009), *L. edodes*, *Tremella fuciformis* (Guo et al., 2004), and *A. bisporus* (Giannenas et al., 2011) have prebiotic activity. Beta-glucans, homo-glucans, and heteroglycans with beta (1→3), beta (1→4), and beta (1→6) glucosidic linkages were thought to be responsible for their bioactivity. Among these, the carbohydrates of the edible mushrooms *P. eryngii*, *L. edodes*, and *Flammulina velutipes*, contain ribose, xylose, fructose, mannose, glucose, and trehalose. In addition, sucrose was observed only in *L. edodes*, whereas both *P. eryngii* and *L. edodes* were constituted primarily of glucose. *F. velutipes* contains mainly xylose (Kim et al., 2009).

Shalini et al. (2017) reported that the fructans from the *Nendran* banana showed highest prebiotic activity score, even higher than commercial FOS (fructose oligosaccharide) and inulin. The action mode of the polysaccharides might be related closely to increased microbial activities and enhanced immune function (Guo et al., 2004). The noncellulosic beta-glucans could be potent immunological stimulators. Among these, some now are used clinically in China and Japan (Chen and Seviour, 2007). Silva et al. (2016) reported that FOS was equally good as glucose to provide an energy source and FOS supported the growth of *L. plantarum* ATCC 14917 with the highest prebiotic score (0.526) followed by *L. casei* (LC-1) (0.222) and *paracasei* ATCC 27092 (-0.051).

Beta-glucans are polysaccharides of D-glucose monomers linked by beta-glycosidic linkages. It is one source of valuable dietary fiber found in cereals, yeast, mushrooms, seaweeds and some bacteria. Chemically, beta-glucans are non-starch polysaccharides with repeating glucose residues in either linear chains or multiply branched structures with the glucose units being branched in several ways depending upon the source of origin. For cereal beta-glucans the chains are completely linear, consisting of consecutively linked (via beta 1-3 linkages) of cellulosic oligomers; i.e. segments of beta 1-4 linked glucose residues (Lazaridou and Biliaderis, 2007). Instead, for microbial beta-glucans, the beta-D-glucopyranose units are linked together through beta-1(1,3) linkages to form a long backbone, whereas side chains mostly arise through beta-(1,6) linkages (Ahmad et al., 2012a). Beta-D-glucans can form large cylindrical molecules containing up to 250,000 glucose units (Vannucci et al., 2013). In 1941, there was the first discovery of a pharmaceutical insoluble yeast crude product called 'Zymosan', which is composed of 50% glucan and other polysaccharides (Pillemer and Ecker, 1941). Beta-glucans are major structural components of the cell walls of brewers' yeast *Saccharomyces cerevisiae*, fungi, and some bacteria. Depending on the source, there are clear differences between beta-glucans in their solubility, molecular mass, tertiary structure, the degree of branching, polymer charge and solution conformation, all of which in turn alter their immune modulating effects (Bohn and BeMiller, 1995; Eccles, 2005). Accordingly, beta-glucans having a beta-(1, 3) chain with beta-(1, 6) branching are more effective than beta-(1, 3) linear chain alone (Bohn and BeMiller, 1995).

CONCLUSION

The present work studied the prebiotic activity score of a carbohydrate-rich extract of three edible mushrooms and commercial prebiotics. The study revealed that an extract of *P. sajor-caju* Produced good prebiotic score when compared to *L. edodes* and *P. Florida* for *L. acidophilus*. The prebiotic activity of mushroom extracts is correlated to the presence of beta-glucans and the fermentation ability of the bacteria.

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