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BACTERIOPHAGE MEDIATED MODULATION OF GUT MICROBIOME RESPONSIBLE FOR COLORECTAL CANCER

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ABSTRACT: The gut microbiome composition directly correlates with the disease's development and thus with an individual's health. According to the published scientific literature, gut microbiota is directly linked with developing diseases such as colorectal cancer. On the other hand, Bacteriophages are the group of viruses that infects the bacterial cells typically and can be utilized to manage the dysbiosis of the gut's microbial population. Here the major focus of this review is to know the applications and processes of bacteriophages in modulating the gut microbiome. According to studies, the modulation of the gut microbiome using bacteriophages has yielded good results. Although a major issue using this technique to modify the gut microbiome is regarding the survival of the phages when coming in contact with the stomach's highly acidic environment. Consequently, effective phage encapsulation is required. Thus, in this review, we focus on treating colorectal cancer using bacteriophage-mediated modulation of the gut microbiota.

INTRODUCTION: The symbiotic microbiota of an organism is present from birth which evolves with age, living environment, and uptake of nutrients [2]. Imbalances in microbiota have been related to the onset of various diseases, including colorectal cancer. The imbalance in the gut microbiota is caused due to several reasons, such as exposure to and consumption of alcohol, dietary changes, or new medications. For example, it was proved that in people with Alcoholic Hepatitis, there was a huge increase in the proportion of *Enterococcus faecalis* in such individuals [4]. A significant progression made by identifying that alcohol increased the permeability of the gut cell membranes, which resulted in the transfer of Cytolisin, a bacterial exotoxin to the liver cells and resulted in the damage of the liver cells [4]. Therefore, alcohol induces the occurrence of Alcoholic hepatitis in individuals. We know that cancers are hereditary in humans. But in the case of colorectal cancer, the scenario is different; only a small proportion of them is hereditary ranging from 10% to 15%, thus showing the responsibility of the gut microbiome in developing colorectal cancer [1].

The bacteriophage modulation of the Gut microbiome can be related to phage therapy which was used before the discovery of antibiotics. But due to the fact that bacteria are developing antibiotic resistance, it's time to shift our focus back to phage therapy [34]. But bacteria can also develop resistance against phages [5]. Phages are of two types; virulent phage and temperate phage. However, virulent phage than temperate phage is

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more suitable because temperate phages may transfer virulent factors or resistance genes [6]. Bacteriophage are extremely specific toward the bacteria cell; they stop their proliferation when they lose their target cells, thus diminishing the chances of a possible infection [7]. The fundamental aim of the present review is to identify the various strategies available for the efficient transfer of phage specific to the gut microbiota causing colorectal cancer.

Gut Microbiome Associated with Colorectal Cancer: Different gut bacteria have been identified, which is directly linked with the induction of colorectal cancer according to the various emerging researches listed in **Table 1.**

TABLE 1: GUT MICROBIOME RESPONSIBLE FOR COLORECTAL CANCER

Name	Mechanism
Fusobacterium nucleatum [19, 29, 30]	The antitumour immune system response is suppressed by a fall in counts of T cells (CD4+) paired with a decreased expression of TOX protein [19]
Bacteroides fragilis (Enterotoxigenic) [20, 30]	Upregulation of the Wnt pathway in association with the proinflammatory MAPK signals due to increased secretion of cytokine along with increased intestinal permeability, due to the binding of metalloproteinase toxin (Zn dependent) to the colonic epithelial cells [20]
Escherichia coli [21, 30]	Bacterium invades the colon's mucosal layer by DNA damage and triggering the Wnt mitogenic signalling pathway, which results in increased permeability of the mucosal layer. Further, breakdown in the DNA repair process facilitates the induction of colorectal cancer [21]
Salmonella typhimurium [22]	The bacterium produces AvrA protein that results in the proliferation of the IL-8 intestinal epithelial cell lining, thereby facilitating the induction of colorectal cancer [22]

The cease in proliferation in the growth of the gut microbiome resulting in dysbiosis is carried out by certain bacteriophages specific to the gut microbiome. **Table 2** depicts the various bacteriophages responsible for eliminating gut microbiome dysbiosis.

TABLE 2: BACTERIOPHAGE RESPONSIBLE FOR THE CEASING THE GROWTH PROLIFERATION OF GUT MICROBES

Name of the Gut Microbe	Name of the Bacteriophage Involved
Fusobacterium nucleatum [23]	Phage M13 [23]
Bacteroides fragilis (Enterotoxigenic) [24]	Phage VA7 [24]
Escherichia coli [15, 16, 25]	Phage T7 [25], Phage ZCEC5 [16], Phage T4 [15]
Salmonella typhimurium [26]	PhageUAB_Phi20 [26], Phage UAB_Phi78 [26], Phage UAB_Phi87 [26]

Challenges Faced During Bacteriophage Loading in Microspheres: The main challenge of targeting phage to the gut microbiome is to protect the phage from the low pH of the gastric juices before reaching the gut microbiome [33]. The virus particles are damaged due to the low pH of gastric juices, as stated by Ma *et al*. in 2008 [8], observed the decrease in the phage viability when bacteriophage Felix O1 were exposed to pH lower than 3.7. Also, the phage used for targeting the gut microbiota should be specific to the gut microbiome; if not, this would damage other good bacteria in our body. Also, the antibodies present in the serum may also inactivate the phage particles before they reach the gut microbiota [9]. Thus, it is evident that simple oral delivery of phage is not helped in this case. An effective solution to the discussed problem is by loading the bacteriophage in biomaterials, as listed in **Table 3.** Another major challenge faced during the bacteriophage loading into the microsphere is the density of bacteriophages being loaded, *i.e.*, a high bacteriophage density is beneficial for the faster reduction in bacterial growth rate [17]. This was also proved by Tanji *et al.* in 2005 for *E. coli* in chemostat where a high phage concentration was able to cease the growth of a high bacterial population 18 successfully. But this shall be a problem when the concentration of the bacteria is low. At that time, the phage concentration may reduce there would be less bacteria cells for the phage to survive. This may reduce the outcome of the phage delivery to the colon even if it is packed in the correct microsphere for their delivery to the colon. Also, the phage in such circumstances might get removed from the colon via the anal tract/Stool. Thus, the bacteriophage should reach the colon at the proper time (microbial dysbiosis), also it is essential for the microspheres to release the phage slowly for a prolonged period of time. In such time, the efficiency of modulation of the gut bacterial

cells using bacteriophages would be high. Bacteriophage are sensitive to dry heat; hence steam sterilization is not a useful option for loading the microsphere with the phage particles for delivery to the gut microbiome [10]. Bacteriophage are also sensitive to high temperatures as it reduces the lytic activity of the virus [10]. The range of temperature at which the bacteriophage remains stable must be taken into consideration while packing the phage into the microspheres. A widely described variety of bacteriophages and their functionality under different circumstances makes it difficult to for assessing the compatibility of phage to biomaterials being used for forming microspheres. However, Rotman *et al.* in 202010described of the three general processes which can be applied while processing the package of phage into the microspheres. They include 1) encapsulation, 2) embedding of the bacteriophage into the biomaterials, and 3) surface adsorption or covalent binding. Rotman *et al.* further concluded that whichever technique is applied for packaging the bacteriophage into the microspheres will depend on the type of biomaterial being used to form such microspheres [10]. How important is the transfer of bacteriophages into the biomaterials, more important is the proper storage of the bacteriophages embedded microspheres. For this, the bacteriophages are embedded into microspheres and then can be dried [11], thus facilitating their storage for a long time at room temperature. This facilitates their easy transportation and storage. The encapsulated phages can be dried with the help of air drying [8], spray drying [12] or lyophilization [13].

TABLE 3: BACTERIOPHAGE ENCAPSULATION USING DIFFERENT APPROACHES FOR DELIVERY TO THE GUT

Name of the Gut Microbe	Name of the Bacteriophage Involved	Bacteriophage Encapsulation Medium
Fusobacterium nucleatum [23]	Phage M13 [23]	Silver nanoparticle [23]
Bacteroides fragilis (Enterotoxigenic) [24]	Phage VA7 [24]	N.D.
Escherichia coli	Phage T7 [25]	Hydrogel [25]
	Phage ZCEC5 [16]	Sodium alginate beads with honey or gelatine [16]
	Phage T4 [15]	Sodium alginate beads with chitosan or polyethyleneimine coating [15]
Salmonella typhimurium	PhageUAB_Phi20[26], Phage UAB_Phi78 [26] Phage UAB_Phi87 [26]	Liposome [26]

N. D: Not Determined: In a study led by Dong *et al.* in 2020 [23], silver nanoparticles were used to deliver phage specific for Fusobacterium nucleatum to the colon. Electrostatically attaching the phage to the silver nanoparticles resulted in the inhibition in the production of immunosuppressive cells and the Fusobacterium nucleatum cells [23]. The main objective behind this study was to rebuild a microenvironment immune to a tumour by blocking the proliferation of the immunosuppressive cells [23].

Phage VA7 is identified as an ideal phage for modulating the population of Bacteroides fragilis population at the gut [24]. Previously, phage VA7 was isolated from the wastewater of Georgia, and showed bactericidal effects on Enterotoxic Bacteroides fragilis, as analyzed by spot test assay [27]. Later, Bakuradze *et al.* in 2021 [24] summarized that an increase in the proliferation of Bacteroides fragilis is associated with the increase in IL-8 levels, causing colorectal cancer. The study used colon epithelial cells infected with enterotoxic Bacteroides fragilis and summarized that phage VA7 was able to reduce the IL-8 levels by reducing the population of Bacteroides fragilis [24]. Therefore, it is essential to deliver the phage to the colon properly.

Liposomes can be used to load the bacteriophage for its delivery to the colon, according to the study conducted by Colom *et al.* in 2015 [26], where three different bacteriophages; Phage UAB_Phi20, Phage UAB_Phi78, and Phage UAB_Phi87, were loaded in liposomes each at a time for attacking *Salmonella typhimurium*. Previously, Collier-Hyams *et al.* in 2002 proved that *Salmonella typhimurium* was responsible for causing colorectal cancer [22]. Liposomes encapsulated phages could survive the low pH of the gastric juice, which was evident when Colom *et al*. in 2015 [26] proved that the Liposomes encapsulated bacteriophage survived the low pH (pH 2.8) of the simulated gastric juice. The liposomes were ruptured, releasing the bacteriophage when it came in contact with the bile salts. Thus, using liposomes to encapsulate the bacteriophage presents two

advantages; it helps the bacteriophage to survive the low pH of the gastric juice. Secondly, it helps in the long-term storage of the phage.

Another method used by Kopač *et al*. in 2021 [25], bacteriophage T7 was encapsulated in a hydrogel composition using HIPE as one of the manufacturing materials of the hydrogel. Escherichia coli was used as a host for the specific phage. The study further found that the bacteriophage T7 encapsulated in hydrogel wereable to withstand the highly acidic pH of the stomach (below pH 3.9). The release of the bacteriophage from the hydrogel was very much specific for pH. The hydrogel network was not completely degraded until the pH for duodenum was achieved (pH above 3.9). Therefore, the phage is protected from the very low pH of acid in the stomach, as well has facilitates the complete release of bacteriophage at the colon when the specific pH of the colon (pH above 3.9) is achieved [25]. Microspheres can be used for loading bacteriophage. The interior of the microspheres is hollow and is not coated with any liquid; thus, bacteriophages can be efficiently packed into the microspheres. During packing of the bacteriophage into the microsphere, the things which need to be taken into consideration are 1) heat sensitivity of the bacteriophages, 2) influence of high temperature on thephage's lytic activity, 3) biomaterials being used for forming the microspheres. From the different literatures available, Sodium alginate and chitosan are most widely used for the preparation of microspheres.

Moghtader *et al*. used Sodium alginate beads with chitosan or polyethyleneimine coating to embed the Escherichia coli phage T4 to harden the beads [15]. The phage release from the microspheres settles after 12 hours in the presence of artificially prepared gastric acid. The study further concluded that chitosan and polyethyleneimine coating delay phage release [15]. Thus, it can be assumed that chitosan and polyethyleneimine-coated microspheres take longer to break; hence, they can be used for microspheres because of their long time of retention of the bacteriophages because of the increased acid resistance due to polycationic coatings. If the viscosity of the alginate is increased using honey/gelatin, it resists the acidic environment (pH= 2) as well as releases the phage completely in 5 hours, in simulated stomach acidic conditions16. Thus, this is a comparatively fast process available for packing and bacteriophage release into the microspheres. The pore size of the microsphere can be decreased due to the addition of chitosan-coated sodium alginate. The reduced pore size decreases the diffusion of protons into the microsphere [8].

TABLE 4: COMPARATIVE ANALYSIS OF VARIOUS METHODS OF PHAGE DELIVERY TO GUT

Medium Used for Phage Transfer	Name of the Phage	Titer Reduction (T.R.)		Time of T.R. (In Hour(S))	Release of Bacteriophage
		N. EN	EN		
Liposomes [26]	UAB_Phi20	5.78$\log_{10}$ PFU/ml	4.8$\log_{10}$PFU/ml	1	74.7% in 1 hour [26]
	UAB_Phi78	8.08$\log_{10}$ PFU/ml	5.4$\log_{10}$PFU/ml	1	92.6% in 1 hour [26]
	UAB_Phi87	7.88$\log_{10}$ PFU/ml	3.7$\log_{10}$PFU/ml	1	56.6% in 1 hour [26]
Hydrogel [25, 31]	T7	N.A.	N.A.	N.A.	100% in 0.5 hour [25, 31]
Sodium alginate beads with honey or gelatin [16, 32]	ZCEC5	2.2$\log_{10}$ PFU/10ml	1$\log_{10}$ PFU/ml	1	100% in 6 hours [16, 32]
Sodium alginate beads with chitosan or polyethyleneimine coating [15]	T4	1.44$\log_{10}$ PFU/ml	0.56 $\log_{10}$ PFU/g	2	100% in 12 hours [15]
Silver Nanopaticles **Error! Reference source not found.**23	M13	N.A.		N.A.	N.A.

PFU: Plaque forming units, N.EN: Non-encapsulated, EN: Encapsulated, N.A.: Not available

DISCUSSION: While using bacteriophage for the modulation of the gut microbiome, it is very much essential to protect the bacteriophage from the highly acidic pH of the gut, the bacteriophage should be highly specific for the microbes in the gut whose growth is being controlled; otherwise, non-specificity of the bacteriophages may result in killing the other good microbe of the body. This shall harm an individual. In addition, responses of the host immune system are said to decrease the proliferation and growth of the phage targeted to the gut. Thus, encapsulating a phage in a proper delivery vessel to the gut is essential.

The various systems discussed above use hydrogels [25], silver nanoparticles [23], liposomes [26,] and sodium alginate beads whose composition is modulated differently each system [14, 15, 16]. A comparison among the different biomaterials available for the encapsulation of the phage, along with their phage release and phage titer reduction is listed in **Table 4**. As identified by Kopač *et al.* [25] hydrogels have proven to be keep phages stable in the low pH of the stomach and further protected the phage+ from the highly acidic gastric juice of the stomach by forming a layer around the phage T7 specific to Escherichia coli of the gut. Poly HIPE was used to increase the crosslinking, increased the integrity of the hydrogel and thus protect the phage from any mechanical pressure or stress during their transport to the gut [25]. The study found no release of bacteriophage at pH less than 3.9. Thus the hydrogel composed with poly HIPE to transfer phage T7 to the gut was successful in protecting the phage from the low pH gastric acid of the stomach, thus, this is why no significant drop in phage titer was observed. The entire phage was released pH above 3.9 [25]. Thus, it is a highly specific medium for releasing phage to the gut.

Among the methods available in literature to us, or the various phage delivery vessels tested, vessels made with sodium alginate beads are the most common. However, as said earlier the manufacturing component of the beads for encapsulation of bacteriophage was modulated every time. The chitosan and alginate are responsible for the prevention of lysis an acidic environment of a very low pH of the stomach and pH of the intestinal juice. For example, in accordance to the work by Moghtader *et al.* in 2016 [15], employed the use of chitosan or polyethyleneimine for coating the sodium alginate beads for the delivery of Escherichia coli phage T4 to the colon [15], whereas Abdelsattar *et al.* [16], during the preparation of sodium alginate beads, employed the use of honey or gelatin to increase the viscosity of the alginate core for the packaging of Escherichia coli phage ZCEC5 [16]. The study further determined the stability of the phage T4 in bile salts for both free and encapsulated bacteriophage.

Encapsulating the bacteriophage in sodium alginate beads increased the stability of the T4 phage and subsequently reduced the phage titer from 1.44 log 10 plaque-forming units/ ml (nonencapsulated) to 0.56 log10 plaque-forming units/g alginate beads after an exposure of 2 hours to the bile salts15. The max phage release was attained within 12 hours, with around 100% phage being released from the microspheres composed of alginate and calcium [15]. On the other hand, Abdel attar *et al.* used a slightly modified composition process for the preparation of the sodium alginate beads, where the increase in the viscosity of the alginate core was focused by using honey or gelatin, resulting in limiting the phage ZCEC5 titer reduction from 2.2 logs 10 plaque-forming units per ml (nonencapsulated) to 1log10 plaque forming units per ml [16]. As a result, the viscocity of the alginate core increases, the intermolecular forces are higher, and thus more stable the beads are. The phage release from the microspheres was approximately 7.5 log10 plaque forming units per ml which accounts for the complete release of bacteriophage ZCEC5 within 6 hour of coming in contact with the artificially prepared gastric juice [16]. The highlighted concern was to make the beads used for encapsulation of the phage more stable to protect the phage from the highly acidic gastric juices.

Liposome prepared using several different lipids with phage UAB_Phi20, phage UAB_Phi78, or phage UAB_Phi87, each at a time at concentrations of 1x 1011 plaque-forming units per ml [26]. Encapsulation of phage in liposomes provided a protective layer to the phage from the acidic gastric juices of the digestive tract. When compared between encapsulated phage in simulated gastric juice to nonencapsulated phage in gastric juice, the phage titer reduction for encapsulated phage was much lesser than for nonencapsulated phage [26].

After a 60 min exposure to artificially prepared gastric acidic conditions (pH 2.8), titer reduction for none capsulated phage UAB_Phi20, UAB_Phi78, UAB_Phi87 were recorded as 5.7 log10 plaque forming units/ ml, 8.0 log10 plaque forming units/ ml and 7.8 log10 plaque forming units/ ml respectively whereas the recorded value of titer reduction reduced to 4.8 log10 plaque forming units/ ml, 5.4 log10 plaque forming units/ ml and 3.7 log10 plaque forming units/ ml for phage UAB_Phi20, UAB_Phi78, UAB_Phi87, respectively after encapsulation26. Thus, liposome-mediated encapsulation of the phage protected the phage from the highly acidic gastric juice. The study further checked the percentage release of the bacteriophage from the liposomes in broiler chickens; after 1 hour the recorded results were as follows 74.7%, 92.6% and 56.6% for phage UAB_Phi20, UAB_Phi78, UAB_Phi87, respectively [26].

Interestingly, the methods used by Dong *et al*. in 2020 for encapsulation of the bacteriophage M13 were slightly different from the processes mentioned above [23]. The main objective was to block the immunosuppressive cells and the growth of *Fusobacterium nucleatum* which is necessary for the generation of an immune system against a tumour in the colon. Silver nanoparticles were employed for binding the phage M13 electrostatically to the nanoparticles [23]. To understand the detoriating effects of using silver nanoparticles attached to M13 Phage vectors on the host, the study injected the nanoparticles into mice intravenously and summarised that the nanoparticles exhibited no deteriorating effect on the host organism (mice) [23].

Thus, intraperitoneal or intravenous delivery of the phage to the colon might help protect the bacteriophage as it bypasses the highly acidic pH of the stomach or low pH gastric juice of other organs. In the case of interaction between enterotoxic Bacteroides fragilis and phage VA7, it is experimentally proven that phage VA7 is specific for Bacteroides fragilis and has the capability of ceasing its growth [24]. However, no such published data is available that has determined the ideal biomaterials for encapsulating the phage and targeting its delivery to the gut. **Table 4** shows that the entire phage is released when sodium alginate beads are used with honey, gelatin, chitosan/polyethyleneimine, and hydrogel to deliver the phage to the gut. Among them, maximum phage release takes place when using hydrogel to encapsulate and deliver phage T7 to the gut. There are currently significant drawbacks to adopting this approach to modulate the gut microbiota that causes colorectal cancer. As we move forward, we will examine the various published literature to connect the unconnected dots, analyze the current constraints, and provide viable remedies.

Limitations and Future Perspectives: There are several constraints when it comes to delivering bacteriophages to the gut for treating colorectal cancer. Such research gaps must be filled in order to develop this procedure and reach the highest feasible efficacy of this method. A description of the many restrictions and potential remedies is provided. Large-scale double-blind clinical trials in animals or humans should be conducted to identify the efficacy of these microspheres in releasing the bacteriophage into the colon. More emphasis should be given to the intravenous or intraperitoneal delivery of phages into the gut. According to the literature available, very little information is available regarding the encapsulation of cocktail of bacteriophage into the microsphere to take control over multiple gut bacteria at a time. Hence more studies should be conducted.

The bacteriophage activity is known to decrease due to a response from the host immune system or may trigger allergic reactions in the body. Therefore, the bacteriophage should always be encapsulated, and their release from the microsphere should be slow or the microsphere should be targeted at a time when microbial dysbiosis prevails to prevent their long-term interaction with the immune system of the host. More research needs to be conducted to bypass the exposure of the stomach's gastric acid as it has been known to reduce the phage titer even if encapsulated in biomaterials. Thus, according to available literature, two different encapsulation methods were identified: using silver nanoparticles and then electrostatically attaching them to the phage or using hydrogels for the delivery of phage to the gut since it promotes no release of phage at

pH below 3.9. More efforts should be given to delivering bacteriophage VA7 (specific to enterotoxic Bacteroides fragilis) to the gut with minimal loss in phage titer and special emphasis on their encapsulation such that such encapsulated phage could easily withstand the low pH, highly acidic gastric acid of the stomach. Further studies need to be conducted to determine the percentage release of bacteriophage from the delivery vessel at a particular time to determine the fastest mode of bacteriophage delivery for modulation of the gut microbiome.

The gut bacterium is responsible for maintaining homeostasis of the gastrointestinal tract. Considering phages that can infect multiple gut bacterium at a time, can transfer virulence factors among other bacteria not responsible for the induction of colorectal cancer, can lead to the development of gastrointestinal diseases. Risk benefit-based analysis should be conducted for phages that infect multiple species of gut bacterium to analyze potential risks, if any, and find solutions for them. A major limitation of phage therapy is that the interactions between bacteriophage and a bacterium, cause the bacterial cell to lyse along with the release of several endotoxins, which can induce the development of several other bacterial infections [28]. Hence, the effects of these endotoxins on the human body should be studied to reduce their harmful consequences. Correct microbial homeostasis is necessary for optimum gut health, and imbalances in the gut microbiota are directly associated with the development of colorectal cancer. More research is needed, focusing on how to stop bacteriophage infection of gut bacteria after equilibrium has been achieved to avoid further gastrointestinal ailment induction.

CONCLUSION: To us, it is now evident that the majority of colorectal cancers are non-hereditary. Any microbial dysbiosis in the gut leads to the development of colorectal cancers. Due to widespread antibiotic resistance, phage therapy has emerged and has given numerous proven results in ceasing the proliferation and growth of several gut microbes responsible for colorectal cancer. However, to make the process more efficient, more research to be conducted with special emphasis given to the preparation of delivery vessels for targeting the delivery of bacteriophage to the gut such that there is a very minute reduction in phage titer when in contact with the acidic gastric acids of the stomach, bypassing the interaction of the phage with the immune system of the host so that the phage titer is not reduced. However, as the phage may kill good gut bacteria, potentially causing adverse health effects in individuals, the phage should be particularly specific for its target microbe.

Nevertheless, many aspects remain questionable. Further analysis of them based on comparing risks and benefits is necessary with large-scale clinical or human trials to resolve them.

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