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RESEARCH ARTICLE

EFFECT OF *BACILLUSCLAUSII* THE PINEAPPLE PROBIOTIC BEVERAGE ON THE BASIS OF PHYSICO-CHEMICAL, SHELF-LIFE STABILITY AND ANTIMICROBIAL ANALYSIS

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Abstract

The pineapple (Ananascomosus) is the world's second-most-produced tropical fruit, with roughly 25.8 million tonnes planted in 2017. Pineapple and pineapple products are well-known for their fragrance and flavour.The probiotic starting culture was *Bacillusclausii* bacterium. The study's vision is to develop a probiotic-rich fermented beverage made from whole pineapple juice, specifically for lactose intolerant persons, and to characterise it in terms of nutritional properties, storage stability, and antibacterial activity.The mixture was fermented with probiotics for 48 hours at 37°C. The shelf-life of the blend was tested using acidity, pH, nutritional and probiotic cell viability, and antibacterial activity.According to the findings, the probiotic pineapple beverage can be stored at 4 °C for 28 days with adequate acceptability and shelf life. The viable probiotic cell counts in the beverage after 14 days were 2.9*10 CFU/mL and 2.2*10 CFU/mL, respectively.In comparison to Vancomycin and Ampicillin, fermented probiotic pineapple beverage demonstrated effective antibacterial action against diarrheal infecting pathogens *S.aureus*.and *S.flexneri*. This study found that pineapple juice can be utilized as a probiotic beverage for diarrhea sufferers, and that it may have good functional and nutritional benefits for human consumption and also suggests that it may have a beneficial health effect on malnourished children in underdeveloped nations.

Introduction:-

Probiotics are microbial feed supplements that are alive. When ingested in adequate quantities, they can improve consumer health by improving the balance of micro flora in the gut(R, 1989).Consumer awareness of functional products, which have the ability to maintain healthy lifestyle, is heading increased use and production of probiotic foods(Roberfroid, 2000).Probiotic bacteria are said to have a variety of health benefits, including antimicrobial, anti-tumour, anti-cholesterol, immunomodulation, anti-diabetic, and treatment of diarrhoea and lactose intolerance(R.Nagpal, 2007).Today, the concept of using meals to promote a sense of well-being, improve health, and reduce illness risk is the new frontier in nutrition studies and associated subjects. In this context, the

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development and contribution of functional foods - prebiotics, probiotics, and symbiotic - must be prioritised as critical pillars of the health-care system.

Pineapple (*Ananascomosus*) is the second-most-produced tropical fruit in the world, with approximately 25.8 million tonnes cultivated in 2017(S.Altendrof, 2017).It contains bromelain enzyme, which is beneficial for digestion, as well as components that contribute to numerous health advantages such as anticancer, antidiabetic, and antihypertensive. Furthermore, it is a good source of vitamins A, B, and C, as well as minerals including calcium, magnesium, potassium, and iron(M.Ahmad, 2015).It is a great source of polyphenolic chemicals like as gallic acid, chlorogenic acid, and ferulic acid, which have been shown to have antioxidative, antimutagenic, and anticarcinogenic properties as well as protective effects against cardiovascular disease and cataracts(S.Y.Baljeet, 2013).Pineapple also contributes dietary fibre (DF) to food matrices, which operate as 'prebiotics' and have a substantial impact on the host's health by selectively boosting the growth and/or activity of certain genera of gut microbiota, including *Lactobacillus* and *Bifidobacterium*(M.DeVrese, 2008).

The concept of using food to generate a sense of well-being, improve one's health, and lessen the risk of illness has become a major breakthrough in the nutrition sciences and related professions(Perricone.M., 2015).Furthermore, the problem is particularly significant due to rising health-care costs, rising life expectancy, and the desire of elderly individuals for a higher quality of life. In addition, the emphasis has switched from therapy to prevention. In this framework, the development and contribution of functional foods—prebiotics, probiotics, and symbiotics—must be encouraged, and they should be considered as fundamental pillars of the health-care system. The global probiotic market is expected to be worth $34.1 billion in 2020 and $73.9 billion by 2030, with an 8.6 percent compound annual growth rate(Jaya Bundele, 2021).

Probiotic drinks have lately arisen as a substantial new market in the global exploitation of milk whey. The food industry has created a variety of fermented items containing probiotic microorganisms. Fermented foods with sufficient quantities of a certain live count of probiotics give several health benefits. Probiotics have a range of health benefits, including enhanced gut health due to pathogen resistance, lower blood cholesterol, relief from lactose intolerance, and a lower risk of cancer(M.K., 2014).Probiotic bacterial cultures inoculated in a variety of dairy and non-dairy beverages are among the dietary matrices that potentially signal a major hazard to probiotic survival. The potential of probiotics to represent living cells in adequate quantities for host intestinal flora adaption is essential in the development of new probiotic drinks(A.B.Shori, 2016).A minimum live cell number of 10^6–10^8 CFU/g of intestinal material is required/recommended to alter the gut environment by probiotic microbes(A.Rzepkowska, 2018).

There are several scientific journals on in vitro and ex vivo studies on non-dairy and dairy foods, but very few have focused on the probiotic organism's survivability in the gastrointestinal state when food matrix is used as a carrier of probiotics(S.J.Hur, 2011)(A.Rzepkowska, 2018).As a result, the produced probiotic will be evaluated for diarrhea prevention and gastrointestinal digesting stimulation, which is required to gain a better understanding of their potential health benefits, particularly for the elderly. Based on this, the current study aimed to develop a probiotic-rich fermented beverage made from whole pineapple juice, as well as characterize it in terms of nutritional properties, storage stability, and antibacterial activity.

Materials and Methods:-

The current research was carried out at BabasahebBhimraoAmbedkar University (BBAU) in Lucknow, at the Food Analysis Laboratory, Department of Food and Nutrition, and the Laboratory of Molecular Microbiology, Department of Environmental Microbiology.

Preparation of the Pineapple Juice:-

Ripe pineapple was chosen, the crown and stem were removed, and the fruit was washed in tap water, hot water, and KMS (Potassium Metabisulphite) to remove any debris. They were cut with a mechanical slicer. Using sterile knives, the pineapple was peeled and the eyes were removed. In a mixer grinder, the prepared slices were crushed, and the juice was extracted by squeezing the crushed mass in a hydraulic press. The juice was filtered through muslin fabric that had been autoclaved. The samples were transferred into a clean sterile glass flask (100mL) containing 8 g of Sucrose, pasteurized at 80°C for 40 seconds, and utilized for inoculation. The juice was kept refrigerated (4±3°C) until it was utilized.

Isolation of Probiotic:-
The probiotic *Bacillusclausii*was extracted from sample(Enterogermina®) in liquid form.For the isolation purposethe culture was grown in Tryptic soya Agar media (Himedia, India) by using Spread Plate method(Aneja.K.R., 2007).The inoculum was grown in tryptic soya broth and cultured at 37°C for 24-48 hours before being utilised as the inoculum for the fermentation mixture.

Inoculation of Probiotic:-
Based on a total viable count of *B.clausii* Bacteria of $7.1*10^7$cfu/mL and a volumetric productive output of 280µL, 100 mL of pasteurised pineapple juice was inoculation into a 250 mL sterilised glass bottle and the blend was incubated at 37°C for 48 hours.

Physico-chemical analysis:-
On equal interval of 7 days for the shelf-life attributepineapple-based probiotic beverage physico-chemical compositionwasanalyzed on basis of biomass determination, total soluble solids byusing digital refractometer,titrable acidity and, protein content by Micro-Kjeldahl method; and pH by using a digital pH meter (ATC model no:6032).

Total phenolic Compound:-
The sample extracts were made according(S.Chandra, 2014)to quantify total phenolic compounds (TPC). In a nutshell, the beverage samples were dried overnight at 105 degrees Celsius. After that, 75 mL of water was used to extract 10 g of material.Ethanol (95 percent v/v) at 40°C for 10 minutes. The solvent extract was preserved. Evaporation takes place at 60 degrees Celsius. The extract was dissolved in around 50 mg of water.5 mL methanol for 45 minutes at 40°C, then centrifugation 1000 rpm.Supernatant was collected after 10 minutes at room temperature.In test tubes, the Folin–Ciocalteu reagent (2.5 mL; diluted 10 times with water) and sodium carbonate (2 mL) (7.5 percent w/v) were added to sample extracts (500 L).. After that, the samples in the tubes were gently mixed by vertexing and incubated at 50degreeC for 5 minutes. A UV-VIS spectrophotometer was used.At a wavelength of 760 nm, the absorption was observed. Finally, the data were expressed in milligrams of 100 g gallic acid equivalents (GAE) (on fresh basis).

Microbiological Analysis:-
For shelf-life study,by repeated ten-fold dilution with sterile peptone water, the number of live *Bacillusclausii*bacteria cells was assessed. Tryptic soy agar was used to plate aliquots of one mL in duplicate. The results were expressed as log Colony Forming Units (CFU)/mL juice after the Petri plates were incubated for 24-48 hours at 37°C.

The number of live bacterial cells was determined using the plate count method. The culture was serially diluted in phosphate buffered saline before being disseminated in triplicate on a TSA plate with 100 mL of the suspension. The plates were counted and the findings were expressed as CFU/mL after being incubated aerobically at 37°C for around 3 days. Spread plate technique was used to test the vitality of the cells in the juice. Every 24 hours, the material was taken aseptically and serially diluted. To obtain countable colonies, serially diluted samples were spread out in TSA plates. Overnight at 37°C, the plates were incubated(Aneja.K.R., 2007).

Viable cell count = (number of colonies)/ (dilution × amount plated) ----------eq. (1):-
The viability of cells was determined by counting the number of colonies grown in the Tryptic soy agar plate using the formula given in equation 1.

Antimicrobial Activity of Probioticated Pineapple Juice:-
Preparation of Pathogenic Strains:-
Pathogenic microorganisms including *Staphylococcusaureus* and *Shigellaflexneri*was taken from Culture Bank, Microbiology Laboratory, BBAU. The stock of bacterial strains was prepared and maintained by transferring a loopful culture of stock cultures to the flask containing specific media broth and incubating at 37°C.

Antimicrobial analysis by Well diffusion method:-
The antibacterial potential of probioticated pineapple juice against pathogenic strains (*S. aureus* and *S. flexneri*) using antibiotics was determined using the agar well diffusion method(Verma, 2013).The pathogenic strains (S. aureus grown on Tryptic soy broth and S. flexneri developed on Luria Bertani broth) were cultured for 24 hours and

incubated at 37°C. The cell suspension was dispersed over the Mueller Hinton agar plates with a sterile spreader to create a lawn of bacterial strains.The plates were left to air dry. To cut uniform wells in the agar, a sterile borer with a diameter of 5mm was utilised. 1 mL fermented juice was placed in a 1.5 mL Eppendorf tube and centrifuged for 5 minutes at 10000 rpm. Antimicrobial activity was tested against *S. flexneri* and *S. aureus* using 150 µL of supernatant as a positive control and positive control as Ampicillin and Vancomycin, respectively.

The plates were incubated for 24 hours at 37°C. A zone of inhibition around the well was detected on the plates.

Statistical Analysis:-
All of the findings of this study are presented as the average of three replicates. Graph pad Prism 9.3.1 (Statistical Package, copyright of IBM Corporation and others) software was used to determine standard deviation and mean for all physicochemical analyses.

Result and Discussion:-

Physico-Chemical Analysis:-
Chemical characteristics such as acidity, total solids (TSS), protein, of the fermented pineapple probiotic blend, as well as biomass determination and TPC for storage capacities, were tested on a weekly basis (Table 1).The value of pH and acidity.The pH of the beverage sample initially increased for up to 21stdays, which could be linked to the sample's strong buffering capacity, particularly in fruit juice(S.Nualkaekul, 2011)(S.Multari, 2020).Furthermore, some Lactobacillus strains have the ability to convert citric acid to lactic acid or autolyze bacterial cells, resulting in the release of peptides into the beverage. Following that, the pH gradually dropped until it reached 4.3 after 28thdays, which might be explained as the beverage's partial buffering was eliminated as the preservation period progressed.The pineapple probiotic had the greatest acidity of 0.66 percent after 21stdays of storage. After fermentation at the 0thday, the titrable acidity of pineapple juice increased by 0.61 percent, indicating that probiotic lactic acid bacteria can ferment the sugars present in probiotic juice together with the (prebiotic) pineapple. At 7thdays, the juice had a high titrable acidity of 0.66 percent. The main cause of this is a drop in pH and a rise in titrable acidity, which can be attributed to both (1). Consumption of sugar (2). Lactic acid in probiotic fruit juice produces organic acid(Moraru, 2007).Lactic acid is created during fermentation, and its concentration rises as fermentation progresses, according to(AH, 1967).

Table 1:- Physico-chemical Analysis.

Parameter	Pineapple Juice					Pineapple Juice fermented with LAB				
	No. of days						No. of days			
	0 day	7 day	14day	21day	28day	0 day	7 day	14day	21day	28day
pH	3.8±0.01	3.9±0.01	3.8±0.01	4.1±0.01	3.5±0.01	3.7±0.01	4.2±0.01	4.4±0.01	4.6±0.01	4.3±0.01
Acidity (LA%)	0.48±0.02	0.56±0.02	0.64±0.02	0.58±0.02	0.56±0.02	0.61±0.02	0.66±0.02	0.64±0.02	0.66±0.02	0.63±0.01
TSS (Brix%)	8.2±0.56	8.3±0.36	8.1±0.45	7.3±0.61	7.6±0.95	13.8±0.92	12.1±0.56	12.3±0.08	12.1±0.78	7.6±0.89
Protein (%)	0.82±0.26	0.79±0.24	0.68±0.14	0.61±0.24	0.58±0.18	0.59±0.13	0.79±0.25	1.05±0.24	1.4±0.20	1.7±0.27
Biomass Determination (%)	0.68±0.01	0.64±0.01	0.59±0.01	0.56±0.01	0.51±0.01	0.52±0.01	0.58±0.01	0.67±0.01	0.72±0.01	0.69±0.01
TPC (mg GAE/100 g)	97±0.15	92.78±0.15	92.78±0.15	90.56±0.15	90.56±0.15	95.43±0.15	89.24±0.15	89.24±0.15	89.24±0.15	89.24±0.15

At the raw stage, the total soluble solid in pineapple juice was 8.2°Brix. Because they were used by bacteria for their growth in pineapple juice, the TSS of the juice increased for a while as the fermentation progressed, but then reduced to 7.6 as time passed.

At 0-day, pineapple juice had an initial biomass of 0.68g/L, while probioticated pineapple juice had an initial biomass of 0.52g/L. With the passage of time, the biomass level of probioticated pineapple juice rises, reaching a

maximum of 21stdays of storage. At 28th days, the biomass in pineapple juice had decreased by 0.69g/L(Nogueria, 2007).As the fermentation process in apple juice progressed, the biomass level decreased. The prebiotic boosted the growth of lactic acid bacteria including *Bifidobacterium*and *Lactobacillus* sp. in meat(Xu, 2003)according to the findings. As reported by(Farinha, 2015)in Bacteriocin synthesis, the introduction of prebiotics enhances cell biomass and specific growth rate while also reducing generation time.

When pineapple juice was inoculated with *Bacillusclausii*, the initial protein content was 0.82 percent at 0 day and decreased to 0.58 percent over time. However, when pineapple juice was inoculated with *Bacillusclausii*, the initial protein content was 0.59 percent and increased to 1.7 percent as fermentation progressed.

The TPC value of alone pineapple juice exhibits 97mg GAE/100 g but with time it decreased to 90.56 mg GAE per 100 g when compared with the TPC value of pineapple juice inoculated with LAB exhibited 95.43 mg GAE per 100 g at 0 day as fermentation occurred TPC decreased with time to 89.24 mg GAE per 100 g.Because the interactions between polyphenols aspolyphenols are only superficially adsorbed on proteins, and proteins are generally non-covalent. The use of sucrose in beverage production would have no effect, determining the TPC content of pineapple beverage, despite the fact that the protein number of beverages varies greatly(C.Thongkaew, 2014).

B.clausii inoculated Pineapple Juice exhibit excellent stability when compared with alone pineapple juice sample at 0th, 7th,14th,21st and 28th day. Similar type of observations was reported in case of pH reported of alone pineapple juice sample depicted 1.2 times decrease with pH of the pineapple juice inoculated with *B. clausii*. In case of acidity depicted of pineapple juice alone was 1.17 times decrease with acidity of the pineapple juice inoculated with pineapple juice. In case of TSS depicted value was 1.4 times decrease with the pineapple juice inoculated with B.clausii. In case of TPC content depicted 1.12 times decrease as compared to TPC value of pineapple juice inoculated with B.clausii.

Microbiological Analysis:-

The total viable cell count (TVC) of the beverage sample was determined after 28 days of storage (Table 2). With the advancement of storage period, the TVC increased significantly.

Table 2:- Total viable count (cfu/mL).

B.clausii	No. of days					
	Control	0 day	7 days	14 days	21 days	28 days
Dilution (10^{-5})	0	$7.1*10^{7}$	$6.3*10^{7}$	$3.7*10^{7}$	$2.9*10^{7}$	$2.2*10^{7}$

The microbial growth pattern indicated that the probiotic bacteria gradually but steadily expanded their population from 0 to 14 days of preservation, after which they began to fall significantly. This finding is consistent with prior studies that found that the TVC of *Lactobacillusreuteri* and *Bifidobacteriumbifidum* in whey-based probiotic beverages decreased after 30 days of storage at 4±1°C. The prepared beverage, on the other hand, had a viable probiotic count that was within a safe range and could be regarded a functional dose for human consumption for up to 28 days at 4°C.

Antimicrobial Activity of the Pineapple Juice:-

The antimicrobial activity (Table 3) of pineapple juice alone exhibited 17mm Zone of inhibition against S. aureus having a MIC 2µg/mL as compared to probioticated pineapple juice exhibited the zone of 14 mm while ZOI (Zone of Inhibition) exhibited by Pineapple juice alone against S.flexneri12mm as compared to probioticated juice (ZOI) is 15mm having a MIC 256µg/mL. It was observed that in both the cases the probiotic pineapple juice also showed antimicrobial activity as Vancomycin and Ampillicin.

Table 3:- Antimicrobial Activity of Pineapple Juice.

Test Organism	***Bacillusclausii***	Control	MIC
S. aureus	15mm	17mm	2µg/mL
S.flexneri	12mm	15mm	256µ/mL

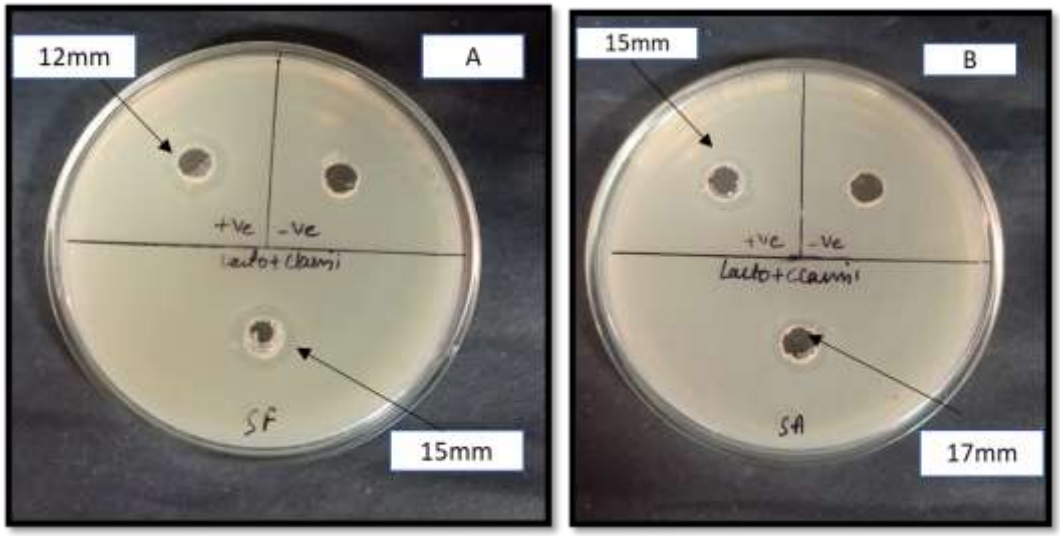

Fig1:- Antimicrobial Activity of Probioticated Pineapple Juice against tested strains. (A). *S.flexneri* (B).*S.aureus.*

Conclusion:-

Pineapple is a prebiotic in and of itself, with a variety of health benefits including antimutagenic, antidiarrheal, antidiabetic, and antihypertensive effects. *Bacillusclausii*have probiotic characteristics, which help to improve gut microbiota and overall health. Especially, this bacteria can be used to modify the pineapple probiotic beverage for lactose intolerant people. Based on these qualities, this research focuses on the findings which could pave the way for the development of a nutritional and functional pineapple beverage.We discovered that fermented beverage showed remarkable results up to 28 days of preservation in terms of pH, acidity, TSS, biomass determination stability test, antioxidant content, viability of probiotic cells, and shelf life at 4°C.Furthermore, fermented pineapple beverage had a higher protein content than probiotic pineapple juice, suggesting that it may have a beneficial health effect on malnourished children in underdeveloped nations. It could also be used for prevention for diarrheapatients. This study adds to the growing body of knowledge about the nutritional and functional qualities of probiotic-based pineapple beverages that are significant to consumers and the food industry.

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