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Efficacy of bigel in improving the viability of probiotic; experimental study

Eficacia de bigel para mejorar la viabilidad de probióticos: un estudio experimental

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ABSTRACT

Gel formulations of oleogel, hydrogel, and oleo-hydrogel (bigel) were evaluated as transdermal drug delivery systems, but recently various studies have proved that gel-based delivery systems are also able to improve the stability and bioavailability of many bioactive food ingredients. The aim of this study was to prepare different formulations of edible bigel and compare their effect on probiotic viability. This work was an experimental research design conducted in Food Technology Laboratory at B.B. Ambedkar University with technical assistance provided by CytoGene Research and Development in Lucknow, India. The experimental work was conducted from January to April 2021. The primary analysing technique was based on quantitative microbial analysis using two nutrient agar media, Reinforced Clostridial Agar RCA and De Man, Rogosa and Sharpe Agar MRS. To these agar medias, two probiotic bacteria was introduced, *Clostridium butyricum* and *Lactic acid bacillus (Lactobacillus sporogenes*) in two different phases, in the first phase (control), the probiotic was free from any type of gel while in the second phase, the probiotic was incorporated with bigel. When the number of colonies formed was compared in both phases, it was observed that the second phase had more colonies, and within the second phase, the best formulation that had maximum number of colonies was 7:3 OG:HG ratio. In RCA media, the results showed that, *Clostridium butyricum* count was raised from 472 to 624 colonies, on the other hand, In MRS media, the *Lactic acid bacillus* count was raised from 13 to 53 colonies. At the physical level, the most stable form of bigel with solid, jelly-like structure and viscoelastic nature was 7:3 OG:HG ratio, and all this work led to a conclusion that, 7:3 OG:HG ratio of bigel is physically and microbiologically ideal to be a potential substance used for coating and encapsulation of probiotic supplement.

Keywords: Bigel, Probiotic, Agar media, *Clostridium butyricum, Lactic acid bacillus.*

RESUMEN

Las formulaciones en gel de oleogel, hidrogel y oleo hidrogel (bigel) se evaluaron como sistemas de administración transdérmica de fármacos, pero recientemente diversos estudios han demostrado que los sistemas de administración basados en gel también son capaces de mejorar la estabilidad y biodisponibilidad de muchos ingredientes alimentarios bioactivos. El objetivo de este estudio es preparar diferentes formulaciones

de bigel comestible y comparar su efecto sobre la viabilidad de los probióticos. Este trabajo fue un diseño de investigación experimental realizado en el Laboratorio de Tecnología de Alimentos de la Universidad Babasaheb Bhimrao Ambedkar con la asistencia técnica proporcionada por *CytoGene Research and Development* en Lucknow, India. El trabajo experimental se llevó a cabo de enero a abril de 2021. La técnica de análisis primaria se basó en el análisis microbiano cuantitativo en el que se utilizaron dos medios de agar nutritivo: Agar Clostridial Reforzado (RCA, por su sigla en inglés) y Agar De Man, Rogosa y Sharpe (MRS). A estos medios de agar, se introdujeron dos bacterias probióticas, *Clostridium butyricum* y bacilo ácido láctico (*Lactobacillus sporogenes*) en dos fases diferentes; en la primera fase (control), el probiótico estaba libre de cualquier tipo de gel mientras que en la segunda fase, el probiótico se incorporó con bigel. Cuando se comparó el número de colonias formadas en ambas fases, se observó que la segunda fase tenía más colonias y, dentro de la segunda fase, la mejor formulación que tenía el máximo número de colonias era la proporción 7:3 OG:HG. En el medio RCA, los resultados mostraron que el recuento de *Clostridium butyricum* aumentó de 472 a 624 colonias, mientras que en el medio MRS, el recuento de bacilos ácido lácticos aumentó de 13 a 53 colonias. A nivel físico, la forma más estable de bigel con estructura sólida y gelatinosa y naturaleza viscoelástica, fue la proporción 7:3 OG: HG. Todo este trabajo llevó a la conclusión de que la proporción 7:3 OG: HG de bigel es ideal desde el punto de vista físico y microbiológico para ser una sustancia potencialmente utilizada para el recubrimiento y la encapsulación de suplementos probióticos.

Palabras clave: Bigel, probiotico, medio de agar, *Clostridium butyricum*, bacilos ácido lácticos.

INTRODUCTION

A hydrogel is a network of polymer chains that are hydrophilic, sometimes found as a colloidal gel in water as a medium, it is highly absorbent, possess a degree of flexibility and can encapsulate chemical systems. The first appearance of the term 'hydrogel' was in 1894 (Bemmelen. 1907). While an organogel/oleogel is a non-crystalline, non-glassy thermoreversible (thermoplastic) solid material composed of a liquid organic solvent, mineral oil, or vegetable oil. The solubility and particle dimensions of the structure are important characteristics for the elastic properties and firmness of the oleogel. Oleogels have potential for use in a number of applications, such as in pharmaceuticals (Kumar and Katare. 2005), cosmetics, art conservation (Carretti and et al. 2005) and food (Pernetti. 2007). The first semisolid formulation of bigel obtained by combining stable oleogel and hydrogel was in 2008 by I.F. Almedida. Formulations of bigel can be introduced in food to enhance the delivery of bioactive components and improve their survival (Zhuang. 2020). Combining these two structured gel systems at high shear produces a biphasic system that has several advantages compared to the pure oleogel or hydrogel alone. It has greater stability because both the continuous and the dispersed phases of the gel systems are semi-solid and therefore immobilized. Upon long-time storage, the capacity of internal phase leaching is decreased and hence, the separation process of phases is delayed. There is more control over bigel properties, not only can the concentration of the gelator be changed but also the proportion of gel phases can be manipulated to tailor the gel for a specific application and enhance its physical and mechanical properties. Each component brings unique

properties to the bigel system and should be taken into account when designing the final product. The most important feature of this bigel is its ability to hold both lipophilic and hydrophilic substances since there is both the lipid and water phase present in the bigel. This gives the advantage of being compatible with a greater variety of bioactive compounds.

The World Health Organization defines probiotics as “live microorganisms which when administered in adequate amounts confer a health benefit on the host”. So, probiotic can easily be explained as an oral supplement or a food product that contains a sufficient number of viable microorganisms that alter the micro flora of the host and has potential beneficial health effects on host. Once the probiotic is administered in the body, they must be able to reach the distal gut (ilium and colon) and get attached to intestinal epithelium. And in order to travel through the GI tract, they must be resistant to acid and bile so they can make their way through the large intestine. The reality is, most of the probiotic supplements on the shelves of the local pharmacy or chemist don’t meet all these criteria.

Probiotic survival in the gut is affected by a number of factors including pH, post-acidification during product fermentation, hydrogen peroxide production, bile, gastric acid, enzyme secretion and the temperatures (Kailasapathy. 2009). Encapsulation is often mentioned as a way to protect bacteria against severe environmental factors (Anal and K.Singh. 2007), (Champagne and P.Fustier. 2007). The goal of encapsulation is to create an environment in which the bacteria will survive during processing and storage and released at appropriate sites (e.g., small intestine) in the digestive tract.

It has been established that some probiotics bind to phospholipid that are major components of the Milk Fat Globule Membrane MFGM (Huppertz, 2009). *Lactobacillus reueri* binds to the MFGM through interaction with protein on the surface of the bacteria. This study also demonstrated that hydrophobic strains of bacteria bound more strongly to the MFGM (Brisson. et al, 210). These non-covalently bound basic proteins that form a structure on the surface of probiotic bacteria are known as S-layer proteins (Deepika and Charalampopoulos. 2010). S-layer proteins on *L. acidophilus* displayed adhesion properties and were found to bind to epithelial cells, thus protecting the probiotic microorganisms (Frece. et al, 2004). And since oleogel is a constituent of bigel that contains phospholipid, bigel can be a potential agent used in coating the surface of probiotic bacteria to be fit for surviving and processing the gut condition like resisting low pH and organic acids.

MATERIALS AND METHODS

Sample preparation: The bigel was prepared from edible components containing an organogel (oleogel) and a hydrogel, the basic constituents of oleogel is a 10% oleogelator for which beeswax was used (10gm) and a 90% continuous phase which was made from soybean oil (90gm). Beeswax was mixed with soybean oil at temperature between 90-100°C. At the same time hydrogel was prepared with 10% hydrogelator for which gelatin was used (10gm) and a 90% continuous phase of water (90gm), gelatin and water was mixed at 40°C. At the time of homogenization, the temperature of gelatin was raised to 60°C and both gels were combined and

homogenized at 24000 rpm for approximately two minutes. The hot mixture was immediately placed in the refrigerator to undergo dilation for 24 hours (Saffold and Acevedo. 2020). Three samples with different ratios of oleogel OG and hydrogel HG was prepared (Figure 1): 1:1 OG: HG, 3:7 OG: HG and 7:3 OG: HG (Bollom. et al. 2020).

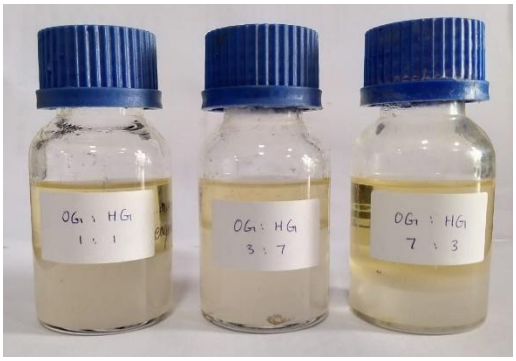

Figure 1: Samples of bigel in different ratios

Probiotic capsules (Aku-Biotic): Instead of isolating probiotic bacteria from food, probiotic supplement capsules were used, so further conclusion can help in determining the best type of gel and which ratio will help the most in encapsulation of the capsules and support bacterial growth.

Each hard capsule contains:

- *Streptococcus faecalis* *30 million*
- *Clostridium butyricum* *2 million*
- *Bacillus mesentericus* *1 milloin*
- *Lactic acid bacillus (Lactobacillus sporogenes*) 50 million

The main two bacteria which were selected to prepare agar media for and conduct experiments on are: *Clostridium butyricum and Lactic acid bacillus LAB.*

Reinforced Clostridial Agar RCA media preparationReinforced Clostridial Agar is used for the cultivation and enumeration of clostridia and based on the original formulation from Hirsch and Grinsted. It can be used to initiate growth from small inocula and to obtain the highest viable count of Clostridia. This medium can be used like the conventional medium for studies of spore forming anaerobes, especially *Clostridium butyricum* in chesse and general for the enumeration and isolation of Clostridia. Casein hydrolysate, beef extract, yeast extract and peptone provide nitrogen, vitamins, amino acids and carbon for growth. Dextrose is the fermentable sugar and sodium chloride ensures osmotic balance. The medium is free from inhibitors and contains cysteine as a reducing agent. Sodium acetate is the buffering agent. See the media composition in Table 1.

Table 1: RCA media composition

Ingredients	Gram/litre
Peptone	10.0
Yeast extract	3.0
Dextrose	5.0
Sodium chloride	5.0
Sodium acetate	3.0
Starch soluble	1.0
L-cysteine hydrochloride	0.5
Agar	13.5

De Man, Rogosa and Sharpe Agar MRS media preparation: Lactobacilli MRS medium is used for the cultivation of all Lactobacillus species and based on the formulation of deMan, Rogosa and Sharpe with slight modification. It supports luxuriant growth of all Lactobacilli from oral cavity, dairy products, foods, faeces and other sources. Proteose peptone and HM peptone B supply nitrogenous and carbonaceous compounds. Yeast extract provides vitamin B complex and dextrose is the fermentable carbohydrate and energy source. Sodium acetate and sodium citrate inhibit Streptococci, moulds and many other microorganisms. Magnesium sulphate and manganese sulphate provide essential ions for multiplication of lactobacilli. Phosphates provide good buffering action in the media. Lactobacilli are microaerophilic and generally require layer plates for aerobic cultivation on solid media. See the media composition in Table 2.

Table 2: MRS media composition

Ingredients	Gram/litre
Peptone	10.0
Yeast extract	5.0
Dextrose	20.0
Sodium citrate	2.0
Sodium acetate	5.0
Magnesium sulphate	0.10
Manganese sulphate	0.05
Dipotassium hydrogen phosphate	2.0
Agar	12.0

Autoclave: Autoclaves operate at high temperature and pressure in order to kill microorganisms and spores. They are used to decontaminate certain biological waste and sterilize media, instruments and lab ware. All the prepared media, distilled water, conical flasks, petri dishes, micropipette, micropipette tips, inoculating L-bar

(spreader) were autoclaved by subjecting them to pressurized saturated steam at 121 °C (250 °F) for around 15–20 minutes.

Serial Dilution: The objective of the serial dilution method is to estimate the concentration (number of colonies, organisms, bacteria, or viruses) of an unknown sample by counting the number of colonies cultured from serial dilutions of the sample, and then back track the measured counts to the unknown concentration. The inoculum tube of the first phase was diluted to 1/10 dilution by adding 0.1gm of solid inoculum (probiotic) to 10ml of distilled water. The second phase (probiotic+bigel) was also diluted to 1/10 dilution. Inoculum tube of both phases were further diluted to 1/10000000 dilution. 1/1000 dilution was selected for *Clostridium butyricum* to spread on RCA media and 1/10000000 dilution for Lactic acid bacillus LAB was spread on MRS media. These dilution factors were selected on basis of their count in the capsules.

Colony-Forming Unit CFU: A colony-forming unit is a unit used in microbiology to estimate the number of viable bacteria or fungal cells in a sample. The number of visible colonies (CFU) present on an agar plate can be multiplied by the dilution factor and divided by volume plated on the media to provide a CFU/ml result.

CFU = No. of colonies in × Dilution factor ÷ Vol. plated on media

RESULT AND DISCUSSION

In the first phase, probiotic sample was diluted and poured on the media (RCA and MRS) to observe the number of colonies formed of both *Clostridium butyricum and Lactic acid bacillus LAB*, as shown in Figure 2a and 2b, their respective CFU was calculated in Table 3 using the formula.

Clostridium butyricum count = 472000 CFU/ml

Lactic acid bacillus count = 1.3×10^9 CFU/ml

Figure 2a: Clostridium butyricum colonies on RCA media (control)

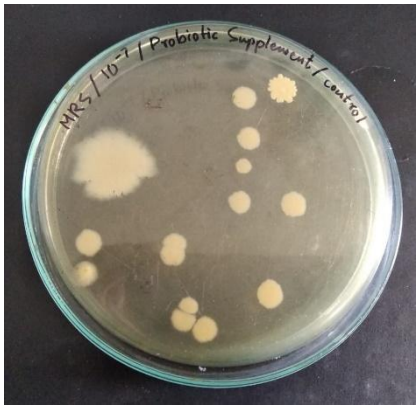

Figure 2b: Lactic acid bacillus colonies on MRS media (control)

Table 3: CFU/ml value for the microbial analysis of probiotic sample (control)

Media	Bacteria	No. of colonies	Dilution Factor	Vol. plated (ml)	CFU/ml
RCA	Clostridium butyricum	472	1000	0.1	4720000
MRS	LAB	13	10000000	0.1	1.3×10^9

In the second phase, bigel was incorporated with probiotic to observe weather the colonies will increase, decrease or remain as it was in the control group. Two ratios of bigel (3:7 OG:HG and 7:3 OG:HG) were used to study which ratio is optimal for bacterial growth.

Figure 3a shows colonies of *Clostridium butyricum* on RCA media, and it obvious how the number of colonies had decreased indicating that, 3:7 OG:HG did not help the probiotic bacteria in increasing its viability.

Figure 3b shows colonies of *Lactic acid bacillus* on MRS media, unlike *C. butyricum, LAB* colonies had grown and more colonies were formed than the control group. In Table 4, CFU of both bacteria were calculated.

Clostridium butyricum count = 472000 CFU/ml

Lactic acid bacillus count = 1.3×10^9 CFU/ml

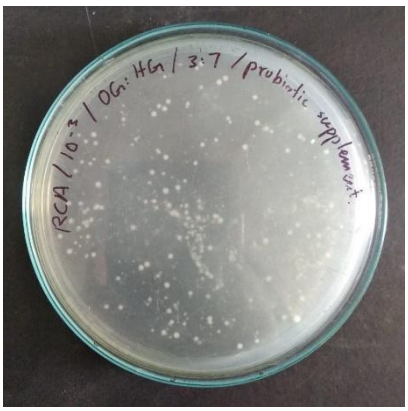

Figure 3a: Clostridium butyricum colonies on RCA media (3:7 OG:HG)

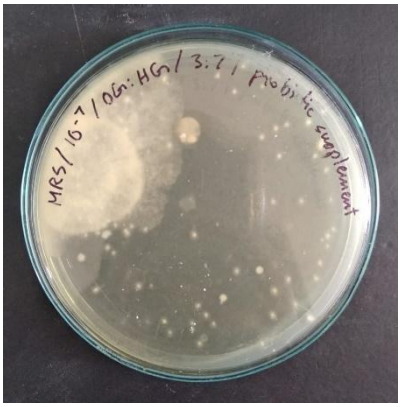

Figure 3b: Lactic acid bacillus colonies on MRS media (3:7 OG:HG)

Table 4: CFU/ml value for the microbial analysis of probiotic 3:7 OG:HG bigel

Media	Bacteria	No. of colonies	Dilution Factor	Vol. plated (ml)	CFU/ml
RCA	Clostridium butyricum	263	1000	0.1	2360000
MRS	LAB	48	10000000	0.1	4.8×10^9

The last sample analysed was of bigel 7:3 OG:HG, Figure 4a shows how this ratio unlike 3:7 helped in improving the microbial growth of *Clostridium butyricum* and increased the number of colonies formed. Figure 4b also showed positive result, in which the growth of LAB was even more than the 3:7 OG:HG bigel. In Table 5 the CFU of both colonies is calculated.

Clostridium butyricum count = 624000 CFU/ml

Lactic acid bacillus count = 5.9×10^9 CFU/ml

Table 5: CFU/ml value for the microbial analysis of probiotic 7:3 OG:HG bigel

Media	Bacteria	No. of colonies	Dilution Factor	Vol. plated (ml)	CFU/ml
RCA	Clostridium butyricum	624	1000	0.1	6240000
MRS	LAB	53	10000000	0.1	5.9×10^9

Figure 4a: Clostridium butyricum colonies on RCA media (7:3 OG:HG)

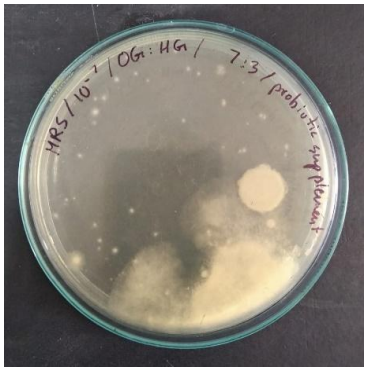

Figure 4b: Lactic acid bacillus colonies on MRS media (7:3 OG:HG)

Bigel of 3:7 OG:HG ratio only enhanced the *Clodtridium butyricum* viability while 7:3 OG:HG had shown to improve the growth and viability of both *Clostridium butyricum and Lactic acid bacillus LAB.*

All the data collected from phase one and two were compared in Figure 5a and 5b.

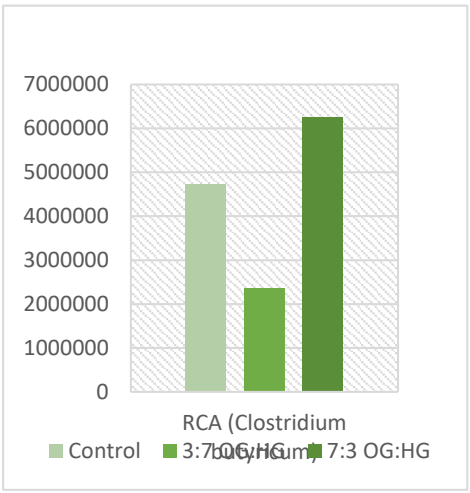

Figure 5a: Comparative chart of CFU/ml values in all RCA media plate

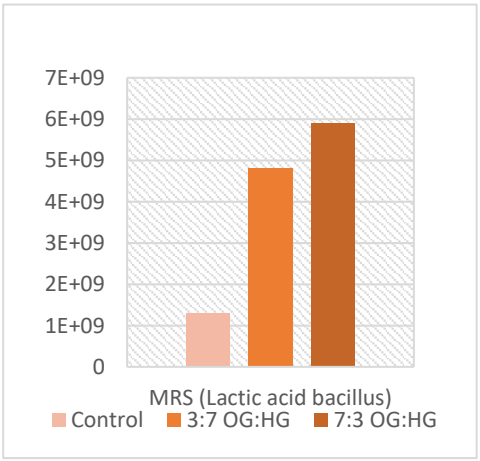

Figure 5b: Comparative chart of CFU/ml values in all MRS media plate

It has been established from this experimental study that, the phospholipid content of oleogel in bigel function as a protective shield and is beneficial for probiotic bacteria as they promote the growth of *Clostridium butyricum* and *Lactic acid bacillus*, the more the ratio of oleogel, better the effect is. Apart from the microbiological point of view, physically, the most stable form of bigel with solid, jelly-like structure and viscoelastic nature was 7:3 OG:HG ratio. All these factors can provide the bacteria a physical barrier and act as a potential coating agent against probiotic capsules, this packaging approach currently receives considerable interest. The bigel system can be further modified and optimized in order to survive the intestinal environment and be applied in coating or encapsulating technique of probiotic supplement.

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