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Microencapsulation of Probiotic, Evaluation for Viability and Cytotoxic Activities of Its Postbiotic Metabolites on MCF-7 Breast Cancer Cell Line

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***Abstract*—**Awareness about probiotic health benefits is increasing tremendously. However, cell viability is often low due to harsh conditions exposed during processing, handling, storage, and gastrointestinal transit. Thus, encapsulation is a promising technique that increases cell viability. The study aims to encapsulate the probiotic, evaluate its viability and cytotoxic activity of its postbiotic on the Michigan Cancer Foundation (MCF)-7 breast cancer cell line. Human and animal raw milk was sampled for lactic acid bacteria. Isolated bacteria were identified using conventional and VITEK 2 systems. The identified bacteria were encapsulated using the spray-drying method. The free and encapsulated probiotic cells were exposed to simulated gastric intestinal (SGI) fluid conditions and different storage conditions for their viability. The properties of the formed probiotic granules, their disintegration time, and the weight uniformity of the microcapsules were tested. Furthermore, the postbiotic of the free cells was extracted, and its cytotoxic effect on the MCF-7 breast cancer cell line was tested through [3-(4,5-dimethylthiazolyl-2)-2,5 diphenyltetrazolium bromide] (MTT) assay. The bacteria isolated were identified as *Lactobacillus plantarum*. The size of the formed probiotic granules ranges within 0.71-1.00 mm in diameter, and disintegration time ranges from 2.14 ± 0.045 to 2.91 ± 0.293 minutes, while the average weight is 502.1 mg. The viability of encapsulated cells stored at refrigerated condition (4ºC) was higher than that of cells stored at room temperature (25 ºC). The encapsulated probiotic cells exhibited better viability after exposure to SGI solution at different pH levels than free cells. The Postbiotic Metabolites (PM) of *L. plantarum* produced a cytotoxic effect that shows significant activity similar to 5FU, a standard antineoplastic agent. The inhibition concentration of 50% growth (IC_{50}) of postbiotic metabolite was consistent with the IC_{50} of the positive control (Cisplatin). *Lactobacillus plantarum* postbiotic exhibited a cytotoxic effect on the MCF-7 breast cancer cell line and could be used as combined adjuvant therapy in breast cancer management. The microencapsulation technique protects the probiotics and maintains their viability.

***Keywords*—**Cytotoxicity effect, encapsulation, postbiotic, probiotic.

I. Introduction

PROBIOTICS are components of bacteria that have been shown to benefit human health. World Health Organization showed that probiotic is a live microorganism that confers a health benefit on the host when administered in adequate amounts [1]. The earlier knowledge about probiotics focused on interactions with indigenous intestinal microbes, thereby improving the host's physiological function [2]. However, recent studies have shown that probiotics transit the gastrointestinal tract to provide broad health benefits to the host [47]. Study had it that, within the past 20 years, there has been growing recognition and attention to the role of the gut microbiome in gastrointestinal health [3]. Tremendous knowledge gained from the study of the human microbiome reveals the association of probiotic bacteria with health benefits [4]. Thus, interest in the global market for probiotic products has increased rapidly in recent years [5].

Substantial evidence had it that probiotic alleviates the symptoms of lactose malabsorption, treat antibiotic-induced diarrhea, including opportunistic enteric pathogens in HIV infection, as well as irritable bowel syndrome, Helicobacter infection, vaginal infection, suppress cancer cells, and reduce blood cholesterol [3], [6], [7].

Cancer remains one of the most common causes of death worldwide [8]. Breast cancer is the most frequent malignancy in women most diagnosed cancer, with an estimated 2.3 million new cases, representing 11.7% of all cancer cases, and a leading cause of global cancer incidence in 2020 [10].

Standard chemotherapeutic medications and conventional treatments are used to treat cancer. However, the safety and stability of these treatment options often fail to achieve a complete cancer remission due to tumor growth, site, stage, metastasis, and nonspecific toxicity to normal cells. When chemotherapy is first delivered, tumors generally decrease by 30% or more, but cancer often mutates and grows fast while acquiring treatment resistance. These medications kill cancer but also destroy healthy cells, develop drug resistance, and cause life-threatening side effects that are often worse than cancer [11].

Microbial-based therapy helps prevent and treat cancer.

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Microbial therapy (viral or bacterial) is derived from naturally occurring live, genetically modified, or purified products of microorganisms that specifically target cancer cells, limit systemic pathogenicity, and boost anti-cancer efficacy [12]. The capacity of probiotics to alter cancer signaling has attracted high interest. It provides therapeutic and preventive effects by downgrading carcinogenic stimulating events [13]. Study had it that PM of probiotic bacteria has equivalent efficacy as live probiotics [14]. A study reported that postbiotics maintained colonic health and thus proposed that postbiotics can be a safer alternative in comparison to live bacteria [15]. A study showed that exopolysaccharide from *L. plantarum* exerts antitumor activity against colon carcinoma cells [16]. Furthermore, *Lactobacillus acidophilus* and *Lactobacillus rhamnosus* had *in vitro* cytotoxic effects against breast and colon cancer cells [17]. Thus, this demonstration suggests that PM is a potential natural antitumor drug.

Most commercially available probiotics are powders or granules produced through wet granulation, spray-drying, and freeze-drying methods. Spray drying and high-temperature application are common production methods, that often induce thermal stress, denature proteins, and cause cell damage [18]. Probiotics should be stable and physiologically active, survive the upper digestive tract in significant numbers, attach to and colonize the intestines, and be viable during storage and shelf life [19]. Many attempts and solutions have been presented to address these obstacles, including a technique focused on creating a physical barrier around probiotic bacteria. In this context, microencapsulation has garnered much attention in recent years [20].

Microencapsulation is a promising technique for bacterial cell protection. Several studies have investigated the protective role of this technique against adverse conditions to which probiotics can be exposed [21], [22]. Microencapsulation is a process in which cells are retained within an encapsulating membrane to reduce cell injury or cell loss in a way that results in appropriate microorganisms being released into the gut [21]. Extrusion is the oldest and most widely used method for microencapsulating probiotics. It is easy to use, has low cost, and allows mild conditions that enable high entrapment of the microencapsulated probiotics. It has been effectively used to encapsulate probiotic bacteria by using biopolymers such as alginates and carrageenan in the presence or absence of minerals (calcium, potassium, etc.) [23]. Furthermore, one of the advantages of microencapsulation using the extrusion method with hydrocolloids is that cells are entrapped within the matrix during the formation of the spheres. At the same time, in other techniques, such as spray drying, freeze-drying, and fluidized bed drying, the microorganisms are entirely released into the product [24].

Thus, this study aims to formulate probiotics using the wet granulation and spray-drying methods, evaluate their viability in simulated gastrointestinal conditions and stability after long-term storage and investigate the cytotoxic effects of its PM against MCF-7 breast cancer cell lines.

II. Method

A. Collection and Handling of Samples

A total of 25 samples of human and animal milk were sampled. Ten milk samples were collected from breastfeeding mothers. Five milk samples were collected from each of the following: cows, sheep, and goats respectively. Samples were collected in well-labeled sterile containers, delivered in the laboratory immediately after collection, and stored at 4 °C. Samples were collected according to international best practices, as stated within the study ethical approval (CMUL/HREC/02/21/811).

B. Isolation and Identification of Bacteria Strain

The milk samples were serially diluted with sterile distilled water, 0.1 ml of the dilution was spread on de Man, Rogosa, and Sharpe (MRS) agar (Oxoid, UK) and MRS agar + cysteine (0.5%) for approval Bifidobacterium. The agar plates were incubated at 37 °C under anaerobic conditions for 48-72 h. Anaerobic jars with anaerobic atmosphere generation bags (Anaerogen, Oxoid) were used to achieve the anaerobic condition. The isolates were obtained by morphological characteristics (colony and cell morphology) on the selective media, and biochemical tests used were Gram reaction, catalase test, production of acid from glucose [25]. The bacteria isolates were finally identified using the Vitek 2 compact system (bioMerieux®) [26]. The identified bacteria isolates were stored in deep MRS agar at 4 ºC.

Ethical approval was obtained from the Health Research Ethics Committee, College of Medicine, University of Lagos (approval number: CMUL/ HREC/02/21/811).

C. Cytotoxicity Activity of Postbiotic Metabolite on MCF-7 Breast Cancer Cell line

1. Extraction of Postbiotic Metabolite

The identified stored (4 ºC) bacteria were activated by subculturing on MRS agar and incubated anaerobically at 37 °C for 24h. After incubation, 500 ml of sterile MRS broth was inoculated with the 24-h culture and incubated anaerobically overnight at 37 °C until an early stationary phase. The culture was centrifuged at 10,000×g for 10 min to separate the cells. The supernatant was collected as a postbiotic metabolite (PM) into two sterile beakers. The pH of the PM in one beaker was adjusted to pH 7.2–7.4 using 5 M NaOH, while the pH (4.8) of PM in the second beaker was left unaltered. The beakers were labeled K1 and K3 and the PM in the two beakers were sterilized by filtration through 0.22 µm polyether sulfone membrane syringe filters respectively. The PM solution was kept at 4 °C until required for cytotoxic analysis. The control sample labeled K2 was sterile MRS broth [27].

2. Cell Source and Medium Preparation

The MCF-7 cell line was purchased from Cell Lines Services (CLS) Germany, in 25 cm^3 culture flasks with a seeding density of 1 x 10^4 cells/cm^2. The cell line propagation media was prepared by supplementing Dulbecco's Modified Eagle Medium (DMEM) (9.4 g) with 1% Fetal Bovine Serum (FBS), 22 g of $NaHCO_3$, 5.9 g of N-2-Hydroxyethylpiperazine-N'-2-

Ethanesulfonic Acid (HEPES) and 1% L-Glutamine in 1000 mL of sterile distilled water. The medium was incubated at 37 ºC for 24 hrs for sterility check [27].

3. MCF-7 Cells Culture, Maintenance and Harvesting

Cells were grown in the supplemented medium in T25 and T75 flasks and maintained in a humidified chamber with 5% CO_2, incubated at 37 ºC until they reached a confluence of 75%. The floating MCF-7 cells were discarded with the culture medium and the cells were washed with Phosphate-buffered saline (PBS). The adhered cells were detached using 0.005% Trypsin solution, re-suspended in a fresh medium, and placed into new flasks.

4. Viability Count, and Seeding of Cells

A 10 µL of the mixture and 10 µL of trypsin blue were pipetted onto a hemocytometer and percentage viability was calculated using the formula:

$$\% \text{ Viability} = [AT / (AT + DT)] \times 100$$

where, AT= total number of living cells, DT= total number of dead cells.

The harvested MCF-7 cells were seeded onto 96-well plates at 1.1 x 10^4 cells/well and then allowed to attach by incubating at 37 °C in a 5% CO^2 incubator for cytotoxicity assay.

5. Challenge of MCF-7 cells with PM

After 24h incubation (attachment) of MCF-7 cells in 96 well plate, cells were treated in triplicates with a two-fold dilution of extracted PM K1 and K3, as well as the negative (K2) and positive (5-fluorouracil (5FU) and Cisplatin) control sample with concentrations ranging from 0-25% (v/v). After 48 hours, 10 µL of MTT solutions (1% w/v) were added to each well and the plates were incubated in the dark for 4 hours. The Optical Density (OD) values were measured at 492 nm with the reference at 630 nm by Elisa reader [27]

Two 96-well plates were used for the assay. Each plate comprises 12 columns and eight rows. All wells had 1.1 x 10^4 cells/mL of MCF-7 breast cancer cells except the wells of the last column. In plate 1, the first three rows, second three rows, and last two rows were treated with K1, K2, and 5FU (5-Fluorouracil) respectively. The first column contains the highest concentration of 100% of samples, after which double-fold dilutions were carried out until the tenth column. Wells in the eleventh column contained the MCF-7 cells and medium (DMEM) while those in the twelfth column contained medium only. All wells contained 100 µL of the medium. The total capacity of each well is 200 µL.

6. Statistical Analysis

Inhibitory concentration (IC50) was calculated using GraphPad Prism Version 9.2.0 (GraphPad Software Inc.). Microsoft Excel 2010 was used in plotting the bar chart.

D. Encapsulation of Probiotic Bacteria

1. Concentration of Probiotic Bacteria Cell Culture

The identified stored (4 °C) bacteria were activated by subculturing on MRS agar and incubated anaerobically at 37 °C for 24h. After incubation, 500 mL of sterile MRS broth was inoculated with the 24-h culture and incubated anaerobically overnight at 37 °C. The cells were harvested by centrifugation at 5000 rpm for 10 min at 4 °C. The cell pellets were washed twice with sodium chloride solution (0.9%) and re-suspended in 10 mL of 0.9% (w/v) sodium chloride solution [28]. A double-fold dilution was used to determine the colony-forming unit per ml (CFU/ml) of the bacteria isolate for the encapsulation. An average of 1x10^9 CFU/ml cell concentration was used as a free cell for microencapsulation.

2. Formulation of Probiotic Using Wet Granulation and Spray-Drying Method

A 100-fold serial dilution was carried out to produce a stock cell containing 85 billion cfu/ml. A 120 µL stock cell was used to prepare 10 billion cfu/ml of 500 g of probiotic granules. To produce 100 capsules of 500 g of probiotic granules, 12 ml stock cell was mixed with 2.0% (m/V) edible skimmed milk. Microcrystalline cellulose, lactose monohydrate, and corn starch were sieved through a 300 µm diameter and 2% (m/m) povidone water solution was added as a binder and blended for 5 min.

The intended dose of *Lactobacillus plantarum* in each capsule (containing 500 mg of formulated granules) is 10 billion cfu. Thus, 10 billion cfu/ml of *Lactobacillus plantarum* contained 120 µL (0.12 mL) of stock cell. The stock probiotic cell is 85 billion cfu/mL. To formulate 100 capsules (containing 500 mg of probiotic granules) 12 mL of the stock probiotic cell was taken and mixed with 23 ml of 2.0% (m/V) skim milk.

To formulate the probiotic, a sieved microcrystalline cellulose, lactose monohydrate impalpable grade, and corn starch were bound together using 2% (m/m) povidone water solution and blended for 5 min. The broth medium containing Probiotics and 2.0% (m/V) skim milk was mixed with the powder mass for 10 min. The wet mass was screened through a 1.70 mm diameter sieve and dried in an oven at 38 ± 2 °C for about 18 h until constant weight is reached. Dried granules were screened through a 1.00 mm diameter sieve. Finally, magnesium stearate (1%, m/m) and aerosil (0.5%, m/m) were added and mixed with the granules. The granules were then filled manually into hard gelatin capsules of size zero [27].

TABLE I
FORMULATIONS OF GRANULES CONTAINING *L. PLANTARUM*

Ingredient	Formulation
Probiotics and 2 % (m/V) skim milk in edible broth medium (mL)	35
Corn starch (g)	30
Lactose monohydrate (g)	38
Povidone (g)	2
Microcrystalline cellulose (g)	30

E. Evaluation of Micromeritic Properties of Granules

1. Percentage Yield

The percentage yield of the granules was determined for drug and was calculated using the following equation as described [28]:

$$\text{Yield} = (M / M_0) \text{ X } 100$$

where, M = weight of granules and Mo = total expected weight of polymer.

2. Angle of Repose

The angle of repose was determined by pouring the granulated blend in a funnel which was raised vertically to obtain a maximum cone height (h).

The radius of the heap (r) was measured, and the angle of repose was calculated as: $\alpha = \tan^{-1} (h/r)$ [28].

3. Bulk Density

Bulk density (ρb) was determined by placing pre-sieved drug excipients blend into a graduated cylinder. The bulk volume (V) and weight of the powder (M) were determined. The bulk density was calculated by using [29]:

$$\text{Bulk density} = \frac{\text{Mass of granules}}{\text{volume}}$$

4. Tapped Density

A known mass of probiotic granules was filled in a measuring cylinder and tapped a fixed number of times. The volume (Vt) and the weight (M) of the blend were measured [29]. The tapped density (ρt) was calculated using the formula:

$$\text{Tapped density} = \frac{\text{Weight of Blend}}{\text{Volume occupied in cylinder}}$$

5. Hausner's Ratio

The powder flow of the probiotic granules was determined using Hausner ratio which is defined as an indirect index of ease of powder flow and is calculated as:

$$\text{Hausner's ratio} = \frac{\text{Tapped density}}{\text{Bulk density}} \text{ [30]}$$

6. Carr's Index

The free flow property of the powder of the probiotic granules was measured by compressibility, an indication of the ease with which a material can be induced to flow is given by % compressibility which is calculated as follows [30]: $C = (\rho t - \rho b) / \rho t * 100$ where ρt = Tapped density and ρb = Untapped bulk density

F. Evaluation of Hard Gelatin Capsule

1. Disintegration Time

One capsule was placed in each of six tubes, the tubes were assembled and suspended in water. Discs were added to each tube, temperature was maintained at 37 ± 2 °C, and assembly was operated for 60 min [31].

2. Weight Uniformity Test

Twenty capsules were selected at random. One capsule was weighed. The capsule was opened, and the contents were removed as completely as possible. The emptied shells were weighed. The net weight of its contents was determined, that was by subtracting the weight of the shells from the weight of the intact capsule. The procedure was repeated with the other 19 capsules. A study showed that the average net weight was determined from the sum of the individual net weights and that the percentage deviation from the average net weight of each capsule was determined [31]. Thus, the deviation of individual net weight should not exceed the compendia limits. The formula used in calculating the deviation (%) was: Deviation (%) = [(Average weight – weight of capsule) / Average weight)] x 100%.

G. Evaluation of the Viability of Probiotic in Simulated Gastrointestinal Fluid

1. Preparation of Simulated Gastrointestinal Fluid

Simulated gastric fluid (SGF) was prepared as previously described [32] with modification. Two different test tubes were filled with 50 ml MRS broth and 1M HCl was added into the broth to adjust the pH to 1 and 1.5 respectively. The solution was sterilized by autoclaving at 121 ºC for 15 min. Pepsin solution (1;3000 µ/g) was sterilized using a membrane filter of pore size 0.22 µm, it was added to sterile MRS broth to a final concentration of 0.3% (v/v).

Simulated intestinal fluid (SIF) was prepared by dissolving 2% (w/w) of bile salt in 500 mL of MRS broth, and the pH was adjusted to 8 using NaOH solution. The prepared solution was sterilized by autoclaving at 121º C for 15 min.

2. Survival Test of Probiotic Bacteria in Simulated Gastrointestinal Fluid Condition

A 9 mL of prepared SGF solution was transferred into sterile test tubes, 1 mL of free cell samples and 1 g of encapsulated cells were added into the separate test tubes and incubated at 37 ºC for 0, 1, 2, and 3 h respectively. The survivability of cells was represented as several viable cells (log cfu/g) counted using the plate count method on MRS agar. After 3 h, the test tubes containing SGF solution of pH 1.5 with free cells and encapsulated cells were centrifuged at 5000g for 15 min. The sediment was suspended in 9 mL of SIF solution and incubated at 37ºC as done for the SGF test. The aliquot sample (1 mL) of free and encapsulated cells was taken after 0, 1, 2, and 3 h and evaluated for cell survivability using the plate count method on MRS agar [32].

H. Evaluation of the Stability of Encapsulated Probiotic

500 mg of encapsulated probiotic granules produced by spray-drying was manually filled in a sterile hard gelatin capsule and stored in a glass container at different storage conditions (4 ºC and 25 ºC) for a storage period of 6 months. Within every two weeks of storage, one capsule of probiotic granules containing 500 mg was withdrawn from the glass container and 1 g of probiotic granules was dissolved in 0.85% (w/v) saline solution, diluted, plated, and incubated at 37 ºC for 24 h to enumerate the viability of cells using plate count method on MRS agar.

III. Result and Discussion

A strain of *Lactobacillus spp.* was isolated from a raw cow milk sample and identified using conventional and Vitek-2

compact system as *Lactobacillus plantarum*. *Lactobacillus* spp. were the commonly isolated bacterial species from raw cow milk. Similarly, an earlier study found a high load of *Lactobacillus* spp. in raw cow milk [25]. The *L. plantarum* was maintained in 20% glycerol/MRS broth suspension frozen at -80 °C. The working stock of the isolate was maintained in MRS deep agar at 4 °C for subsequent use.

A. Viability of MCF-7 Cell

1. Percentage MCF-7 Cell Viability

Before the cytotoxic test, the MCF-7 cell was examined under a microscope, and the viability was calculated as: % viability = [3.21 x 106/ (3.21 x 106) + (1.0 x 104)] x 100 = 99.689%. Thus, the % viability is 99.7%.

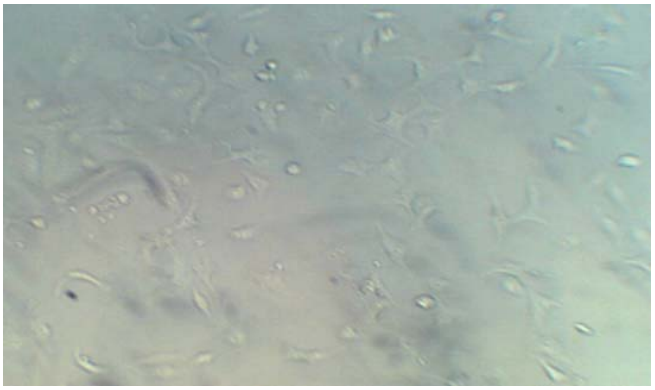

Fig. 1 MCF-7 cells under a microscope (Magnification: 10x)

2. Cytotoxic Effect of PM (PM) on MCF-7 Cell

Fig. 2 shows the cytotoxic effects of K1, K2, K3, 5FU and CIS on the MCF-7 cells from concentration of 0% to 25% after 48 hours. At 0%, there were no samples or positive controls applied, therefore all the bars are at a baseline of 100 (as shown on the vertical axis). From 0.2% to 25%, 5-Fluorouracil shows the highest cytotoxic effect by going below the baseline, as low as 55. Following 5-fluorouracil in cytotoxicity effect on MCF-7 cell is K3 (the PM with non-adjusted pH). Cisplatin, an antineoplastic agent, also shows cytotoxic effects at all concentrations. K1 and K2 showed no significant cytotoxic effect. K1 is the PM with adjusted pH (7.3), while K2 is sterile MRS broth that serves as a negative control sample.

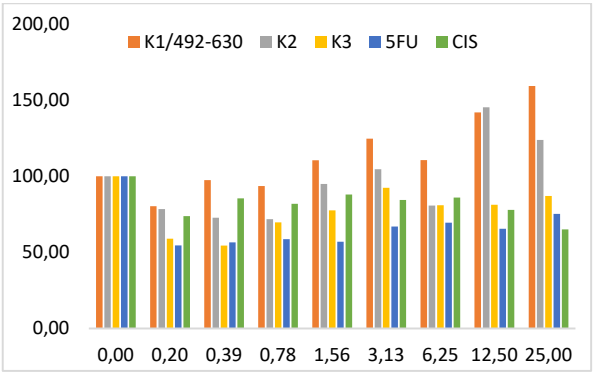

Fig. 2 The cytotoxic effects of K1, K2, K3, 5FU and Cisplatin

From Fig. 2, the 'y-axis is the baseline for the measurement of cytotoxicity. Values below 100 show a cytotoxic effect while values above 100 represent a lack of cytotoxic effect The 'x' axis is the concentration of PM from '0' to '25'%.

The result of this study showed that PM K3, produced by *L. plantarum* exhibited a cytotoxic effect on MCF-7 breast cancer cells at all concentrations as demonstrated in Fig. 2. The result concurs with a previous study [33]. The cytotoxic effect produced by PM K3 was almost consistent with the cytotoxic effect of 5 Fluorouracil (positive control), an antineoplastic used in the chemotherapy of breast cancer, colorectal cancer, gastric cancer, pancreatic cancer, and glioblastoma multiform [33]. An earlier study showed that the administration of probiotics during anticancer therapy yielded clinical results, as it improved gut dysbiosis in cancer patients [34]. However, the PM with adjusted pH (7.3), showed no cytotoxic effect. This lack of cytotoxicity could be due to possible interaction between the PM and the 5 M sodium hydroxide used to adjust the pH. While the negative control (K2), (sterilized MRS broth) appeared generally neutral possibly because there was no PM in the solution. Similarly, the cell type-dependent cytotoxic effects of PM in this study conformed with the findings accordingly [35]. The cytotoxic effect of the PM was time- and dose-dependent and no cytotoxicity activity was noted for the MRS medium which was used as negative control against various cancer cell lines.

The result of the inhibitory concentration of 50% growth (IC_{50}) as calculated using GraphPad Prism Version 9.2.0 (GraphPad Software Inc.) was shown in Table I.

K3 has the lowest IC_{50} value of 0.0344, followed by Cisplatin (0.2483), then K1, 5-Fluorouracil, and K2 has the highest IC_{50} value of 0.9257.

TABLE II
IC_{50} VALUES OF K1, K2, K3, 5FU AND CISPLATIN

Cancer cell	K1	K2	K3	5FU	Cisplatin
MCF-7	0.3926	0.9257	0.0344	0.4339	0.2483

Notes: Human breast cancer cells (MCF-7) are anchorage-dependent cells. The highest PM concentration being tested on MCF-7 is 25% (v/v). The values were detected after 48 hours of incubation.

The inhibition concentration of 50% growth (IC_{50}) measures the potency of the postbiotic against the breast cancer cell. Thus, IC_{50} determines the quantity of postbiotics required for *in vitro* inhibition of breast cancer cells by 50%. The biological component could be an enzyme, cell, cell receptor, or microorganism as earlier reported [36]. From the result of the IC_{50}, Cisplatin, a positive control has the lowest value of 0.2483 followed closely by K3 (the postbiotic metabolite with non-adjusted pH and greatest cytotoxic activity), then K1 (the postbiotic metabolite with adjusted pH). Fluorouracil, another positive control, has an IC_{50} value of 0.4339 while K2 (the negative control, showed no cytotoxic activity).

B. Probiotic Granules

Probiotic granules were formulated using the wet granulation method. Granules within a size range of 0.71-1.00 mm in

diameter were obtained. A 500 mg of the granules were weighed and filled into hard gelatin capsule shells. Size zero hard gelatin capsules were obtained as shown in Fig. 3 B.

Fig. 3 (A) Encapsulated *L. plantarum* granules and (B) encapsulated *L. plantarum* in capsule shell

1. Properties of Probiotic Granules

The properties of the granules were evaluated. These properties include Percentage Yield (%), Angle of Repose (θ), Bulk Density (gm/cm^3), Tapped Density (gm/cm^3), Hausner's Ratio and Carr's Index (%).

The Percentage Yield (%) of the granules is 97.82% which shows that only 2.18% of the total granules expected was lost during the wet granulation process. The Angle of Repose (θ) of the granules is 19.85^o. The United States Pharmacopeia states that angles less than 31 ^{o}C have an excellent flow [37]. In several branches of science, the angle of repose has been used to characterize the flow properties of solids. Thus, the Angle of repose is a characteristic related to inter-particulate friction or resistance to movement between particles [38]. The bulk density was found to be 0.590 ± 0.008. The bulk density of a powder is the ratio of the mass of the unused powder sample and its volume as well as the inter-particulate void volume. [39]. It was found to be 0.701 ± 0.012 gm/cm^3 for all formulations. Both Bulk Density (gm/cm^3) and Tapped Density (gm/cm^3) are used in the determination of Hausner's Ratio. In this work, the Hausner's ratio was found to be 1.187 ± 0.018. The USP states that Hausner's ratio of less than 1.19 is "Good". Hausner's ratio is a common method used to envisage the characteristics of powder flow [40].

In pharmaceutics, Carr's index is often used as an indication of the compressibility of a powder. In a free-flowing powder, the bulk density and tapped density would be close in value, therefore, Carr's index would be small. [41]. A Carr's index greater than 25 is considered to be an indication of poor flowability [42]. From the result of this study, Carr's index is 15.7 which is by far less than 25. This shows that the flow property is "Good".

TABLE III
EVALUATION OF GRANULES/CAPSULE PROPERTIES

S/N	Granules/Capsule Property	Mean ± Standard Deviation (n = 3)
1	Percentage Yield (%)	97.82 ± 0.03
2	Angle of Repose (θ)	19.85 ± 0.432
3	Bulk Density (gm/cm^3)	0.590 ± 0.008
4	Tapped Density (gm/cm^3)	0.701 ± 0.012
5	Hausner's Ratio	1.187 ± 0.018
6	Carr's Index (%)	15.738 ± 1.290
7	Disintegration time (min)	2.37 ± 0.091

2. Disintegration Time

Disintegration time test and weight uniformity were used to evaluate the Probiotic hard gelatin capsules formulated. Disintegration does not imply a complete solution of the capsule or even of its constituent. One capsule was used in each of the six tubes of the basket using water as the immersion fluid, maintained at 37 ± 2 °C. At the end of 15 minutes time limit of operating the disintegrating apparatus, all the capsules must have disintegrated completely, or else the test will be repeated with 12 additional capsules of which not less than 16 of the total 18 capsules must have disintegrated [43]. From the result of this study, the disintegration time for a hard gelatin capsule was found to be in the range of 2.14 ± 0.045 to 2.91 ± 0.293 minutes which is by far less than 15 minutes. This means that all six capsules have passed the disintegration on the first attempt. The result is in line with the study where the disintegration time for a hard gelatin capsule was found to be in the range of 2.5 ± 0.816 to 3.33 ± 0.471 min [44].

3. Weight Uniformity Test

From the weight uniformity result in this study, the highest deviation from the mean was 0.4% deviation. Study showed that capsules fulfill its weight uniformity test if the mass of individual capsules is outside the limits of 85-115% of the average mass. However, the capsules fail to comply with the test if 1 individual mass is outside the limits of 75-125% of the average mass [44]. Comparing the result from this study with the Pharmacopeia requirement, the capsules have passed the weight uniformity test.

TABLE IV
EVALUATION OF WEIGHT UNIFORMITY

S/N	Weight (mg)	Deviation (%)
1	501.2	± 0.1882
2	500.4	± 0.3475
3	504.1	± 0.3893
4	501.0	± 0.2280
5	502.8	± 0.1304
6	501.7	± 0.0886
7	502.5	± 0.0707
8	501.3	± 0.1683
9	503.2	± 0.2101
10	503.2	± 0.2101
11	503.8	± 0.3296
12	503.3	± 0.2300
13	501.3	± 0.1683
14	502.3	± 0.0309
15	501.2	± 0.1882
16	500.4	± 0.3475
17	501.5	± 0.1284
18	501.9	± 0.0488
19	503.5	± 0.2698
20	502.3	± 0.0309
Average weight = 502.1		

C. Survival of Lactobacillus plantarum in Simulated Gastrointestinal Fluid Condition.

The survival of *L. plantarum* exposed to different pH values of simulated gastric and intestinal fluid conditions was

evaluated as shown in Table IV. The exposure time to SGF solution has a more significant effect on the viability of free than encapsulated cells. At pH 1 of SGF solution, free cells were reduced to 1.03 log CFU/g after 3 h of exposure time whereas the viability of encapsulated cells was found to be 5.82 log CFU/g at the end of the analysis. Similarly, at pH 1.5 the viability of free and encapsulated cells was found to be 1.87 and 6.32 log CFU/g respectively. Thus, the reduction in survival was slightly lesser at pH 1.5 compared to pH 1. The reduction in survivability of free cells was found to be more than double compared to the reduction of encapsulated cells. The survivability was found to be reduced for lower pH values while increasing the exposure time. This indicates that the encapsulated cells were able to maintain viability in gastric conditions. It could be due to the impermeable structure of microcapsules formed by starch and skim milk and produced at high temperatures. Similarly, our result concurs with the reported study [35].

The survivability of 1.05 log CFU/g was observed in free cells, while encapsulated cells maintained survivability of 5.64 log CFU/g at the end of 3 h of exposure time under SIF conditions. Thus, the SIF solution in our study has a minor effect on the survival of encapsulated probiotic cells compared to free cells. A similar result was previously reported [32] during survivability test of encapsulated spray-dried probiotic powder in simulated gastric and intestinal conditions at different pH levels (1, 1.5, and 2).

TABLE V
SURVIVABILITY OF FREE AND ENCAPSULATED PROBIOTIC CELLS UNDER SIMULATED GASTROINTESTINAL CONDITIONS

	SGF				SIF	
Time, h	pH-1.0		pH-1.5		pH-8.0	
	Free cell (FC)	Encapsulated cell (EC)	Free cell (FC)	Encapsulated cell (EC)	Free cell (FC)	Encapsulated cell (EC)
0	9.21 ± 0.38	8.96 ± 0.26	9.01 ± 0.08	8.86 ± 0.16	1.87 ± 0.22	6.28 ± 0.25
1	6.05 ± 0.12	7.90 ± 0.15	6.49 ± 0.05	8.25 ± 0.14	1.54 ± 0.20	6.02 ± 0.15
2	3.13 ± 0.08	6.87 ± 0.10	4.32 ± 0.3	7.40 ± 0.12	1.22 ± 0.17	5.80 ± 0.07
3	1.03 ± 0.04	5.82 ± 0.09	1.87 ± 0.26	6.32 ± 0.30	1.05 ± 0.08	5.64 ± 0.05

Note: values are expressed as mean ± standard deviation.

D. Viability of Encapsulated Probiotics under Different Storage Condition

After manufacturing, the probiotic products must be transported from the manufacturing site and survive storage in a given condition within periods often exceeding 12 months [45]. During storage, the probiotic cells can be exposed to environmental stressors such as temperature, water, oxidation, pH, and light [46]. Membrane lipid oxidation is one of the undesired effects encountered during storage [37]. Altogether, low temperatures and low humidity contribute to higher survival rates during storage.

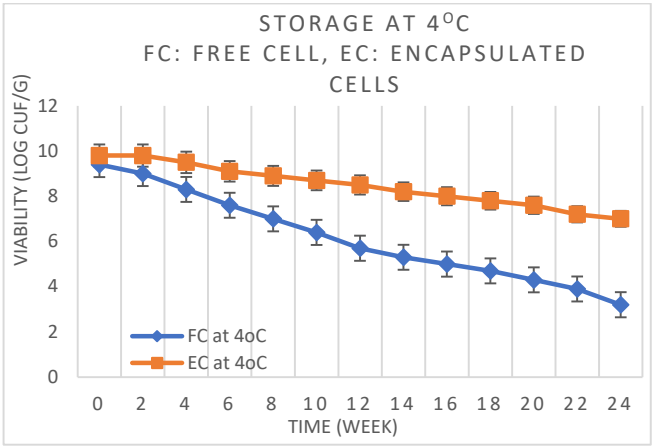

Fig. 4 Viability of encapsulated cell (EC) and free cells (FC) during storage period at 4 °C

In this study, the free *L. plantarum* cells and encapsulated cells were stored at room (25 °C) and refrigerated (4 °C) temperatures. Free and encapsulated cells were tested for viability for every week up to 12 weeks. The viability of both free and encapsulated cells from the starting day of storage period were presented in Figs. 4 and 5.

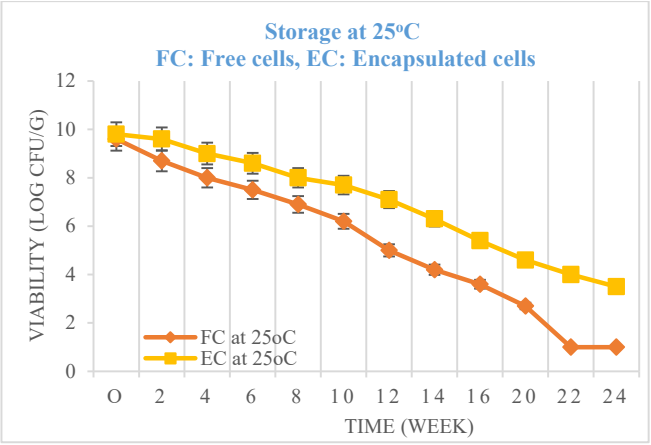

Fig. 5 Viability of encapsulated cell (EC) and free cells (FC) during storage period at 25 °C

It was found that the viability of free cells stored at 4 and 25 °C was less than 4 log CFU/g after 12 weeks. However, the encapsulated capsules stored at 4 °C achieved the highest viability among free and encapsulated cells stored at 25 °C and free cells stored at 4 °C.

The loss of viability of free and encapsulated cells at 25 °C is probably due to the oxidation of membrane lipids and denaturation of proteins that lead to the degradation of macromolecules in bacterial cells [46]. However, the viability of encapsulated cells was higher compared to the free cells. The reason could be because the probiotic cells encapsulated in

protective carriers stabilize the cellular structures and thus reduce the environmental stresses by restricting molecular movement.

The cells stored at temperatures close to above 0 ºC reduce the rate of unfavorable chemical reactions.

IV. Conclusion

Probiotics have a vast range of beneficial properties and gained increasing medical importance over the last decade. Multiple studies including animal models and human breast cancer cells have shown the antitumor effects of probiotics. In this study, the probiotic was formulated by encapsulation using the spray drying method, and the postbiotic was tested for cytotoxicity of the MCF-7 Breast Cancer Cell Line. The result of the survivability and viability test showed that the encapsulated probiotic was stable in gastrointestinal fluid and maintained viability after 6 months at 4 ºC and 25 ºC. Thus, microencapsulation is a technique that provides a physical barrier and protection under harsh conditions. This study also showed that PM produced by *Lactobacillus plantarum* exhibited cytotoxic effects on MCF-7 breast cancer cells; therefore, probiotic containing *L. plantarum* are useful as adjuvant therapy in breast cancer management. The prevalence of breast cancer among women is extensively on the rise despite current therapeutic methods. So, finding novel and safe preventive and therapeutic methods is highly important because of resistance to some chemical drugs.

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References

[1] H. E Sidjabat and L. Blackall, “One health Probiotics” Microbiology Australia, 2020, https://doi.org/10.1071/ma20016.

[2] C. Ma et al., “Probiotic consumption influences universal adaptive mutations in indigenous human and mouse gut microbiota,” Communications Biology 2021 4:1, vol. 4, no. 1, pp. 1–12, doi: 10.1038/s42003-021-02724-8.

[3] Su, Grace L., Ko, W. Cynthia, Premysl Bercik, Yngve Falck-Ytter Shahnaz Sultan, Weizman V. Adam, and Morgan L. Rebecca, "AGA Clinical Practice Guidelines on the Role of Probiotics in the Management of Gastrointestinal Disorders." Gastroenterology, 2020, https://doi.org/10.1053/j.gastro.2020.05.059.

[4] S. K. Kim et al., “Role of Probiotics in Human Gut Microbiome-Associated Diseases,” J. Microbiol. Biotechnol, vol. 29, no. 9, pp. 1335–1340, Sep. 2019, doi: 10.4014/JMB.1906.06064.

[5] U. Wendel, “Assessing Viability and Stress Tolerance of Probiotics-A Review,” Front Microbiol, vol. 12, Jan. 2022 doi:10.3389/FMICB.2021.818468.

[6] S. Choi, Y. J. Hwang, M. J. Shin, and H. Yi, “Difference in the Gut Microbiome between Ovariectomy-Induced Obesity and Diet-Induced Obesity,” J. Microbiol. Biotechnol., vol. 27, no. 12, pp. 2228–2236, Dec.2017, doi:10.4014/JMB.1710.10001.

[7] G. D’Ettorre et al., “Probiotic supplementation promotes a reduction in T-cell activation, an increase in Th17 frequencies, and a recovery of intestinal epithelium integrity and mitochondrial morphology in ART-treated HIV-1-positive patients,” Immun Inflamm Dis, vol. 5, no. 3, pp.244–260, Sep.2017, doi: 10.1002/IID3.160.

[8] F. Cardoso et al., “Early breast cancer: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow- up†,” Ann Oncol, vol. 30, no. 8, pp.1194–1220, Aug. 2019, doi: 10.1093/ANNONC/MDZ173.

[9] H. Sung et al., “Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries,” CA Cancer J Clin, vol. 71, no. 3, pp. 209–249, May 2021, doi:10.3322/CAAC.21660.

[10] F. Cardoso et al., “European Breast Cancer Conference manifesto on breast centres/units,” Eur J Cancer, vol. 72 pp. 244–250, Feb. 2017, doi:10.1016/J.EJCA.2016.10.023.

[11] S. Chaluvadi, A. T. Hotchkiss, and K. L. Yam, “Gut Microbiota: Impact of Probiotics, Prebiotics, Synbiotics, Pharmabiotics, and Postbiotics on Human Health,” Probiotics, Prebiotics, and Synbiotics: Bioactive Foods in Health Promotion, pp. 515–523, Oct. 2016, doi: 10.1016/B978-0-12-802189-7.00036-8.

[12] M. Sedighi et al., “Therapeutic bacteria to combat cancer; current advances, challenges, and opportunities,” Cancer Med, vol. 8, no. 6, p.3167, Jun. 2019, doi: 10.1002/CAM4.2148.

[13] V. Sankarapandian et al., “An Update on the Effectiveness of Probiotics in the Prevention and Treatment of Cancer,” Life, vol. 12, no. 1, Jan.2022, doi: 10.3390/LIFE12010059.

[14] A. H. Rad, L. A. Maleki, H. S. Kafil, H. F. Zavoshti, and A. Abbasi, “Postbiotics as promising tools for cancer adjuvant therapy,” Adv Pharm Bull, vol. 11, no. 1, pp. 1–5, 2021, doi: 10.34172/APB.2021.007.

[15] Konstantinov SR, Kuipers EJ, Peppelenbosch MP. Functional genomic analyses of the gut microbiota for crc screening. Nat Rev Gastroenterol. 2013; 10:741–5 Foo HL, Loh TC, Law FL, Lim YZ, Kufli CN, Rusul G. Hepatol. Nature publishing group.

[16] J. Angelin and M. Kavitha, “Exopolysaccharides from probiotic bacteria and their health potential,” Int J Biol Macromol, vol. 162, pp. 853–865, Nov. 2020, doi: 10.1016/J.IJBIOMAC.2020.06.190.

[17] H. U. Celebioglu, Y. Erden, and H. B. Ozel, “In vitro cytotoxic effects of lactobacilli grown with lime honey on human breast and colon cancer cells,” Food Biosci, vol. 41, p. 101020, Jun. 2021, doi: 10.1016/J.FBIO.2021.101020.

[18] K. C. Baral, R. Bajracharya, S. H. Lee, and H. K. Han, “Advancements in the Pharmaceutical Applications of Probiotics: Dosage Forms and Formulation Technology,” Int J Nanomedicine, vol. 16, pp. 7535–7556, 2021, doi:10.2147/IJN.S337427.

[19] K. Venema, J. Verhoeven, S. Verbruggen, L. Espinosa, and S. Courau, “Probiotic survival during a multi-layered tablet development as tested in a dynamic, computer-controlled in vitro model of the stomach and small intestine (TIM-1),” Lett Appl Microbiol, vol. 69, no. 5, pp. 325–332, Nov. 2019, doi: 10.1111/LAM.13211.

[20] D. de A. Paula, E. M. F. Martins, N. de A. Costa, P. M. de Oliveira, E.B de Oliveira, and A. M. Ramos, “Use of gelatin and gum arabic for microencapsulation of probiotic cells from Lactobacillus plantarum by a dual process combining double emulsification followed by complex coacervation,” Int J Biol Macromol, vol. 133, pp. 722–731, Jul. 2019, doi: 10.1016/J.IJBIOMAC.2019.04.110.

[21] S. Razavi, S. Janfaza, N. Tasnim, D. L. Gibson, and M. Hoorfar, “Microencapsulating polymers for probiotics delivery systems: Preparation, characterization, and applications,” Food Hydrocoll, vol.120, p. 106882, Nov.2021, doi: 10.1016/J.FOODHYD.2021.106882.

[22] H. Liu et al., “Protective approaches and mechanisms of microencapsulation to the survival of probiotic bacteria during processing, storage a gastrointestinal digestion: A review,” Crit Rev Food Sci Nutr, vol. 59, no. 17, pp.2863–2878, Sep. 2019, doi:10.1080/10408398.2017.1377684.

[23] N. Choudhury, M. Meghwal, and K. Das, “Microencapsulation: An overview on concepts, methods, properties and applications in foods,” Food Front, vol. 2, no. 4, pp. 426–442, Dec. 2021, doi:10.1002/FFT2.94.

[24] L. Huan, W. C. Steve, C. Maoshen, I. Yue, L. Rong, X. Feifei & Z.Fang (2019) Protective approaches and mechanisms of Microencapsulation to the survival of probiotic bacteria during processing, storage and gastrointestinal digestion: areview; Critical Reviews in Food Science and Nutrition, https://doi.org/10.1080/10408398.2017.1377684.

[25] Y. Taye, T. Degu, H. Fesseha, and M. Mathewos, “Isolation and Identification of Lactic Acid Bacteria from Cow Milk and Milk Products,” Scientific World Journal, vol. 2021, 2021, doi:10.1155/2021/4697445.

[26] Y. B. Soemarie, T. Milanda, and M. I. Barliana, “Isolation, Characterization, and Identification Candidate of Probiotic Bacteria Isolated from Wadi Papuyu (Anabas testudineus Bloch.) a Fermented Fish Product from Central Kalimantan, Indonesia,” Int J Food Sci, vol.2022, 2022, doi:10.1155/2022/4241531.

[27] L. O. Chuah et al., “Postbiotic metabolites produced by Lactobacillus plantarum strains exert selective cytotoxicity effects on cancer cells,”

BMC Complement Altern Med, vol. 19, no. 1, pp. 1–12, Jun. 2019, doi:10.1186/S12906-019-2528-2/TABLES/3.

[28] D. N. Pujara and B. R. Parmar, "Formulation and Evaluation of Hard Gelatin Capsule of Losartan Potassium," Inventi Rapid: Pharm Tech, vol. 2013, no. 2, pp. 1–4, 2013, (Online). Available: www.inventi.in.

[29] B. D. Kumar, and N. Sharma. "Design, Formulation and Evaluation of Fast Disintegrating Tablets of Glipizide."2019. https://doi.org/10.22270/jddt.v9i3-s.2806

[30] P. Hrishav Das, and B. Nath. "Formulation and Evaluation of Oral Fast Disintegrating Tablet of Ibuprofen Using Two Super Disintegrants." International Journal of Current Pharmaceutical Research; Vol 9, Issue 4, 2017, https://core.ac.uk/download/482244375.

[31] United States Pharmacopeia and National Formulary (USP 36- NF 31), "Disintegration 701," in United States Pharmacopeia Convention, Rockville, MD, 2013, pp. 305–307.

[32] D. Arepally, R. S. Reddy, and T. K. Goswami, "Studies on survivability, storage stability of encapsulated spray dried probiotic powder," Curr Res Food Sci, vol. 3, pp. 235–242, Nov. 2020, doi 10.1016/J.CRFS.2020.09.001.

[33] H. K. Tan, H. L. Foo, T. C. Loh, N. B. M. Alitheen, and R. A. Rahim,"Cytotoxic effect of proteinaceous postbiotic metabolites produced by Lactobacillus plantarum I-UL4 cultivated in different media composition on MCF-7 breast cancer cell," Malays J Microbiol, vol. 11, no. 2, pp.207–214, Jun. 2015, Accessed: Oct. 12, 2023. (Online) Available: https://doaj.org/article/fc6f9a748a19405790ab20aae5179e2e.

[34] L. K. Ursell, J. L. Metcalf, L. W. Parfrey, and R. Knight, "Defining the human microbiome," Nutr Rev, vol. 70, no. 1, pp. 38–44, Aug. 2012, doi: 10.1111/J.1753-4887.2012.00493. X.

[35] Stasiak-Róza´nska, L.; Berthold-Pluta, A.; Pluta, A.S.; Dasiewicz, K.; Garbowska, M. Effect of Simulated Gastrointestinal Tract Conditions on Survivability of Probiotic Bacteria Present in Commercial Preparations. Int. J. Environ. Res. Public Health 2021, 18, 1108; https://doi.org/10.3390/ijerph18031108

[36] Hoetelmans RM. "IC50 versus EC50". PK-PD relationships for anti-retroviral drugs. Amsterdam: Slotervaart Hospital. Archived from the original on 2017-05-28 – via U.S. Food and Drug Administration.

[37] S. Huang, F. Gaucher, C. Cauty, J. Jardin, Y. L. Loir, R. Jeantet et al., Growth in hyper-concentrated sweet whey triggers multing, stress tolerance and spray drying survival in *Lactobacillus casei* BL23: from the molecular basis to new perspectives for sustainable probiotic production. Front. Microbiol.9:2548, doi: 10.3389/fmicb.2018.02548.

[38] N. Kabilan, M. Murugesan, T. Balasubramanian, S. Geethalakshmi. "Physico-Chemical Analysis of Siddha Drug Poora parpam - A Comparative Evaluation between Natural and Synthetic Source", Scholars Research Library, European Journal of Applied Engineering and Scientific Research, 2017, 5(2):6-14.

[39] A. Punitha, S. Visweswaran, N.J. Muthukumar, "Physico-chemical evaluation and hptlc finger print of siddha poly herbal formulation "Swasa kudori mathirai" 2015.https://core.ac.uk/download/482239090.pdf.

[40] Bulk Density and Tapped Density of Powders; Stage 6 Harmonization Official August 1, 2015. https://www.usp.org/.../gen-chapter/bulk_density

[41] M.A. Kaleem, M. Z. Alam, M. Khan, M., Jaffery, S. H. I., & Rashid, B. An experimental investigation accuracy of Hausner Ratio and Carr Index of powders in additive manufacturing processes. Metal Powder Report. doi:10.1016/j. mprp.2020.06.061.

[42] United States Pharmacopeia National Formulary (USP 36- NF 31), "Disintegration 701," in United States Pharmacopeia Convention, Rockville MD, 2013, pp. 305–307.

[43] A. M. Agiba, S. S. Abul-Ella, Rehab A. Abd El-Monem, "Pharmaco-technical Development and Optimization of Multilayered Tablets: An Updated Industrial Review with Emphasis on Bilayer Tablets", International Journal of Applied Pharmaceutics ISSN- 0975-7058 Vol 13, Issue 4, 2021. DOI: https://dx.doi.org/10.22159/ijap.2021v13i4.41528.

[44] U. Wendel, "Assessing Viability and Stress Tolerance of Probiotics", Front. Microbio., Vol.12- 2022; https://doi.org/org/10.3389/fmicb.2021.818468

[45] W. Fiore, S. Arioli and S. Guglielmetti, The neglected microbial components of commercial probiotic formulations. Microorganisms 2020, doi:10.3390/microorganisms8081177

[46] S. Han, Y. Lu, J. Xie, Y. Fei, G. Zheng, Z. Wang, J. Liu, L. Lv, Z. Ling, B. Berglund, M. Yao and L. Li (2021) Probiotic Gastrointestinal Transit and Colonization After Oral Administration: A Long Journey; Front. Cell. Infect. Microbiol. 11:609722. doi:10.3389/fcimb.2021.609722.