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ANTICANCER, ANTIOXIDANT AND ANTIVIRAL ACTIVITY OF NOVEL NANO-PARABIOTIC *WEISSELLA PARAMESENTEROIDES* MN2C2

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ABSTRACT

Probiotics, or beneficial bacteria, rely on their viability to offer health benefits. The concept of "paraprobiotics," suggesting that non-viable bacteria may offer health benefits similar to live probiotics, has challenged this notion. In some cases, administering live probiotics to individuals with weakened immune systems can be risky. Paraprobiotics could be a viable alternative in such scenarios. Paraprobiotics refer to the use of inactivated microbial cells or cell fractions to promote health benefits. Due to their extended shelf life, safety profile, and various beneficial effects such as cholesterol reduction, immune modulation, anti-inflammatory properties, and anti-cancer potential, paraprobiotics have gained significant attention. The main objective of this study was to develop parabiotics and nano-parabiotics using a cost-effective waste medium and assess their in vitro biological activities, including antioxidant, anticancer, and antiviral properties, for potential functional food applications. The probiotic strain Weissella paramesenteroides MN2C2 was cultured in a low-cost sweet whey medium (LCSWM), and the resulting culture was separated into supernatant and heat-killed cells (HK), which were then sonicated to produce sonicated heat-killed (SHK) nano-parabiotics. The biological activities of HK and SHK were evaluated in vitro, including their antioxidant, anticancer, and antiviral activities. Furthermore, the safe and biologically active concentrations were determined for incorporation into functional dairy products such as yogurt, along with sensory evaluation. The HK and SHK parabiotics were successfully prepared using LCSWM and characterized as nano-formulations with sizes of 618.7 nm for HK and 456.4 nm for SHK, as determined by dynamic light scattering (DLS) and Transmission Electron Microscopy (TEM). The IC50 values for the antioxidant activities of HK and SHK were determined to be 38.36 µg/mL and 37.61 µg/mL, respectively. Both HK and SHK demonstrated a safe concentration of 1.1 mg/mL with a selectivity index (SI) of 2.2 against colorectal adenocarcinoma (Caco-2) cells, and SHK exhibited a 99.99% reduction in virus titers against Coxsackie virus B3 (CVB3). Finally, a safe dose of SHK was incorporated into yogurt as a functional food with acceptable sensory attributes.

Keywords: Probiotics, Parabiotics, Nano-Parabiotics, Antioxdant, Anticancer, Antiviral, Functional Food

INTRODUCTION

Probiotics, considered as functional food, improve the host's health by producing chemical compounds that play physiological roles in human body metabolism **(Roshdy, 2024).** However, a number of questions about their use have been raised in recent years. Probiotic product storage and industrial processing, in particular, continue to present technological problems since they may seriously compromise cell viability. Live microbe safety needs to be considered, especially when giving them to susceptible populations like the elderly and immune-compromised individuals. Due to these limitations, interest in novel products based on probiotics that are not viable, including postbiotics and paraprobiotics, has increased.
Paraprobiotics, specifically, are defined as “non-viable, inactivated microbial cells that confer a health benefit to the consumer.” They have the capacity to modulate both the innate and adaptive immune systems, possess anti-inflammatory, antiproliferative, and antioxidant characteristics, and have an antagonistic effect on pathogens. *Weissella paramesenteroides* MN2C2, a probiotic bacterium, has shown high potential as a health promoter by producing various bioactive compounds such as anticancer enzymes and antioxidant polysaccharides **(Amer *et al*., 2021)**. Furthermore, paraprobiotics have the potential to demonstrate improved safety, provide technological and practical advantages, and be incorporated into products meant for older adults and those with weakened immune systems. These characteristics present a significant opportunity to lead the industry with innovative, safer, and more stable nutraceuticals or functional meals **(Siciliano *et al*., 2021)**. Parabiotics are non-viable microbial cells, crude cell extracts, or inactivated probiotics that provide benefits to human or animal consumers when administered in sufficient amounts **(Nataraj *et al*., 2020).** Parabiotics can serve as an alternative to probiotics for high-risk patients or those with underlying conditions that make it challenging to consume live bacteria **(Song *et al*., 2019; Kim *et al*., 2021)**. In comparison to probiotics, parabiotics possess characteristics such as a safe profile, extended shelf life, stability in the digestive system, resistance to hydrolysis, and non-toxicity **(Akter *et al*., 2020).** Recently, there has been a growing interest in the use of parabiotics as pharmaceuticals, with ongoing research focusing on their potential for cancer prevention and treatment **(Rad *et al*., 2020).** Many *in vitro* studies have reported that heat-killed bacteria show significant cytotoxicity in cancer cells via caspase-mediated apoptosis **(Subramaniam *et al*., 2016)**. Heat-killed *L. plantarum I-UL4, L. brevis,* and *L. paracasei* exhibit significant selective cytotoxicity in breast and colorectal cancer (CRC) cells by inhibiting proliferation and inducing apoptosis **(Chuah *et al*., 2019, Karimi *et al*., 2019)**. Consuming paraprobiotics may be a viable option in certain situations. Paraprobiotics refer to the use of inactivated (non-viable) microbial cells or cell fractions to support a consumer's health. Due to their extended shelf life, safety, and beneficial effects such as lowering cholesterol, modulating biological responses, enhancing immunity, and possessing anti-inflammatory and anti-proliferative properties, paraprobiotics have gained significant attention. These attributes suggest that, despite the exact underlying mechanisms remaining unclear, paraprobiotics could play a crucial role in promoting specific physiological activities and improving the consumer's health **(Akter *et al*., 2020)**.
The survival rates of patients undergoing chemotherapy or radiotherapy have increased in recent decades due to significant advances in drug therapy, a better understanding of cancer pathogenesis, and increased awareness of the importance of early disease diagnosis in the population **(Siegel *et al*., 2016).** The evolution of cancer treatment has resulted in the development of personalized and more selective therapies.
However, the high cost of some modern therapeutic approaches limits patient access to precision medicine in specific cancer centers, especially in developing countries, where most cancer-related deaths are concentrated **(Lee *et al*., 2018; WHO, 2021)**. Therapeutic regimens used worldwide are still based on non-selective cytotoxic drugs and promote unwanted side effects that compromise therapeutic efficacy and can contribute to increased mortality due to toxicities **(Wallington *et al*., 2016).** Among these toxicities, mucositis affects the entire gastrointestinal tract and is accompanied by pain, vomiting, and diarrhea, usually leading to treatment interruption or a reduction in chemotherapy dose intensity. Clinical management of gastrointestinal mucositis is symptomatic, and there is still no preventive or curative treatment available **(Van Sebille *et al*., 2015)**.

The intestinal microbiota in healthy individuals plays a crucial role in maintaining intestinal homeostasis, providing protective effects on epithelial integrity. This mechanism involves bacterial interaction with toll-like receptors and activation of the NF-kB signaling pathway, which helps prevent mucosal damage and promotes cell repair and regeneration **(Stringer, 2013; Touchefeu, 2014).** Furthermore, the mucus produced by intestinal epithelial cells and the expression of intercellular junction proteins are vital components of the intestinal barrier that protect against pathogens **(Ulluwishewa, 2011).**

Anticancer drugs can lead to dysbiosis, causing alterations in intestinal permeability and inflammation. Utilizing bacteria-based formulations to restore balance in the intestinal microbiota is acknowledged for its health-promoting effects and can help counteract the negative effects of chemotherapy on the gastrointestinal tract **(Lu, 2019).** There is a diverse range of bacteria-containing products available, including probiotics, prebiotics, symbiotics, postbiotics, and paraprobiotics **(Molska, 2019).**

The dairy industry may be particularly interested in paraprobiotics due to their stability over a broad pH and temperature range, allowing them to be added to highly acidic foods and before thermal processing without losing their functionalities. Their inclusion helps prevent harmful changes such as high acidity following fermentation in yogurt without altering the product's sensory qualities **(Barros *et al*., 2020).** While paraprobiotics are naturally present in yogurt, their levels are uncontrollable, which does not guarantee a consistent physiological response in vivo (Cuevas-González *et al*., 2020).

This study aims to investigate the antioxidant, anticancer, and antiviral properties of heat-killed (parabiotics) *Weissella paramesenteroides* MN2C2 and its nano-formulation. Additionally, the study focuses on preparing dairy functional food using nano-parabiotics.

MATERIAL AND METHODS

Microorganism and culture conditions

Our study team earlier obtained the strain from buffalo colostrum 48 hours after delivery, and they identified it as *Weissella paramesenteroides* MN2C2, which is registered in the gene bank with the accession number MK530206 **(Fayed *et al*., 2019)**. De Man-Rogosa-Sharpe (MRS) broth medium was used to inoculate the organism (10^6 CFU/mL), which was then cultured aerobically at 37°C for 24 hours at pH 6.5 in a static incubator. After the incubation period was over, the culture was combined with a 40% glycerol solution, and the resulting suspension was kept at -80°C until it was needed **(AOAC, 2012)**.

Analysis of sweet whey

The analysis of the tested byproduct sweet whey **(Yin *et al*., 2010)** was performed at the Analysis and Consultation Unit, Domain of Evaluation and Remediation of Hazardous Industrial wastes, National Research Center, Dokki, Egypt. The analysis methodology was performed according to the Standard Methods for the Examination of Water and Wastewater **Fayed *et al*. (2019).**

Tested low-cost sweet whey medium and growth conditions

This study examined whey's capability to maintain *Weissella paramesenteroides* MN2C2's substantial growth and, as a result, enable the tested strain to produce strongly biological agents **(Yin *et al*., 2010)**. The medium used contained untreated whey/yeast extract medium, which was made up of yeast extract (g/L of untreated whey), $MgSO4.2H_2O$ (0.2), and $CaCO_3$ (1.5), adjusted to pH 6.5 **(Fayed *et al*., 2019)**, with MRS medium serving as the control.

Preparation of Parabiotics (HK)

In order to prepare the heat-killed bacteria, 2% (10^8 CFU/mL) inoculum of bacterial was inoculated in a medium MRS and incubated for 48 h at 37˚C in aerobic conditions. The culture was then centrifuged for 20 min at 4000 rpm/5 min and the supernatant was discarded. The precipitate was washed with phosphate buffer (pH 7.2), following which 2 mL of serial distilled water was added to the bacterial cells precipitate and the resulting suspension was heated at 98˚C for 10 min to kill the bacteria. In order to ensure the inactivation of the bacteria, the heated bacterial suspension was re-cultured, and after ensuring that the bacteria did not grow in the culture medium, the sediment was freeze-dried. The heat-killed bacteria in this study were based on a modification of the method **(Ou *et al*., 2011)**.

Preparation of Nano-parabiotics sonicated Heat killed (SHK)

The nanoparticles of parabiotics prepared according to **Taurozzi *et al*. (2012)** method with modifications by sonication of the freeze-dried power 45% for 5 min (Cole-parmer instruments, CPX750, USA) on ice bath then lyophilization to produce nano-parabiotics.

Characterization of Nano-parabiotics (SHK)

Transmission Electron Microscopy (TEM)

The morphological characteristics of the formed nanoparticles were detected by Transmission Electron Microscopy (TEM). The imaging was carried out using Electron probe micro-analyzer (JEM-1400-USA). The film samples prepared on the TEM grid and allowed to dry then the images of the nanoparticles were taken as described by **Chen and Subirade (2005)**.

Dynamic light scattering (DLS)

To measure the size distribution of the formed nanoparticles; the dynamic light scattering (DLS) (NICOMP 380 ZLS, PSS, Santa Barbara, CA, USA) was used **(Cicci, 2017)**.

Biological applications of HK and SHK

Antioxidant activity using DPPH

Radical scavenging assay using 2,2-diphenyl-1- picrylhydrazyl (DPPH) method was applied to detect the activity of HK, SHK samples **(Yin *et al*., 2010)**. Different concentrations of both crude and partially purified EPS-NPs samples (40, 80, 120,160, 200, 240 µg/mL) were prepared in methanol. After that, 100 µL of each sample were added to 900 µL of DPPH and mixed well then incubated in the dark for 30 min. The decrease in absorbance was measured at 517 nm against a control without sample.

$$\% \text{ Free radical scavenging} = [(A \text{ control} - A \text{ sample})/A \text{ control}] \times 100$$

Where A Sample is the absorbance of DPPH solution with sample and A control is the absorbance of DPPH solution in methanol without sample.

In vitro anticancer activity

The cytotoxicity assay was evaluated in the Embryology and Cell Culture Lab, Faculty of Agriculture, Cairo University Research Park (CURP). Lung cancer (A549), Breast cancer (MCF-7), Colorectal adenocarcinoma (Caco-2), Hepatocellular carcinoma (HepG-2), and Human Lung normal (Wi38) cell lines were used. A concentration of 10^3 cell/well was inoculated in 96-well microtiter plates containing Roswell Park Memorial Institute (RPMI 1640) medium then incubated at 37ºC for 24 h under 5% CO2 incubator. Then a fresh, serum free, medium was added followed by the addition of different concentrations of the tested samples (HK and SHK) (0.5-10.0 $mgml^1$) then the plates were incubated for further 48 h against negative control (cells alone). After that, 40 µL of 2.5 µg/mL/L MTT (3-(4, 5-dimethylthiazol-2-yl)- 2,5-diphenly tetrazolium bromide) were added to each well and incubated at 37ºC for 4 h. Amount of 200 µLof sodium dodecyl sulphate (10% SDS) was added to each well and incubated overnight to stop the reaction. Doxorubicin standard (50 µg /mL) was prepared to give 100% lethality (positive control). The absorbance was measured at 540 nm using a microplate multi-well reader **(Thabrew *et al*., 1997)**.

$$\text{Viability \%} = [\text{Reading of extract/Reading of negative control} - 1] \times 100$$

Selectivity index (SI)

High SI values (> 2) indicate selective toxicity of the tested samples towards cancer cells, while low SI values (< 2) reveals that the tested substances are broadly toxic to normal cells **(Awang *et al*., 2014)**. Each SI value was calculated by: SI = IC50 normal cell/IC50 cancer cell.

Antiviral activity by measuring of cytopathic effect

A safe dilution from HK and SHK samples was prepared to be evaluated against coxsackie virus type B3 (CVB3) infection. Activated CVB3 was diluted in culture medium in a range of 10^{-4}–10^{-7}; then 50 µL from each dilution was incubated with an equal volume of samples at 37°C for 1 hour in microtiter plate using CO_2 incubator against control (without HK and SHK). The samples were used at concentrations of 1.5 and 3 mg/mL. Then, a fresh medium was added to the treated and untreated virus and then the plates were incubated at 37°C in CO_2 incubator for 72 hours. The cytopathic effect was observed under an inverted microscope and virus titration was calculated as 50% tissue culture infection dose (TCID50) as described by **Shaheen *et al*. (2015)**. Differences between the values of treated and untreated virus gave the reduction in virus titer.

Application of probiotic SHK

Milk

Fresh buffalo's milk, used for the manufacture of yoghurt, was obtained from the Faculty of Agriculture, Cairo University. The approximate chemical composition

of this milk is as follows (g/100 g): 4.00 protein, 7.00 fat, 0.80 ash, 17.00 total solids, 5.00 lactose, and 82% water.

Tested probiotic strain starter culture

The probiotic strain, used to ferment the buffalo's milk, was previously isolated by the research team from buffalo colostrum after 48 h of delivery and identified as *Weissella paramesenteroides* MN2C2 then stored in the gene bank under the accession number of MK530206 **(Amer *et al.*, 2021)**.

Control probiotic strains starter culture

Control probiotic yoghurt fermentation culture (ABT), commonly used by yoghurt manufacturing industries in Egypt, was acquired from the Dairy sciences department, Faculty of Agriculture, Cairo University, Egypt (Chr. Hansen's, Denmark). The ABT culture contains inocula of *Lactobacillus acidophilus*, *Bifidobacterium bifidum* and *Streptococcus thermophilus*.

Preparation of yoghurt

Fresh buffalo's milk was subjected to heat treatment at 85°C for 10 min, then left to cool at 42°C **(Fayed *et al.*, 2019)**, then transferred into 100 mL cups and categorized as C, T1. First of all, category C cups, which represented the control samples, were inoculated with 2.5% of ABT yoghurt culture. On the other hand, T1 cups were inoculated with a mixture of 2.5% of ABT yoghurt culture and SHK (1.1 mg/mL Conc.). The inoculated milk samples were incubated at 40°C for 4 h then refrigerated at 4 ± 1°C.

Yoghurt evaluation

Quality evaluation

The quality of the obtained yoghurt samples was evaluated through the measurement of the total viable count (TVC) of bacteria in each sample as well as the estimation of both the resulting pH and the acidity of these samples. The TVC of the treated samples was performed by serially diluting 1 mL from each sample in saline solution (0.9%) then spreading each appropriate dilution onto triplicates of MRS agar plates, which were incubated for 48 h at 37 °C. After the incubation period, the colonies formed on each plate were counted. The pH value of each sample was also measured using a digital laboratory Jenway 3510 pH meter, UK. Finally, titratable acidity (TA) was also measured according to **AOAC (2012) method**.

Sensory evaluation

Fresh samples of yoghurt were sensory evaluated by 50 members of both the Chemistry of Natural and Microbial Products and those of the Dairy science Departments, National Research Centre, Cairo, Egypt. The yoghurt was graded for its taste and flavor (out of 60 points), its body and texture (out of 30 points), and its color and appearance (out of 10 points) according to the score card method suggested by **Keating and White (1990)**.

Statistical analysis

All of the determinations, reported in this study, were performed in triplicate, and the results were presented as mean values.

RESULTS AND DISCUSSION

Low-Cost Sweet Whey Medium (LCSWM)

The dairy waste releases a lot of whey, which is heavy in energy protein and milk sugar, into the environment, polluting it and harming the economy. Whey has a variety of uses, including the generation of lactic acid and growing lactic acid bacteria. The results at Table (1) and Figure 1 showing that the probiotic strain *W. paramesenteroides* MN2C2 can grow on low-cost sweet whey medium (LCSWM) without significant difference record in compare with stander MRS medium for all lactic acid bacteria. The results showed that the time 48 hour was the highest microbial count of bacteria through using LCSWM. These results were in agreement with **Miloud, (2017)** who reported that sweet whey can be used as culture medium for lactic acid bacteria.

Table 1 Low-cost sweet whey medium (LCSWM)

Growth parameters	24h		48h		72h	
	LCSWM	MRS	LCSWM	MRS	LCSWM	MRS
pH	4.6±0.2	4.5±0.2	4.5±0.2	4.4±0.3	4.4±0.2	4.3±0.3
Acidity	0.99±0.3	1.00±0.2	1.00±0.3	1.16±0.2	1.16±0.2	1.25±0.2

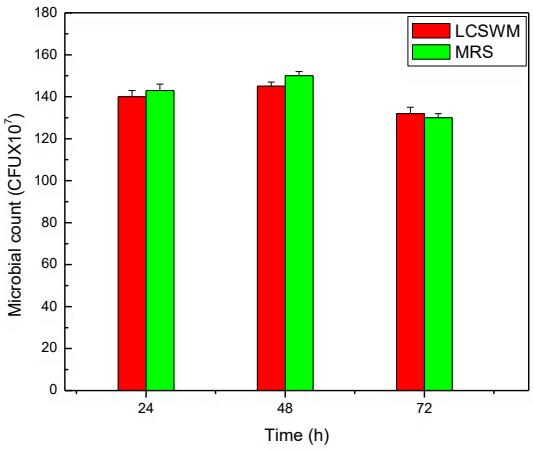

Figure 1 Low-cost sweet whey medium (LCSWM)

Preparation and characterization of (HK) and (SHK)

The evaluation of mean diameter of the particles HK and SHK as main character were determined by dynamic light scattering (DLS) (NICOMP 380 ZLS, PSS, Santa Barbara, CA, USA). Also, The HK and SHK were evaluated for their size and shape characteristics by Transmission Electron Microscopy (TEM) and Scanning Electron Microscopy (SEM).

The first evaluation of particle size for HK was by mean diameter of the particles HK as 618.7 nm (Figure 2) and SHK was 456.4 nm (Figure 3) as main character were determined by dynamic light scattering (DLS) (NICOMP 380 ZLS, PSS, Santa Barbara, CA, USA).

The HK and SHK were also evaluated for their size and shape characteristics by Transmission Electron Microscopy (TEM) and Scanning Electron Microscopy Figure 4 for HK which showed the clusters and clouting of cells with large aggregates with large size than the SHK at Figure 5 which showed good distribution and homogenous of lysis cells after sonication with smaller size less than 100 nm.

As a result, it was possible to clearly see differences in the mean diameter results between the two analysis methods. This discrepancy may be explained by the fact that higher particle sizes are recorded by the DLS analysis since it analyzes the hydration layer and polymer shells of the particles. Furthermore, for samples with varying particle sizes and shapes, the results can be misleading due to the significant overlay of big particle scattering over that of tiny particle scattering **(Fissan *et al.*, 2014)**. Additionally, the sample characteristics have an impact on the DLS measurements; as a result, disparities in results between TEM and DLS sizes were seen **(Makvandi *et al.*, 2019)**.

In addition, **Hoshyar *et al.*, (2016)** state that for nanoparticles (NPs) to avoid leaking into blood capillaries quickly, their diameter should not be less than 200 nm, and it should not be less than 10 nm to prevent being captured by fixed macrophages. Also, nanotechnology applications at the field of probiotics and prebiotics showed many benefits on nutrition and human health applications **(Dangi *et al.*, 2023)**.

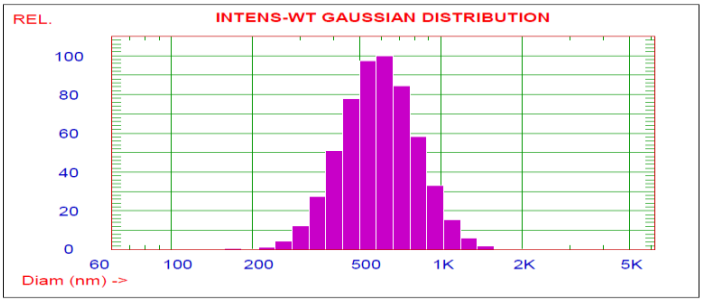

Figure 2 HK particle size.

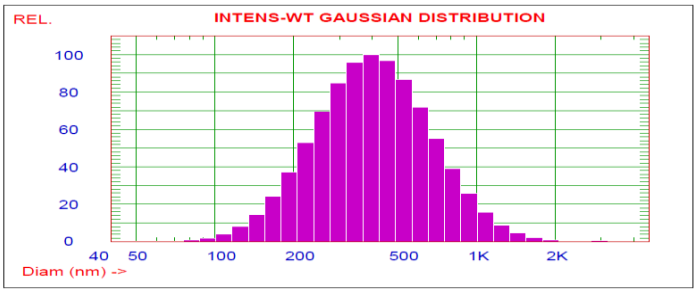

Figure 3 SHK particle size.

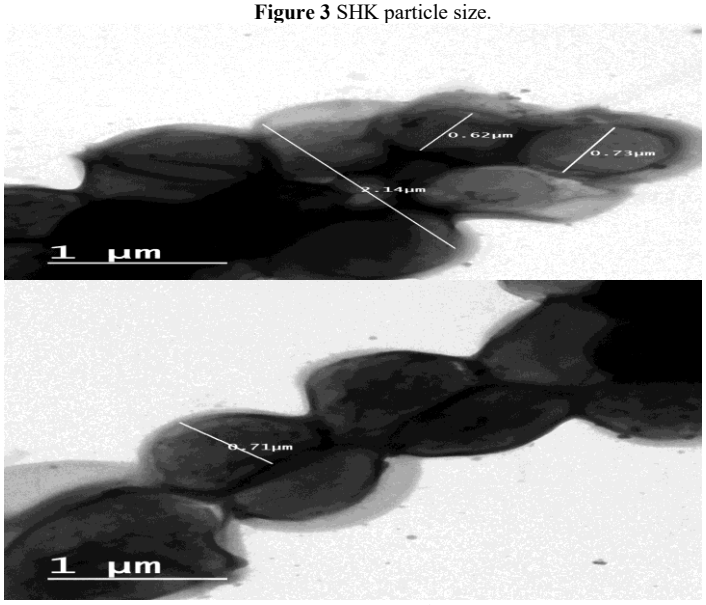

Figure 4 TEM of HK

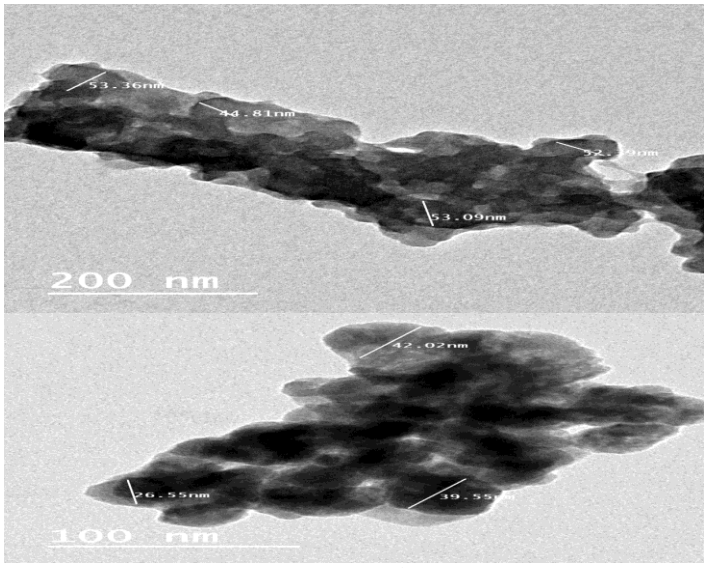

Figure 5 TEM of SHK.

Different biological applications of HK and SHK

Antioxidant activity by DPPH Radical Scavenging method

The results in Table 2 showed that the scavenging activity of 63.94 % was recorded when the HK sample concentration of 120µg/mL was used. Therefore, the concentration of HK required to scavenge 50% of the DPPH free radicals (IC50) was calculated as 38.36µg/mL. While SHK showed that the scavenging activity of 62.96% at conc. was 120µg/mL with IC50 37.61µg/mL.
According to **Jang *et al.* (2018)**, the results suggested that live or heat-killed *L. plantarum* Ln1 isolated from kimchi might be useful as an antioxidant probiotic.

Table 2 Antioxidant activity of crude and partially purified EPS-NPs

Sample		DPPH %	IC50 (µg /mL)
HK	80	61.38±0.25	38.36±0.25
	100	63.45±0.27	
	120	63.94±0.32	
SHK	80	60.18±0.29	37.61±0.35
	100	62.28±0.22	
	120	62.96±0.21	

Anticancer activity of HK and SHK

As shown in Table (3), the both HK and SHK did not show cytotoxic activity against breast cancer (MCF-7) cell line; but displayed moderate activity against liver cancer (HepG-2) with IC50 of 1.4 and 1.5 mg/mL, respectively. Although, the cytotoxic activity decreases from HK to SHK at colorectal adenocarcinoma (Caco-2) after preparation at nano form from 2 to 1.1 mg/mL but the selectivity index (SI) increased to 2.2. SI values could be increased by removing the toxic compound(s) using certain chemical method(s) **(Sufian *et al.*, 2013; Indrayanto *et al.*, 2021)**. Also, it is worth mentioning that low selectivity index indicates that the cytotoxic activity is attributed to cytotoxicity rather than activity against the cancerous cells themselves **(Saetung *et al.*, 2005 and Indrayanto *et al.*, 2021)**. In contrast, high selectivity index should offer the potential of safer therapy.
The results were in agreement with **Kim *et al.* (2021)** that, the antitumor effects of Lactobacillus strains and heat-killed Bifidobacterium on human colorectal cancer RKO cells in xenograft models, both in vitro and in vivo. Initially, an MTT test and flow cytometry were used to investigate the cytotoxic and apoptotic effects of 11 distinct strains, respectively. Therefore, they suggest that the use of the combination of MG5346 and MG4584 as parabiotics could effectively inhibit the growth of colorectal cancer.

Table 3 *In vitro* anticancer activity of HK and SHK

Cell lines	HK		SHK	
	IC50 (mg/mL)	Selectivity Index (SI)	IC50 (mg/mL)	Selectivity Index (SI)
Caco-2	2	0.41	1.1	2.2
HepG-2	1.4	0.59	1.5	1.5
MCF-7	0	ND	0	ND
A549	2.4	0.34	0	ND
Wi-38	0.82	1	2.2	1

Antiviral activity of EPS

To reduce or inhibit the viral reproduction without adverse effect on host cell viability, a low toxicity medicine with broad-spectrum activity was recommended **(Harrison, 2020)**. The antiviral activity of HK and SHK was detected against Coxsackie virus B3 (CVB3) (Table 4). Both HK and SHK have potent antiviral activity which reached 3–4 logs reduction in virus titers with lethal percentage ranged from 99.95% to 99.99%, respectively. SHK a substantial high inhibitory effect against CVB3, with 4 Log reduction of the virus yield at 3.0 mg/mL, in another wards, SHK achieved 99.99% reduction of viral titer. According to **Jung *et al.* (2017)** an increase in alveolar macrophage cells in the lungs and airways, early activation of virus-specific antibodies, decreased levels of pro-inflammatory cytokines, and innate immune cells were all associated with protection from heat-killed *Lactobacillus casei* DK128 (DK128).
Importantly, the mice that were protected against primary viral infection with heat-killed DK128 pretreatment developed later heterosubtypic immunity against secondary virus infection. Studies employing knockout mice models suggested that B cells and partially CD4 T cells, but not CD8 T cells, were necessary for protection against influenza virus via heat-killed DK128 pretreatment. Also, the research supports the theory that heat-killed LAB might be created as anti-virus probiotics by shedding light on how hosts can be given innate and adaptive protection through heat-killed DK128 therapy to guard against influenza virus.

Table 4 Antiviral activity of HK and SHK against CVB3

Sample	Conc. (mg/mL)	Virus titers alone	Virus titers with EPS	Reduction value	Inhibition %
HK	3.0	10^6	$10^{2.5}$	$10^{3.5}$	99.95
SHK	3.0	10^6	10^2	10^4	99.99

Yoghurt quality evaluation

While the Total Viable Count (TVC) of each sample increased over the time of the 15-day storage period showed in Figure (6). In the initial samples taken from categories control (C), SHK at 1.1 mg/mL (T1), the (TVC) was recorded at 83 and 65 CFU x10^7, respectively. However, over the storage duration, these results progressively increased to 258 and 198 CFU x10^7, respectively.

Table 5 shows that the rise in titratable acidity levels was also connected with the bacteria numbers in each sample. After a period of 15 days of storage, T1's acidity value of 1.14 but C was 1.18. That means no change after adding SHK to yoghurt. Conversely, variations in the pH levels of every experimental sample both stored and fresh were also noted. Fresh samples from categories C and T1 had pH values of 4.65 and 4.94, respectively. Over the course of the storage period, these values decreased dramatically, reaching 4.43 and 4.45 for tested samples from categories named C and T1. The results of **Fayed *et al.* (2019)** and **Ismail *et al.* (2020),** who observed an increase in probiotic strain and starter culture viable counts over storage durations, were supported by these observations. The rise in pH and acidity levels was also connected with the bacteria numbers in each sample. **Assem, *et al.* (2019)**, who discovered that the acidity rose with storage time, corroborated these findings. Also, **El-Shafei *et al.* (2018)** demonstrated that the pH values in all samples declined across the storage time.

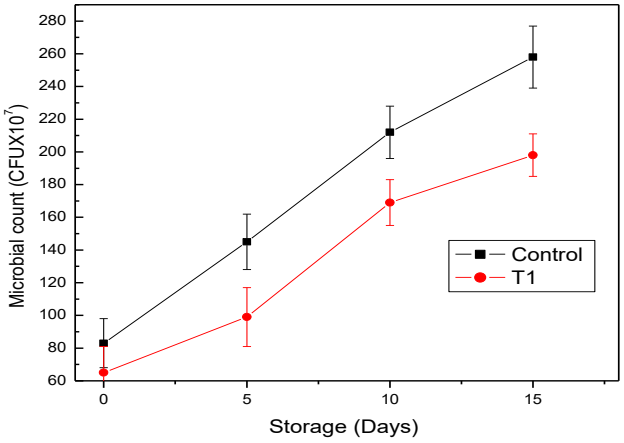

Figure 6 Effect of different storage period of yoghurt samples on the microbial total count

Table 5 Effect of different storage period of yoghurt samples on: A. TVC, B. pH, and C. Acidity

Treatments	Storage (Days)	pH	Titratable Acidity (TA)
Control	Fresh	4.65±0.2	0.87±0.3
	5	4.61±0.3	0.98±0.2
	10	4.54±0.2	1.00±0.2
	15	4.43±0.2	1.18±0.2
T1	Fresh	4.94±0.2	0.77±0.3
	5	4.84±0.3	0.90±0.3
	10	4.64±0.2	1.08±0.2
	15	4.45±0.2	1.14±0.2

Sensory evaluation

Dairy products' shelf life and quality are reflected in their sensory attributes. The fresh T1 samples were close to the fresh C samples in term of taste and similar at color and appearance. But the samples were at resulting in a total score of 92 and 91 % for the sensory evaluation of C and T1, respectively, as shown in Table 6. The body and texture of the T1 samples were superior to those of the C samples for increasing the viscosity. The dairy industry may be especially interested in paraprobiotics because of their stability over a broad pH and temperature range, which enables them to be added to highly acidic foods and before thermal processing without losing their functionalities. Their addition avoids harmful alterations like high acidity during fermentation in yogurt because it doesn't alter the sensory evaluation of the product **(Barros *et al.* 2020)**.

Table 6 Sensory evaluation of fresh yoghurt at 5 ± 1ºC

Treatments	Taste and Flavor (out of 40 points)	Body and Texture (out of 40 points)	Color and appearance (out of 20 points)	Total (out of 100 points)
C	37±0.3	37±0.2	18±0.3	92±0.3
T1	35±0.2	38±0.3	18±0.2	91±0.2

CONCLUSION

Probiotics are live bacteria that offer health benefits, but "paraprobiotics" are non-viable bacteria that may provide similar advantages without risks to those with weakened immune systems. This study used a cost-effective waste medium to create paraprobiotics from Weissella paramesenteroides MN2C2, evaluating their antioxidant, anticancer, and antiviral properties for potential functional food applications. The paraprobiotics were successfully prepared and characterized as nanoparticles, showing promising biological activities. Incorporating them into yogurt resulted in a safe and well-received functional food product.

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