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A First Report on Buttermilk Isolate *Dipodascus armillariae and* its Improved Strain DAM 5 as a Novel Probiotic

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ABSTRACT: A new era in the probiotic family is emerging with the identification of many fungal strains as probiotics. Fungi are excellent candidates for probiotic flora due to their distinct cellular structures and enhanced capacity to survive in the hostile environment of the gastrointestinal system. Here, probiotics formulated using buttermilk isolate of *Dipodascus armillaria* and its caffeine improved variant (DAM 5) were compared with conventional strain of *Lactobacillus acidophilus*. Probiotic characterization of isolates revealed pH 1 tolerance, high antimicrobial activity and increased lactic acid production by DAM5. In this endeavour, an effort was made to create nutritionally enhanced probiotic drink, using the medicinal plant extracts of *Alternanthera sessilis* & *Eclipta prostrata*, with the chosen strains and assessed for their capacity to endure in or ferment these plants' leaf extracts. Two weeks fermented plant extracts revealed improving acidity, low pH, higher turbidity, antimicrobial & antioxidant activity and an increase in energy content of the chosen isolates. *A. sessilis* proved to be a best plant substrate compared to *E. prostrata*. Statistical analysis revealed the use of DAM 5 to be significant in probiotic formulation compared to other isolates. Extended study improving their shelf life can be better future prospect.

Keywords: Probiotics, Alternanthera sessilis, Eclipta prostrata, Dipodascus armillaria, DAM 5.

INTRODUCTION

One of the key challenges faced in the 21st century is the need to feed a rapidly increasing human population through a balanced diet, despite increasingly limited natural resources. Additionally, recent nutritional advancements have demonstrated that diet also plays an important role in the prevention and possible cure of various diseases by regulating multiple physiological functions. Also, the knowledge about the advantageous effects of the probiotic microorganisms and the foods that contain it gains recognition with the increase in lifestyle-related diseases Benucci *et al.* (2022); Roy and Kumar (2018). Probiotics are live bacteria or yeasts that, when given in sufficient proportions, provide a health benefit to the host. To date, with the growing interest in health consciousness, the concept of probiotic foods has gotten a lot of attention. The mechanism by which probiotics help the host is by the production of microbial inhibitory substances, blocking their adhesion sites, competing for nutrients degrading toxin receptors and stimulating immunity Mathew, (2019). A large number of probiotic species and strains belong to the genera *Lactobacillus* and *Bifidobacterium*. Other groups of the LAB (*Streptococcus* and *Enterococcus*), *Bacillus*, *Propionibacterium*, and the yeast *Saccharomyces* are being used as probiotic microorganisms. Probiotic strains have a high acid and biletolerance, which contributes to their ability to survive passage through the GI tract. These are intrinsic characteristics of the strain, which can be improved by the protective action of carrier foods and/or by the presence of nutrients. However, due to issues with lactose intolerance, milk allergies, or excessive cholesterol/fat content, many sections of society continue to lack access to probiotics, so a suitable supplement is required (Nagpal *et al.*, 2012). The present study was to identify yeast from probiotic sources like curd, improve the strain using natural agents like caffeine and analysed for its probiotic efficacy so that it can be applied in production of probiotic enhanced nutritional foods.

MATERIALS AND METHODS

A. Isolation of Lactic Acid producing microorganism

The medium De Man, Rogosa and Sharpe (MRS) (Chennai, Tamil Nadu), was used as a selective growth medium for the isolation of lactic acid producing bacteria/yeast. The pH of the media was adjusted between 6.2-6.6 and sterilized by autoclaving at 15 lbs pressure (121ºC) for 15 minutes, then poured into sterile petri plate. Isolation of microbes from store bought buttermilk was done by the serial dilution technique. Appropriate dilution was spread on MRS

agar plates at pH 6.5. These plates were incubated at 37ºC for 24 hrs. The individual colony was picked up and again streaked on MRS agar for further purification (Khyati, 2021). Colony morphology of pure cultures was recorded for study.

B. Screening of the isolates

(i) Gram staining: Gram staining is an ancient and reliable microbial identification technique that is used to differentiate two types of microorganisms based on their different cell wall constituents and thereby differences in the colouring of cells either violet and pink. The smear of the isolated colony was submersed in a crystal violet staining reagent for 60 seconds. Slides were washed gently with water for 2 seconds. Then smears were flooded with mordant dye (Gram's iodine) for 1 minute. Again, the slides were washed in a gentle and indirect stream of water for two seconds. These were further flooded with the decolorizing agent (Ethanol, 95%). It was continuously added dropwise to the slide until the decolorizing agent becomes clear. Further, safranin solution was poured over the slides for 1 minute for counterstaining of cells. Then these were washed under tap water until a clear solution appears in the effluent. The slides were dried for some time and observed under the high-resolution microscope. The gram-positive bacteria appear violet in colour and gram-negative bacteria appear pink in colour (Khyati, 2021).

(ii) Light microscopy. The pure culture of the isolates obtained was observed under light microscopy under oil immersion (1000X) magnification. A loopful of culture in saline was prepared as a wet mount on a clean glass slide, covered with a cover slip and observed under microscope.

(iii) Molecular identification. The selected isolates with probiotic properties were subjected to molecular characterization at genus level. The best isolate among selected isolates was identified at species level by using 18S rRNA sequencing. The primers used were: 5'TCCTGAGGGAAACTTCG3' and 5'ACCCGCTGA ACTTAAGC3'.

C. Analysis of Probiotic properties of isolate

(i) Tolerance to stimulated low pH of human stomach. The ability of isolates to sustain at low pH was tested using distilled water adjusted to pH levels 1.0, 2.0 and 3.0 using hydrochloric acid along with sterile distilled water at pH 6.5 as control. The different sets of test tubes were inoculated with isolated cultures and after 1-hour interval 1ml sample was taken out and allowed to incubate at 37ºC. The sets of tubes were further serially diluted and poured on MRS agar and incubated at 37ºC for 24-48 h and colony counts were recorded (Mathew, 2019).

(ii) Antimicrobial activity. Modified agar well diffusion method was used to detect antimicrobial activities of cell free supernatant (CFS) produced from the isolates. Antibacterial activity was determined against Escherichia coli. All of LAB isolates were incubated for 48 hour at 37°C. After incubation cells were removed by centrifugation. The indicator organism is inoculated in nutrient broth and incubated at 37°C for 5- 6 hours. The incubated organisms were swabbed on to the MHA (Muller – Hinton Agar) plates using swab and the CFS (Cell Free Supernatants) were used as antimicrobial agents. Using sterile tips, the CFS was poured into the well of about 50µL and kept for incubation at 37°C for 24 hours. Antimicrobial activity was evaluated by measuring zone of inhibition against the test organisms.

(iii) Lactic acid production. The pure cultures of the isolates were cultured in MRS broth for 24 hrs at 37ºC. The centrifuged supernatant of the broth cultures was recorded for lactic acid production by acid- base titration against 0.1N Sodium hydroxide using phenolphthalein indicator (Mathew, 2019).

D. Development of improved strain of isolate

The selected yeast strain with probiotic properties (*Dipodascus armillaria*) was subjected for improvement by treating with caffeine of varying concentrations: 0.01Mm, 0.05mM, 0.1Mm, 0.5mM, 1mM. The CFU count and turbidity was periodically analysed to check whether caffeine is inhibiting the growth or not. The probiotic characteristics of the improved isolates were also analysed as previously discussed in Section 3.

RESULTS AND DISCUSSION

A. Isolation of Probiotic microbe for screening

Five strains were obtained from buttermilk. The individual bacterial isolates which show different morphology were sub cultured in order to obtain pure cultures. The pure isolates 5 colonies named as C1, C2, C3, C4 and C5 were maintained at 4ºC in refrigerator for further studies. The colony morphology recorded was correlated with gram staining/light microscopic studies whose results obtained were shown in Table 1. C4 isolate was identified to be yeast owing to its colony morphology and pseudohyphae structures seen under light microscopy (Fig. 1- 3).

Table 1: Morphological results of the isolates.

Isolates	Gram Staining	Colony Morphology
C1	Gram positive rods	Transparent, small colonies
C2	Gram positive rods	White, small colonies
C3	Gram negative cocci in tetrads	White, small colonies
C4	Gram positive large rods. Pseudohyphae in wet mount	Flat, creamy-white colonies
C5	Gram positive rods	Creamy, small colonies

Fig. 1. Isolated C4 colony; flat, creamy-white colonies.

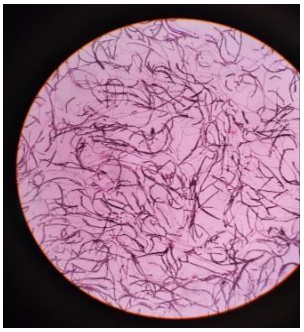

Fig. 3. Gram's stain of C4 isolate.

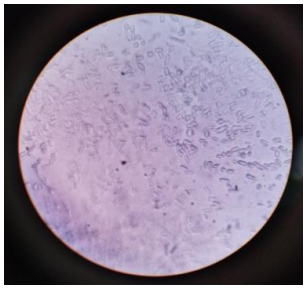

Fig. 2. Wet mount microscopy of C4 isolate.

B. Molecular identification of C4 isolate

18s rRNA sequencing results of the C4 isolate that fulfilled the criteria for probiotic was analysed for its phylogeny and the yeast was identified as *Dipodascus armillaria.* This is a first report of this isolate to be obtained from buttermilk in India and hence the sequence was published in GenBank, NCBI - Accession number OQ518922.

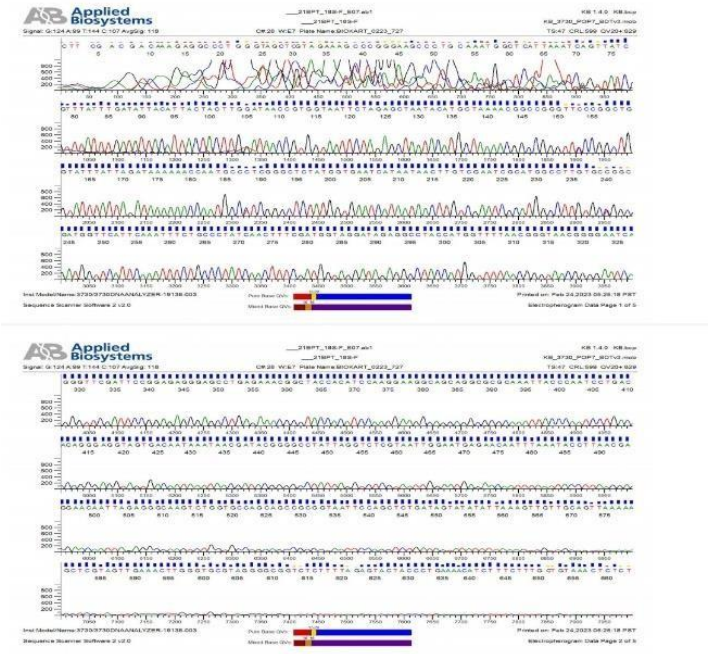

Fig. 4. Electropherogram of *Dipodascus armillaria.*

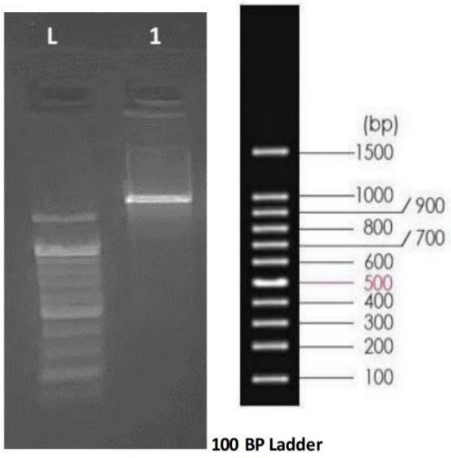

Fig. 5. PCR band of *Dipodascus armillaria.*

C. Development of improved strain of the C4 isolate

Dipodascus armillariae and its five different variants that were improved with different concentrations of caffeine were analysed for its probiotic characteristics. Estimation of lactic acid and turbidity shows 1mM caffeine treated strain could produce more lactic acid and is more turbid compared to the other caffeine treated strains and *D. armillaria strain* (i.e., no caffeine) (Table 2).

Table 2: Development of Improved strains of *D. armillaria.*

Concentration of caffeine	CFU Count	Acidity (in%)	Turbidity (at 600nm)
0.01mM	36×10^4	0.23%	1.9011
0.05mM	51×10^4	0.24%	1.9486
0.1mM	62×10^4	0.24%	1.9646
0.5mM	75×10^4	0.50%	2.0740
1mM	107×10^4	0.7%	2.1008
No caffeine	24×10^5	0.6%	0.381

The improved variety *Dipodascus armillaria* treated with 1mM caffeine (namely, DAM 5) also exhibited the following probiotic properties of pH tolerance and antimicrobial activity.

D. Low pH Tolerance

Low pH tolerance test carried out for the isolates showed that DAM 5 strain is more tolerant to pH 1 showing 56×10^4 CFU/ml followed by *D. armillariae* with 46×10^4 CFU/mL compared to control.

Fig. 6. DAM 5 showing high pH tolerance at 1, followed by *D. armillaria.*

E. Antimicrobial Activity

Highest antimicrobial activity was showed by DAM 5 with 23mm zone of inhibition compared to other isolates which was 16mm for *D. armillaria* and control.

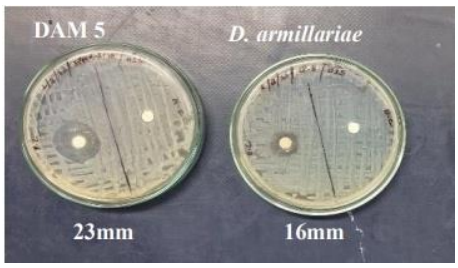

Fig. 7. DAM 5 showing high anti- Escherichia activity with a zone of inhibition of 23mm.

The present study had thus aimed to isolate and improve yeast strain from buttermilk sample. Since yeasts co-exist among traditional fermented dairy products, it is believed that yeasts also may have some probiotic properties (Lama and Tamang 2022). It's said that Dipodascus are one among the ten abundant yeast strains found in the gut (Desnilasari *et al.*, 2020). This particular yeast strain is normally present in fermented foods and has also been found in fermented 'Hairy' Tofu (Maas *et al*., 2023). This research is a first report of this yeast variety as a buttermilk isolate in India. The major selection criterions for a probiotic includes, resistance to low pH and antimicrobial activity (Alkalbani *et al.,* 2022). Both Dipodascus as well as DAM 5 strain survived in low pH, latter being highly tolerant at pH 1 showing more CFU/mL and higher antimicrobial activity. Use of caffeine being a natural stimulant effective in probiotic strain improvement is also thus proven as 1mM concentration showed 16% increase in lactic acid production in 24 hours of study. The action of caffeine in the strains of diploid yeast *Saccharomyces* sp. has been studied in early days by Kiefer in 1975 in gene interference mechanism that gives an insight for us in our present study.

CONCLUSIONS

In the present study entitled an attempt has been made to isolate an efficient potential probiotic yeast strain from buttermilk, its improvement with caffeine and further evaluation of their essential probiotic properties. Presence of *Dipodascus armillariae* in buttermilk has been revealed and its utilization as a probiotic becomes novel and significant as the results concluded higher pH tolerance (at pH 1), better antimicrobial efficacy (23mm) and enhanced lactic acid production (0.6%). Use of caffeine being a natural stimulant effective in probiotic enhancement is also proven as 1mM concentration showed 16% increase in lactic acid production in 24 hours of study. Hence, due to these properties this particular improved variety *Dipodascus armillaria* treated with 1mM caffeine (namely, DAM 5) was identified to be used in future for probiotic product formulations.

FUTURE SCOPE

The developed strains can be utilized to develop probiotic functional food using medicinal plants. Use of this strain in probiotic juice preparation on commercial scale may benefit the consumers, especially those who are lactose intolerant and sensitive to milk-based products. Use of caffeine as natural stimulants in strain improvement can also be extended for other beneficial microorganisms. The study has actually been extended to develop nutritionally enhanced probiotic drink, using the medicinal plant extracts of *Alternanthera sessilis* & *Ecliptaprostrata*, with the chosen strain DAM 5 and assessed for its capacity to endure in or ferment these plants' leaf extracts. The study is still under process whose results can be correlated on completion. Additionally, fermented plant products are cholesterol

free, low-cost and may provide better nutrition and health.

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Conflict of Interest. None.

REFERENCES

Alkalbani, N. S., Osaili, T. M., Al-Nabulsi, A. A., Olaimat, A. N., Liu, S. Q., Shah, N. P. & Ayyash, M. M. (2022). Assessment of yeasts as potential probiotics: A review of gastrointestinal tract conditions and investigation methods. *Journal of Fungi*, *8*(4), 365.

Benucci, G. M. N., Wang, X., Zhang, L., Bonito, G., & Yu, F. (2022). Yeast and lactic acid bacteria dominate the core microbiome of fermented 'hairy' tofu (Mao tofu). *Diversity*, *14*(3), 207.

Desnilasari, D., Agustina, W., Putri, D. P., Iwansyah, A. C., Setiaboma, W., Sholichah, E., & Herminiati, A. (2021). The characteristics of probiotic drink based on moringa leaves juice. *Journal Teknologi Dan Industri Pangan*, *32*(1), 9-15.

Khyati, O. (2021). Studies on microencapsulated probiotic starter cultures for the development of functional beverages. *Shodhganga*.

Lama, S., & Tamang, J. P. (2022). Isolation of yeasts from some homemade fermented cow-milk products of Sikkim and their probiotic characteristics. *Fermentation*, *8*(12), 664.

Mathew, S. (2019). Isolation & characterization of probiotic bacteria and develop probiotic enriched food, *Shodhganga*.

Maas, E., Penders, J., & Venema, K. (2023). Fungal-bacterial interactions in the human gut of healthy individuals. *Journal of Fungi*, *9*(2), 139.

Nagpal, R., Kumar, A., & Kumar, M. (2012). Fortification and fermentation of fruit juices with probiotic lactobacilli. *Annals of microbiology*, *62*, 1573-1578.

Roy, P., & Kumar, V. (2018). Functional food: probiotic as health booster. *J. Food Nutr. Popul. Health*, *2*(2), 12.

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