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Chapter 1: Introduction

Abstract:

In spite of having many advantages, administration of a drug through the oral route only allows less than 10% absorption of the drug, which has low solubility or low cell membrane permeability, or both through the gastrointestinal (GI) tract, especially small intestine[1]. Therefore, it has many disadvantageous consequences, including substantial financial loss due to the requirement of at least ten times more drugs than the body originally require[2–5]. Moreover, oral drug administration usually requires multiple doses due to the faster metabolism of drug molecules in the GI tract. In addition to that, excessive use of drugs shows undesired toxicity in various parts of the digestive tract[6,7]. Henceforth, reduction of drug use is necessary without compromising the effectiveness of the drugs. This thesis introduces a novel drug delivery approach integrating nanotechnology with microbial factors, offering a new paradigm for targeted and sustained drug release.

We report the development and deployment of Bacterioboat, which consists of surface-encapsulated mesoporous nanoparticles on metabolically active *Lactobacillus reuteri,* as a drug carrier suitable for oral administration[8]. The porous chitosan nanoparticle layer serves as a protective barrier, shielding the bacteria from any potential adverse effects of the loaded drug while preserving the integrity of the delivery system during its passage through the gastrointestinal (GI) tract. Importantly, the bacteria remain alive and metabolically active throughout the process, retaining their capacity for biofilm formation, which is crucial for effective colonisation and sustained drug release.

The BB system's versatility extends beyond drug delivery. It can attach dietary supplements, such as vitamins and proteins, as well as other beneficial compounds or enzymes, to the microbes. This allows for a sustained release and enhanced bioavailability of these compounds, providing added health benefits without any negative side effects. Furthermore, the BB system can be easily formulated into various oral dosage forms, including tablets, capsules, or oral suspensions. This adaptability ensures that the system can be consumed conveniently and effectively for therapeutic purposes or to deliver desired health benefits.

1.1 Drug Delivery System

Drug delivery systems play an important role in modern medicine by ensuring that therapeutic drugs reach their intended sites in the body quickly and securely. Traditional medication delivery systems, while generally successful, have several problems, including low bioavailability, non-targeted distribution, and negative side effects[2,3]. Recent advances in drug delivery technology seek to address these constraints by providing more accurate and efficient treatment alternatives. One of the core principles governing the effectiveness of drug delivery is ADME, an acronym for Absorption, Distribution, Metabolism, and Excretion[9,10]. Understanding these pharmacokinetic processes is essential for developing effective drug delivery systems. A drug enters the bloodstream from its site of administration. Factors influencing absorption include the drug's physicochemical properties, the formulation, and the route of administration. Once absorbed, the drug is transported via the circulatory system to various tissues and organs. The distribution phase is influenced by factors such as blood flow, tissue permeability, and the binding of the drug to plasma proteins. For example, orally

administered drugs must navigate the acidic environment of the stomach and the enzymatic activity in the intestines, which can significantly impact their bioavailability[11,12]. While the Lipophilic drugs tend to distribute widely and accumulate in fatty tissues, hydrophilic drugs may remain largely within the extracellular fluid. The body chemically modifies the drug during the metabolism, primarily in the liver, through enzymatic activity. This phase often results in the conversion of the drug to more water-soluble metabolites that can be easily excreted[13]. Metabolism can activate prodrugs or, conversely, inactivate active drugs. Enzymes like cytochrome P450 play a significant role in this process, and genetic variations in these enzymes can lead to inter-individual differences in drug response[14,15]. Excretion is the final phase, where the drug and its metabolites are eliminated from the body. The primary routes of excretion are renal (via the kidneys) and biliary (via the liver and bile). Factors affecting excretion include renal function, urine pH, and the drug's molecular size and polarity[14].

The advantages of understanding and optimising ADME processes in drug delivery are manifold. By enhancing absorption, drugs can achieve higher bioavailability, leading to more consistent therapeutic effects. Effective distribution ensures that the drug reaches its target sites in sufficient concentrations. Metabolism can be leveraged to prolong the drug's activity through the use of prodrugs or to reduce toxicity. Efficient excretion mechanisms prevent the accumulation of drugs and their metabolites, minimising potential side effects.

However, there are also challenges associated with ADME.

Depending upon the routes of drug delivery, the following table classifies the types of drug delivery through examples.

Different Drug Delivery Routes with examples

Drug Admin route	Form of administration	Importance	Example of Drugs
Oral route	Tablets, Capsules, Liquids	Easy, Non-invasive, painless	Paracetamol, Albendazole, aspirin, Metronidazole,
Intravenous	In solution	100% Absorption, High Bioavailability	5- Fluorouracil, Dextrose, Doxorubicin, Paracetamol
Intraperitoneal	In solution/ suspension	Slow release, High bioavailability	Insulin, heparin, antibiotics, etc
Intramuscular	In solution/ suspension	Very slow release, Immune activation	Chlorpromazine, Morphine, Lorazepam, Codeine.
Intra pulmonary	Aerosol	Very fast absorption, Very Bioavailability	Corticosteroids and long-acting beta-agonists
Trans-dermal & intra-dermal	In solution/ suspension	Slow release, Low bioavailability	Anti-anginal drugs, Oil and ointments
Tropical admin.	Cream, solution, suspension etc	Local Application: no systemic exposure.	Eyedrops, eardrops, Povidone-iodine
Buccal or Sublingual	Tablets	Slower absorption and enzyme activation	GTN, Buprenorphine, Desaminooxytocin

Poor absorption can limit a drug's efficacy, necessitating higher doses or alternative delivery methods. Unfavourable distribution can result in sub-therapeutic concentrations at the target site or toxic levels in non-target tissues. Variability in metabolism due to genetic differences can lead to unpredictable drug responses, necessitating personalised dosing regimens. Additionally, inefficient excretion can cause drug accumulation and increased risk of adverse effects[16]. A thorough understanding of these pharmacokinetic principles allows for optimising therapeutic regimens, enhancing drug efficacy and safety while mitigating potential drawbacks. The Biopharmaceutics Classification System (BCS) is a vital framework that categorises drugs based on their aqueous solubility and intestinal permeability, significantly impacting the choice of drug administration routes. The BCS classification plays a crucial role in determining the appropriate route of administration for drugs[17]. Understanding the interplay between solubility, permeability, and stability helps in designing effective drug delivery systems and selecting the optimal route to ensure therapeutic efficacy. This system, developed by the FDA, aims to streamline drug development and regulatory approval processes by predicting the in vivo performance of oral drug products[18]. The BCS classification provides a framework for understanding how different drugs behave when administered orally and guides the formulation strategies to stabilise or optimise drug delivery; class I drugs typically do not require complex formulation strategies due to their favourable solubility and permeability, leading to stable and predictable absorption profiles[19]. Their predictable absorption profile makes oral tablets and capsules the most convenient and effective delivery forms. Examples: Paracetamol (acetaminophen) and propranolol. Class II drugs often require solubility enhancement techniques, such as surfactants, solid dispersions, or particle size reduction, to stabilise their absorption and ensure adequate bioavailability. While these drugs can permeate biological membranes effectively, their absorption is limited by their low solubility in GI fluids. Dissolution rate is the rate-limiting step for their absorption[20]. Although oral administration is still common, additional formulation strategies are often required to enhance solubility and dissolution. Techniques include micronisation, solid dispersions, and the use of solubilising excipients. Achieving consistent bioavailability can be difficult, and variability in GI conditions can affect drug absorption. Formulation enhancements are crucial to ensure adequate therapeutic levels[13]. Examples: Ketoconazole and ibuprofen. Class III drugs benefit from permeability enhancement strategies, such as the use of absorption enhancers or encapsulation in nanoparticles, to overcome their limited ability to cross biological membranes. These drugs dissolve readily in GI fluids but struggle to cross the intestinal mucosa due to low permeability. Even with solubility not being a limiting factor, ensuring consistent and efficient absorption remains challenging. The variability in permeability across different patients can lead to inconsistent therapeutic outcomes. Oral administration is possible but may require permeability enhancement strategies. Formulations such as prodrugs, permeation enhancers, or nanoparticle encapsulation can improve absorption[19]. Examples: Cimetidine and acyclovir. Class IV drugs face the greatest challenges for oral absorption due to both poor solubility and permeability[19]. They are often poorly absorbed when taken orally, resulting in low bioavailability. Non-oral routes are often preferred for Class IV drugs to bypass GI limitations. Intravenous (IV) administration is common as it ensures 100% bioavailability by directly delivering the drug into the systemic circulation[21]. Other routes, such as intramuscular, subcutaneous, or inhalation, may also be considered based on the drug's properties and therapeutic needs. Examples: Paclitaxel and furosemide. Formulations such as lipid-based systems, nanoparticles, or prodrugs are often necessary to improve their bioavailability [22]. However, these strategies can be complex and may not always achieve consistent results. Although there are different routes of administration, the most common and preferred route is the oral route of

administration for drug delivery as it is a non-invasive, convenient and easy-to-use route of drug delivery.

1.2 Oral route for drug delivery

Because oral drug delivery is convenient, patient-friendly, and economical, it is still the gold standard for administering medications[1]. Research shows that around 80% of all drugs are designed to be taken orally, demonstrating how commonplace it is[1]. Compared to other routes, this one has many important benefits, such as self-administration, non-invasiveness, painlessness, and convenience. Oral administration poses notable hurdles for several medicines with limited GI tract permeability, which may restrict their therapeutic efficacy. Acid sensitivity and enzymatic hydrolysis Low-permeability drugs have difficulty passing through the intestinal barrier and getting into circulation, lowering their bioavailability. The percentage of a medication that enters the body and travels to its location of action is referred to as bioavailability. The therapeutic impact of the medicine was ultimately limited by low bioavailability, which is reflected in a smaller percentage of the supplied dose reaching its target [2,3]. Moreover, medications that are not absorbed and excreted in faeces could contaminate the Environment and cause microbes to become resistant to antibiotics [23,24]. Oral bioavailability of some cancer medications, such as 5-Fluorouracil (5-FU), employed in this trial, is frequently restricted for oral drug delivery.

Factors Influencing the Oral Drug Delivery as Choice of Administration Route

- **Bioavailability**
  Ensuring the drug reaches therapeutic levels in systemic circulation is crucial. Poor bioavailability through the oral route may necessitate alternative delivery methods[25].

- **Stability**
  Drugs that are unstable in the GI environment, such as those susceptible to degradation by stomach acid or enzymes, are less suitable for oral administration[25].

- **Patient Compliance**
  Oral administration is generally preferred due to convenience, but this must be balanced against the drug's pharmacokinetic properties[26].

- **Onset of Action**
  For rapid therapeutic effects, non-oral routes such as intravenous injection may be preferred[21,27].

- **Formulation Feasibility**
  The practicality of developing a stable and effective formulation for the intended route plays a significant role in decision-making[28].

- **Adequate Bioavailability**
  Medications classified under **BCS Class I** and **Class II** often show good to moderate bioavailability when taken orally. Even drugs in **Class III** and **Class IV** can be formulated to improve their absorption profiles, making oral dosing feasible in most cases[22].

- **Chronic Use**
  Medications for chronic conditions (e.g., diabetes, hypertension, and hypercholesterolemia) are better for oral administration since they often require daily or long-term use. Oral dosing improves patient compliance and overall quality of life[29].

Oral drug delivery faces several limitations, as explained by the Biopharmaceutics Classification System (BCS) and ADME (Absorption, Distribution, Metabolism, and Excretion) properties. BCS Class I drugs, despite their high solubility and permeability, may suffer from first-pass metabolism, reducing their effective concentration. BCS Class II drugs have high permeability but low solubility, requiring advanced formulations to enhance dissolution, leading to variable absorption. BCS Class III drugs, although highly soluble, face poor permeability challenges, necessitating the use of permeability enhancers or prodrugs to improve absorption. BCS Class IV drugs, with low solubility and permeability, present the most significant challenges, often requiring complex delivery technologies to achieve therapeutic levels. Additionally, ADME factors further complicate oral delivery[10,30]. Absorption can be affected by gastrointestinal pH, motility, and interactions with food or other drugs[31]. Distribution can be limited by protein binding and tissue permeability[32]. Metabolism, primarily in the liver, can lead to significant drug degradation before reaching systemic circulation[15]. Finally, excretion processes, mainly renal, can affect drug half-life and therapeutic effectiveness. The oral route has several limitations, especially for low solubility drugs that lead to low half-life in the GI tract, only 3-5% of BSC-III and IV drugs' absorptions leading to Low bioavailability, and it becomes non-suitable for GI-tract inflamed patients[33]. Therefore, while oral delivery is convenient and preferred, careful consideration of these limitations is required to ensure effective and consistent therapeutic outcomes[22].

1.3 Examples of Medications Commonly Administered Orally Rather Than Intravenously and Their Classification According to the Biopharmaceutics Classification System (BCS)

Why orally administered?

Certain medications are commonly administered orally for their ease of use, patient compliance, and suitable pharmacokinetic properties. The Biopharmaceutics Classification System (BCS) is a scientific framework that classifies drugs based on their solubility and permeability[34], further helping to understand why specific medications are favoured for oral administration. Below are some of the examples of oral drugs, i.e., Paracetamol, Aspirin, Metformin, Levothyroxine, Omeprazole, Simvastatin, and Amoxicillin, their reasons for oral use, and their BCS classifications.

Paracetamol (Acetaminophen)

Paracetamol is a widely used analgesic and antipyretic. It is readily absorbed in the gastrointestinal tract, providing predictable bioavailability and systemic distribution. Its oral administration allows easy home use for managing pain and fever without requiring medical supervision. Paracetamol falls

under BCS Class I (high solubility, high permeability), making it ideal for oral administration as it easily dissolves and passes through the gastrointestinal membrane, ensuring effective absorption[35].

Aspirin (Acetylsalicylic Acid)

Aspirin is commonly taken orally for its antipyretic, anti-inflammatory, and antiplatelet effects. The oral route is preferred due to its rapid absorption in the stomach and small intestine, ensuring quick therapeutic action. Its frequent use for long-term cardiovascular prevention is practical when taken orally. Aspirin is classified under BCS Class I, characterised by high solubility and high permeability, ensuring that it dissolves rapidly and is efficiently absorbed when taken orally[36].

Metformin

Metformin, the first-line treatment for type 2 diabetes, is taken orally due to its high efficacy in controlling blood glucose levels[37]. It is well absorbed in the gastrointestinal tract and poses minimal risk of hypoglycaemia, making it ideal for long-term use. Oral administration allows patients to self-administer the drug regularly without requiring intravenous infusion. Metformin is categorised under BCS Class III (high solubility, low permeability). While it is highly soluble in water, its absorption is limited by its low permeability, which means most of the drug's action occurs via gastrointestinal absorption rather than systemic circulation[38].

Levothyroxine

Levothyroxine is a synthetic thyroid hormone taken orally for the treatment of hypothyroidism [39]. Its long half-life and predictable pharmacokinetics make oral administration ideal for long-term hormone replacement therapy. Oral dosing simplifies the treatment process, as the drug is absorbed on an empty stomach and allows for once-daily dosing. Levothyroxine is considered BCS Class II (low solubility, high permeability). It has limited solubility in the gastrointestinal tract but is highly permeable, which enables effective absorption into the bloodstream when appropriately formulated[40,41].

Omeprazole

Omeprazole, a proton pump inhibitor, is used to manage acid reflux and peptic ulcers[42]. Oral administration is preferred since the drug directly affects the parietal cells in the stomach, reducing acid production. It is taken orally before meals to maximise its absorption and therapeutic effect, avoiding requiring intravenous administration in most cases. Omeprazole falls under BCS Class IV (low solubility, low permeability). This presents challenges for oral absorption, but specific formulations (e.g., enteric-coated capsules) have been developed to enhance their bioavailability[40].

Simvastatin

Simvastatin is used to lower cholesterol levels and reduce the risk of cardiovascular disease[43]. Oral administration is preferred for long-term use, as it allows patients to take the medication daily without medical supervision. It is efficiently absorbed through the gastrointestinal tract, making oral dosing the most practical option. Simvastatin is classified under BCS Class II (low solubility, high permeability). While it is not highly soluble, its good permeability ensures effective systemic absorption after oral administration[40].

Antibiotics (e.g., Amoxicillin)

Amoxicillin and other common antibiotics are often administered orally due to their effective absorption in the gastrointestinal tract and ability to maintain therapeutic concentrations[44]. Oral antibiotics are particularly useful for outpatient treatments, allowing patients to manage bacterial infections at home without requiring intravenous intervention. Amoxicillin is categorised under BCS Class I (high solubility, high permeability). This means it is highly soluble and easily absorbed, which makes oral administration straightforward and effective[45].

1.4 Examples of Medications Administered Intravenously Rather Than Orally with Their Biopharmaceutics Classification System (BCS) Classification

Why Intravenous route is preferred over oral route?

Intravenous (IV) administration is required for certain medications due to poor oral bioavailability, rapid inactivation in the gastrointestinal tract, or the need for immediate therapeutic effects. The Biopharmaceutics Classification System (BCS) classifies drugs based on their solubility and permeability, helping to understand why intravenous administration is preferred over oral dosing[46]. Below are examples of such medications, i.e.,5-Fluorouracil (5-FU), Vancomycin, Heparin, Dobutamine, and Morphine, their BCS classifications and reasons for intravenous use.

5-Fluorouracil (5-FU)

5-Fluorouracil (5-FU) is a chemotherapy drug used to treat various cancers[47]. Intravenous administration is required due to its poor oral bioavailability. Oral 5-FU is rapidly metabolised in the liver and gastrointestinal tract by dihydropyrimidine dehydrogenase (DPD), significantly reducing its effectiveness. Intravenous administration bypasses this first-pass metabolism, ensuring the drug reaches therapeutic concentrations in the bloodstream. It is a BCS Class III (high solubility, low permeability) drug. 5-FU is partially soluble, and its low permeability through the gastrointestinal membrane results in poor bioavailability when administered orally, necessitating intravenous delivery [48,49].

Vancomycin

Vancomycin is a glycopeptide antibiotic used to treat severe infections caused by Gram-positive bacteria, including methicillin-resistant *Staphylococcus aureus*[50]. It is administered intravenously for systemic infections because it is poorly absorbed orally. Oral vancomycin is only used for localised gastrointestinal infections like *Clostridioides difficile* colitis. Intravenous administration ensures adequate systemic concentrations to treat severe infections. It is a BCS Class IV (low solubility, low permeability) drug. Vancomycin's poor solubility and low permeability make oral administration ineffective for treating systemic infections, requiring IV administration for effective treatment[51].

Heparin

Heparin is an anticoagulant used to prevent and treat thromboembolic disorders[52]. Due to its large molecular weight and susceptibility to enzymatic degradation in the gastrointestinal tract, oral administration is impossible. Intravenous or subcutaneous administration is necessary to achieve the required anticoagulant effect in the bloodstream. BCS Class IV (low solubility, low permeability). Heparin has low solubility and permeability, meaning it cannot be absorbed effectively from the gastrointestinal tract, requiring intravenous or subcutaneous administration for efficacy[53].

Dobutamine

Dobutamine is a sympathomimetic drug used to manage acute heart failure and cardiogenic shock[54]. It is administered intravenously to achieve rapid therapeutic effects. Oral administration is not feasible due to its short half-life and rapid metabolism in the liver, making IV delivery essential for continuous control of cardiac output in critical care situations. BCS Class I (high solubility, high permeability). Although dobutamine has favourable solubility and permeability characteristics, its rapid hepatic metabolism and short half-life require continuous IV infusion for effective therapeutic action[55].

Morphine (for Acute Pain)

Morphine, a potent opioid analgesic, is administered intravenously for rapid relief of acute and severe pain, such as post-surgical or trauma-related pain[27]. While oral formulations are available for chronic pain, intravenous administration provides immediate action, which is critical in acute care settings where prompt pain control is needed. BCS Class I (high solubility, high permeability). Morphine is well absorbed when administered orally, but for acute pain management, the intravenous route is preferred due to its rapid onset of action and more controlled delivery[56,57].

Rationale for Intravenous Administration with BCS Classification

- **Poor Oral Bioavailability**
  Medications like 5-FU and vancomycin have poor oral bioavailability due to rapid degradation or poor absorption in the gastrointestinal tract. Their BCS Class III and Class IV classifications explain their need for intravenous delivery, bypassing these barriers to ensure effective therapeutic levels in the bloodstream[30,49].

- **Rapid Therapeutic Action**
  Intravenous administration ensures immediate drug delivery into the systemic circulation, which is essential for drugs like dobutamine and morphine in acute or emergency situations. Oral administration would lead to delayed absorption and a slower onset of action, which is unsuitable for critical care[21].

- **Avoidance of First-Pass Metabolism**
  Drugs like 5-FU undergo extensive first-pass metabolism when taken orally, significantly reducing their efficacy. Intravenous administration bypasses the liver and gastrointestinal tract, allowing these medications to exert their therapeutic effects more consistently[58].

- **Precise Dosing and Control**
  Intravenous administration allows for precise control of drug concentrations in the bloodstream, which is particularly important for medications like heparin and 5-FU with narrow therapeutic windows. This method allows clinicians to adjust doses in real time, ensuring both efficacy and safety[55].

In conclusion, intravenous administration is often the preferred route for medications with poor solubility, low permeability, or rapid metabolism, as classified by the BCS system[27]. These properties limit their effectiveness when taken orally, making intravenous delivery crucial for achieving desired therapeutic outcomes in acute and long-term treatments.

1.5 Choice of Bacteria / Microbe for BB preparation

Probiotic bacteria have been selected for UDDS in this thesis due to their unique properties, particularly their ability to survive in the gastrointestinal tract and form biofilms, which enhance drug delivery efficiency. *Lactobacillus reuteri*, *Lactobacillus rhamnosus*, and *Bifidobacterium longum* offer numerous advantages. These probiotics are generally recognised as safe (GRAS) and can endure the harsh conditions of the gastrointestinal environment, such as acidic pH and bile salts[59]. Their ability to produce biofilms provides an additional layer of protection, helping to shield therapeutic agents from degradation as they pass through the digestive system[59–61].

For example, *Lactobacillus reuteri* not only demonstrates resilience in the gastrointestinal environment but also forms biofilms that promote the sustained and localised release of anti-inflammatory agents, which is beneficial in treating gastrointestinal diseases like colitis[62]. This biofilm-producing capability helps extend the retention of the bacteria and the associated drug, enhancing drug delivery efficiency and prolonging therapeutic effects. Similarly, *Lactobacillus rhamnosus* is known for its biofilm formation[63], which aids in the targeted delivery of therapeutic agents to the colon, while *Bifidobacterium longum* has been explored in cancer therapy, utilising its ability to adhere to the intestinal mucosa and deliver chemotherapeutic drugs directly to tumour sites[64].

The ability of probiotics to form biofilms also enhances their interaction with the gut lining, which allows for increased residence time and better absorption of the therapeutic agents. For instance, *Lactobacillus plantarum*, known for its biofilm-producing capability, has been used to deliver therapeutic proteins for treating inflammatory bowel disease (IBD)[65]. Additionally, the biofilm-forming nature of probiotics like *L. reuteri* contributes to their immune-modulating effects, further supporting their role in drug-delivery systems designed for immune-related therapies.

Furthermore, using probiotics, which form biofilms, aligns with the trend toward biocompatible and biodegradable drug carriers. This reduces the risk of toxicity and immune rejection compared to synthetic systems. Probiotic biofilms offer a stable and controlled environment for drug release, as demonstrated in studies using *Lactococcus lactis* for oral vaccine delivery[66]. The ability of probiotics like *L. reuteri* to form biofilms makes them highly effective in protecting drugs from premature degradation and ensuring more precise and efficient delivery. Overall, the combination of biofilm formation, safety, and enhanced bioavailability makes probiotic bacteria an innovative choice for oral drug delivery systems.

The normal gut microflora can be used for the preparation of the UDDS that produces biofilm as the production of biofilm is required in the mechanism of the stability of UDDS in the gut that causes the release of drug over a longer period of time and extends the window of the drug to many folds which causes the better and almost complete absorption of the dosage through the oral route. So, we choose biofilm-producing *Lactobacillus reuteri* to prepare the universal drug delivery system as it is the typical gut inhabitant in humans and is also used in probiotics[67].

1.6 Chitosan nanoparticles - as a vehicle for drug delivery

We have used deacetylated chitosan to synthesise a novel microbial drug delivery system, which we have named Bacterioboat, which consists of Bacteria and chitosan nanoparticles. Chitosan is the second most abundant polymer extracted from the sea shells. It is non-toxic, abundantly and readily available, easily stored, stable at room temperature, and a low-cost polymer having the least immunological responses[1]. Nanoparticles of chitosan are already being synthesised and are being used to deliver various drugs[68]. It is non-interactive to the maximum number of drugs.
Chitosan is derived from chitin through partial deacetylation, resulting in a polymer where varying proportions of the N-acetyl groups have been replaced by amino groups (NH_2). This gives chitosan a positively charged character under acidic conditions[69].

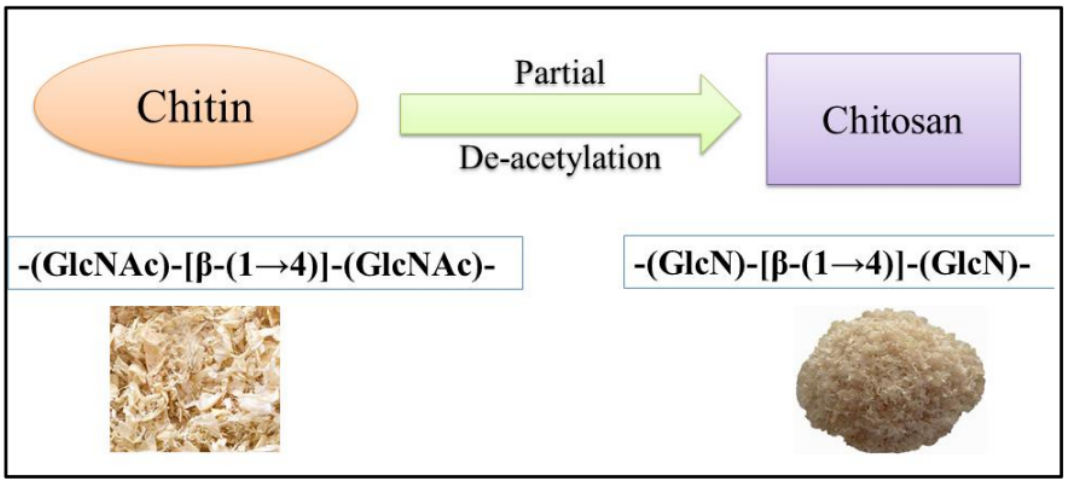


Figure 1: Representation of the chitosan source from partial de-acetylation of Chitin.

GlcNAc stands for N-acetylglucosamine for chitin, and GlcN represents glucosamine units, which are deacetylated forms of GlcNAc for chitosan[70].
Chitosan solution creates chitosan nanoparticles through a series of chemical interactions. The formation of these nanoparticles typically involves ionic gelation, where chitosan's positive charge, due to its amino groups, interacts with negatively charged cross-linkers like sodium tripolyphosphate (TPP)[1]. First, chitosan is dissolved in an acidic solution to protonate the amino groups, making the polymer positively charged [71]. Then, TPP is added to the chitosan solution. The negative phosphate groups of TPP electrostatically interact with the positive amino groups of chitosan, causing gelation and forming chitosan nanoparticles[72]. These nanoparticles are stabilised by the electrostatic forces between chitosan and TPP, creating a stable, cross-linked network. The size and stability of the chitosan nanoparticles can be optimised by adjusting the chitosan-to-TPP ratio, pH, and ionic strength of the solution, which affect the degree of cross-linking and the properties of the nanoparticles.

Additionally, functional groups or therapeutic agents can be incorporated into the chitosan solution if needed[73,74].

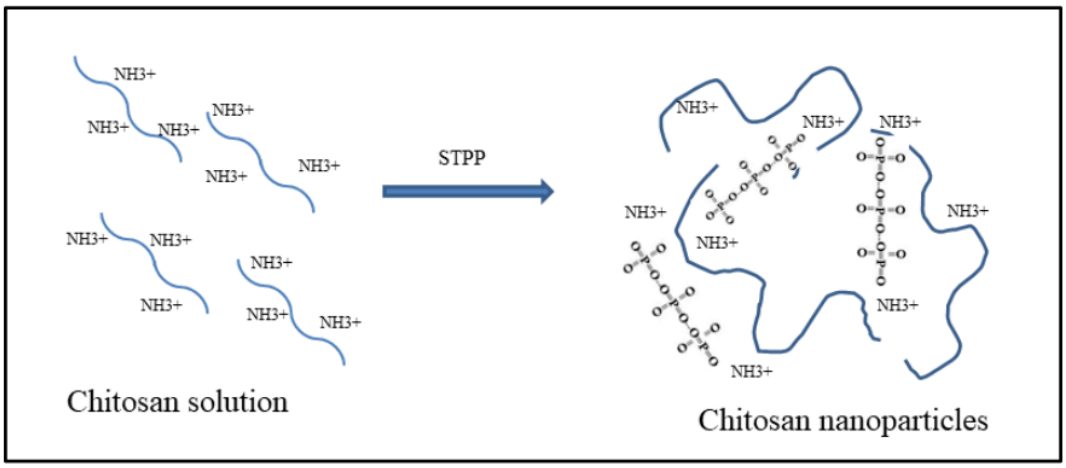


Figure 2: Generic representation of the crosslinking of Chitosan with STPP to form Chitosan nanoparticles

LC50 value of chitosan underscores its low toxicity, particularly in animal models, highlighting its potential as a safe and effective biopolymer for medical, pharmaceutical, and environmental applications. In toxicological studies using mice, which serve as common model organisms, the LC50 value for chitosan has been more precisely determined. Research indicates that the LC50 value for orally administered chitosan in mice is approximately 16 g/kg body weight[75]. This relatively high value suggests that chitosan has low toxicity in mice, supporting its potential for safe use across various applications, making it suitable for a wide range of biomedical and industrial applications.[72]

1.7 Choice of Anti-cancerous Drug

Orally administered anticancer drugs face significant challenges related to low bioavailability, primarily due to poor solubility, first-pass metabolism, efflux mechanisms, pH variability, and enzymatic degradation[76,77]. Addressing these issues through advanced formulation strategies and drug modification techniques is crucial for improving the therapeutic efficacy and clinical outcomes of oral anticancer therapies. Continued research and innovation in this field are essential to overcome these barriers and enhance the effectiveness of oral cancer treatments.

Oral administration of anticancer drugs is a preferred route due to its convenience, patient compliance, and potential for long-term therapy. However, despite these advantages, the effectiveness of orally administered anticancer drugs is often compromised by low bioavailability.

We opted for 5-fluorouracil (5-FU) as our model drug for developing a universal drug delivery system, which we named Bacterioboat (BB). 5-Fluorouracil (5-FU) is a widely used chemotherapeutic agent in treating various cancers, including colorectal, breast, and head and neck cancers[48]. Traditionally administered intravenously, efforts have been made to develop oral formulations of 5-FU to improve patient convenience and compliance. However, the oral administration of 5-FU is fraught with

challenges, primarily related to its pharmacokinetic properties and bioavailability. 5-Fluorouracil (5-FU) is predominantly administered intravenously rather than orally due to several significant pharmacokinetic and pharmacodynamic challenges that hinder its effectiveness when taken by mouth. One of the primary reasons is its poor bioavailability. When 5-FU is administered orally, it undergoes extensive first-pass metabolism by the enzyme dihydropyrimidine dehydrogenase (DPD) in the gastrointestinal tract and liver[49]. This metabolic process drastically reduces the amount of active drug that reaches the systemic circulation, rendering oral administration inefficient. Moreover, the absorption of 5-FU from the gastrointestinal tract can be highly variable among patients due to differences in gastrointestinal pH, the presence of food, and individual variations in DPD enzyme activity. This variability can lead to inconsistent therapeutic drug levels, complicating dose optimisation and reducing treatment efficacy.

Another critical issue with oral administration is the narrow therapeutic window of 5-FU. The need to achieve therapeutic concentrations without causing significant toxicity is challenging. Oral doses required to overcome low bioavailability often result in high peak plasma concentrations, which increase the risk of severe adverse effects, such as gastrointestinal toxicity, myelosuppression, and mucositis. These toxicities can be debilitating and limit the feasibility of oral 5-FU administration, particularly in patients who are already weakened by their disease[78,79].

While 5-FU is highly effective when administered intravenously, its oral bioavailability and solubility are significantly limited. To address this issue, we chose a drug that typically causes gastrointestinal irritation and has low bioavailability to demonstrate the BB system's potential for such medications.

Cancer remains a serious health challenge, with increasing incidence due to factors such as fertilisers, industrial pollutants, and radioactive exposure[80]. Patients undergoing cancer treatment often require prolonged medication, which can lead to gastrointestinal tract irritation from repeated doses[81]. Therefore, 5-FU was selected as our model drug to showcase the BB system's effectiveness in cancer treatment.

1.8 Mechanism of Action of 5-FU

5-Fluorouracil (5-FU) is a pyrimidine analogue that can be mistakenly incorporated into RNA and DNA in place of uracil or thymine. This misincorporation disrupts the normal biosynthesis and function of nucleic acids, thereby interfering with essential cellular processes and leading to cell death.[82,83]

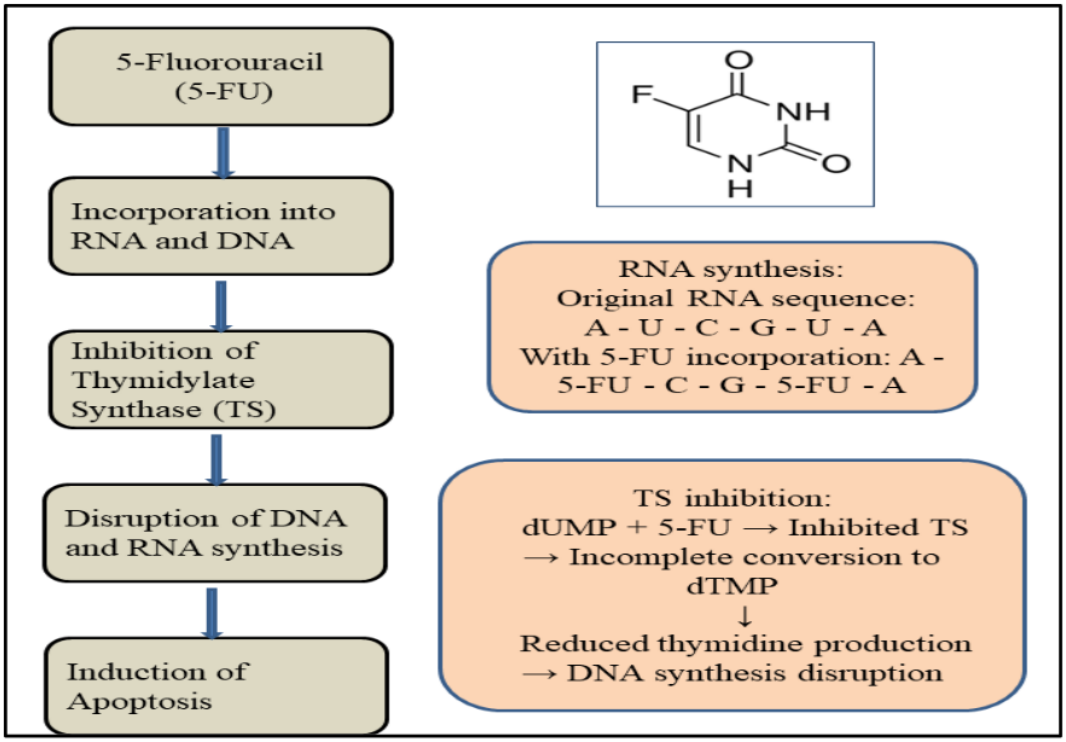


Figure 3: Flowchart showing the mechanism of the action of the 5-FU drug

- **5-Fluorouracil (5-FU)**
  Starts the process as the chemotherapy drug is administered to the patient.

- **Incorporation into RNA and DNA**
  5-FU is converted into active metabolites that are incorporated into both RNA and DNA during their synthesis.

- **Inhibition of Thymidylate Synthase**
  5-FU inhibits the enzyme thymidylate synthase (TS), which is crucial for the production of thymidine, an essential component of DNA.

- **Disruption of DNA and RNA synthesis**
  The inhibition of thymidylate synthase and incorporation of 5-FU into nucleic acids disrupt the normal synthesis of DNA and RNA, leading to errors and abnormalities in nucleic acid structure.

- **Induction of Apoptosis:** The disruption of nucleic acid synthesis, accumulation of aberrant nucleotides, and cellular stress responses trigger apoptosis pathways, leading to programmed cell death of cancer cells.

Chapter 2: Literature Review, hypothesis and objectives

Nanotechnology provides an unparalleled opportunity to improve medication delivery by designing and fabricating nanoscale carriers and devices. Nanoparticles, liposomes, and dendrimers can encapsulate medications, preserve them from degradation, and then deliver them to particular cells or tissues in the body [84]. Furthermore, nanomaterials may respond when exposed to external stimuli or physiological signals, allowing the controlled release of drugs at the intended site of action[7]. The UDDS uses nanotechnology to facilitate the precise and efficient delivery of medication while simultaneously minimising systemic toxicity and off-target effects.

2.1 Nanoparticles-based drug delivery system

The use of nanoparticles in drug delivery systems provides a dynamic method for administering drugs, utilising a variety of nanoparticles like polymeric and inorganic nanoparticles[26]. Because of their biocompatibility, biodegradability, and various chemical structures, natural polysaccharides, such as chitosan, alginate, hyaluronic acid, and cellulose derivatives, are being investigated for their potential use as drug delivery vehicles[85,86]. These polymers offer benefits like improved medication absorption, controlled-release formulations, targeted drug delivery, and improved patient compliance.

2.1.1 Chitosan nanoparticles-based drug delivery system

Chitosan nanoparticles offer versatile drug-loading strategies. Drugs can be encapsulated within the nanoparticle core through various techniques like ionic interactions or hydrophobic interactions [6]. Alternatively, drugs can be conjugated to the chitosan nanoparticles surface via covalent bonds[87]. The chosen method influences the release profile of the drug. By altering the composition or structure of chitosan nanoparticles, researchers can design sustained release systems for drugs requiring prolonged action or triggered release systems for targeted delivery upon specific stimuli. Chitosan nanoparticles have emerged as a potential platform for delivering anticancer drugs like doxorubicin. These nanocarriers provide substantial benefits over conventional drug delivery systems, as evidenced by numerous studies[88].

Chitosan nanoparticles significantly enhanced the solubility of doxorubicin, a chemotherapeutic agent, improving drug bioavailability and therapeutic efficacy [89,90]. Further, chitosan nanoparticles efficiently encapsulate doxorubicin, resulting in sustained drug release and reduced systemic toxicity[90]. The potential of these nanoparticles for targeted drug delivery has been extensively explored, successfully conjugating folic acid to chitosan nanoparticles and facilitating selective drug accumulation in folate receptor-overexpressing cancer cells[91]. Another research shows the role of chitosan nanoparticles in overcoming drug resistance by altering intracellular drug distribution and inhibiting drug efflux pumps[92]. In terms of clinical translation, studies have shown promising results.

Chitosan-insulin nano-formulations are evaluated for their sustained release and modulation of inflammatory responses, showcasing their potential to enhance burn wound healing through prolonged therapeutic effects [93]. This research examines degradable nano armour for probiotics, enabling sustained and targeted drug release to enhance inflammation-targeted delivery[94]. These outcomes

underscore the ability of chitosan nanoparticles to be a valuable tool in cancer therapy. Further research is needed to optimise nanoparticle formulations, explore different drug combinations, and conduct larger-scale clinical trials to fully realise the therapeutic benefits of this technology.

2.1.2 Polymeric nanoparticles-based drug delivery system

Polymeric nanoparticles (PNPs) have emerged as a pivotal component in drug delivery systems, primarily due to their ability to enhance drug bioavailability, protect drugs from degradation, and provide controlled release of drugs. These nanoparticles, typically 10 to 1000 nm, are made from biodegradable and biocompatible polymers such as poly lactic-co-glycolic acid (PLGA), chitosan, and polycaprolactone (PCL). Their versatility facilitates the encapsulation of various pharmaceutical compounds, thereby improving their solubility and stability[87,95].

Another research shows enhanced intracellular delivery of anticancer drugs using PNPs, significantly improving the therapeutic index[87]. Using PLGA nanoparticles in targeted drug delivery systems substantially reduces systemic adverse effects by facilitating the accurate distribution of therapeutic substances to designated locations inside the body[96]. These findings underscore the potential of PNPs in achieving precise and efficient drug delivery, especially for conditions requiring targeted therapy, such as cancer[96].

Advances in surface modification techniques have further enhanced the functionality of PNPs. By attaching targeting ligands like antibodies, peptides, or folic acid, PNPs can achieve higher specificity for diseased cells, minimising the impact on healthy tissues. This targeted delivery approach has been shown to improve the therapeutic outcomes of various drugs while reducing off-target effects[87,97].

Chitosan, a naturally derived polymer, has been extensively researched for its mucoadhesive properties, which enhance drug absorption across mucosal tissues[85]. Additionally, polymeric nanoparticles have the capability to cross biological barriers like the blood-brain barrier, presenting new possibilities for treating central nervous system disorders[98]. Polymeric nanoparticles offer auspicious solutions for drug delivery challenges by enhancing solubility, delivering controlled release, and enabling specifically targeted delivery. Ongoing research and development in this field are expected to further optimise these systems for a wide range of therapeutic applications, potentially transforming the drug delivery landscape.

2.1.3 Inorganic nanoparticles-based drug delivery system

Inorganic nanoparticles, like gold, have unique surface functions for drug conjugation or targeted ligand attachment, facilitating exact regulation of drug administration and dissemination. These systems provide flexibility and customisation for encapsulating, targeting, and releasing therapeutic agents, improving patient outcomes[99].

Inorganic nanoparticles have emerged as highly versatile tools with transformative potential spanning numerous fields, including health, environmental science, and electronics. Oxide and quantum dots offer distinct advantages for medical applications[99]. Inorganic nanoparticles (INPs) have garnered significant attention in drug delivery systems due to their unique properties, including high surface area, tunable size, and the potential for functionalisation with various therapeutic agents[100]. Key materials used for INPs include gold, silver, silica, and iron[101].

Gold nanoparticles (AuNPs) are marked for their biocompatibility and facile surface modification[101]. Extensively researched for cancer therapy, AuNPs enhance drug delivery and provide photothermal therapy[102]. Another research showed that AuNPs could be conjugated with chemotherapeutic drugs, targeting ligands, and imaging agents, creating a multifunctional platform for diagnosis and treatment[101].

Silver nanoparticles (AgNPs) are particularly noted for their antimicrobial properties[99,103]. They have been effectively used to deliver antibiotics and other antimicrobial agents. AgNPs can disrupt bacterial cell membranes and enhance the efficacy of traditional antibiotics, making them valuable in treating resistant infections. Another research has demonstrated the potential of AgNPs in healing diabetic wounds[104–106]. Their work emphasises the ability of the nanoparticles to facilitate wound recovery through antimicrobial activity to enhance cellular proliferation and migration in diabetic conditions[107,108]. AgNPs are versality deployed for drug delivery as well as biochemical analysis and serum analysis[109]. Anthelmintic properties of silver nanoparticles and solid lipid nanoparticles have been used to treat helminth infections orally[110,111].

Solid lipid Nano formulations are evaluated for their targeted drug delivery and oral drug delivery for the various drugs efficiently[112,113]. Solid lipid nanoformulations are deployed for the sustained release of Albendazole[111].

Silica nanoparticles offer high pharmaceutical loading capacity and regulated release characteristics. They can be synthesised with mesoporous structures, allowing for the encapsulation of a wide range of drugs[99].

Insulin–cobalt core-shell nanoparticles as an innovative solution for targeted bioimaging and diabetic wound healing. By incorporating cobalt ions with insulin, these nanoparticles enhance quantum yield, allowing for effective receptor-targeted imaging while also promoting wound healing. They demonstrate strong protein–metal interactions, high stability, and effective drug release, making them promising in bioimaging and therapeutic applications for managing diabetic wounds[114]. Synthesis of insulin–nickel fluorescent quantum clusters that specifically detect lead ions at very low concentrations both in vitro and in cell-free environments and facilitate wound healing, which can be tracked due to their bright fluorescence[115].

Quantum dots (QDs) are semiconductor nanoparticles with unique optical properties, making them useful for imaging and theranostic applications. QDs could be used to track the delivery and release of drugs in real-time, providing valuable insights into the drug delivery process and enabling simultaneous treatment and monitoring of diseases[116,117].

The development of insulin-protected copper quantum clusters synthesised for targeted bioimaging applications. These quantum clusters, stabilised within an insulin matrix, exhibit high fluorescence and enhanced stability, essential for specific receptor targeting. Quantum clusters with target specificity are effective for tissue-specific imaging applications[118]. The unique interaction between copper ions and insulin protein allows precise localisation that can be used for drug delivery and diagnostic imaging applications[119]. Another research used the insulin-infused bimetallic nano-subcluster composed of copper and silver, optimised for wound healing applications. These exhibit insulin-loading efficiency and controlled drug-release properties, making them well-suited for applications in sustained-release drug delivery. With their combined anti-inflammatory, antimicrobial, and cellular growth-promoting characteristics, these nanoparticles present a promising approach for managing diabetic wounds[108].

Carbon dots (CDs) have emerged as a versatile nanomaterial in drug delivery and wound healing due to their tunable surface functionalities, biocompatibility, and optical properties. Their nanoscale size allows CDs to penetrate biological barriers effectively, enhancing targeted drug delivery and retention at the treatment site and becoming a better alternative to quantum dots[120]. CDs can be engineered with surface modifications, such as functional groups or coatings, to facilitate drug loading, increase stability, and enable controlled release, which is particularly beneficial in therapeutic delivery systems. Additionally, CDs exhibit intrinsic photoluminescence, allowing real-time monitoring of the drug distribution and uptake, an advantageous feature for tracking therapeutic efficacy in vivo[121,122].
In wound healing, CDs demonstrate remarkable properties as bio-adhesive materials. The integration of CDs into sealants or nano-glues enhances adhesion-cohesion properties and promotes cellular regeneration. Recent studies have explored CDs in hybrid formulations with other polymers, such as gelatin or tannic acid, creating bio-adhesive matrices that support drug and growth factor delivery. Furthermore, CDs exhibit reactive oxygen species (ROS) modulation capabilities, enabling them to generate and scavenge ROS as needed, aiding in wound sterilisation and accelerating tissue repair. Consequently, CDs represent a promising platform for advanced drug delivery and wound healing applications, addressing drug release challenges and wound site recovery[123].
Nanosized sealants enable superior adhesion-cohesion properties for advanced wound healing beyond conventional adhesives[124]. Through controlled hydrothermal pyrolysis, dopamine, phloroglucinol, and glutaraldehyde are transformed into carbon dots with gelatine, creating an efficient nano glue with strong adhesion that supports insulin delivery and offers ROS modulation for wound healing, including diabetic models[123].
IONPs could be functionalised with therapeutic agents and directed to specific sites within the body using external magnetic fields, thereby enhancing the precision of drug delivery. INPs can be administered through various drug delivery routes, including oral, intravenous, intranasal, and transdermal methods[125]. For instance, AuNPs and SiNPs are often delivered intravenously to target tumours directly[126]. IONPs are typically administered intravenously for targeted drug delivery and imaging, while QDs are used in both intravenous and intranasal routes for imaging and tracking purposes[117,126].
In summary, inorganic nanoparticles provide a multifaceted and potent platform for pharmaceutical delivery, enabling targeted, controlled, and efficient treatment of various diseases. Ongoing research continues to optimise their properties and expand their applications, holding promise for future advancements in nanomedicine, but many nanoparticles exhibit cytotoxicity, which can cause adverse effects on human cells and tissues. For instance, some metal nanoparticles, such as silver and cadmium, have been associated with oxidative stress and inflammation. Comprehensive toxicological studies are needed to evaluate the long-term safety of these nanoparticles, particularly for biomedical applications[16].

2.1.4 Magnetic Nanoparticles

MNPs, often composed of iron oxide (Fe_3O_4 or γ-Fe_2O_3), can be coated with various biocompatible materials such as silica, polymers, or dextran to enhance stability and functionality in biological environments. This coating not only protects the core but also allows for the conjugation of therapeutic agents, targeting ligands, and imaging molecules[99]. Magnetic nanoparticles can be employed for magnetic drug targeting by delivering drugs to specific locations within the body using an external

magnetic field. By attaching therapeutic agents to the surface of magnetic nanoparticles, it is possible to guide these particles to targeted tissues or tumours, enhancing the precision of drug delivery. This method minimises off-target effects and enhances the therapeutic efficiency of the medication.
For instance, magnetic nanoparticles have been used in targeted delivery of anticancer drugs, with the magnetic field guiding the nanoparticles to tumour sites[127].
Magnetic nanoparticles can also be used in thermal therapy or magnetic hyperthermia, where they are heated by alternating magnetic fields to induce localised hyperthermia[128].
This research has been focused on the development and optimisation of MNP-based drug delivery systems. Their research illustrates the potential of these nanoparticles to be functionalised with targeting ligands to enhance their specificity and uptake by diseased cells. Additionally, their studies have explored the controlled release mechanisms, where the drug is released in response to specific stimuli such as pH changes, temperature, or magnetic fields[99].
A notable application of MNPs is in the treatment of cancer. MNPs can be loaded with chemotherapeutic drugs and directed to the tumour site, where an alternating magnetic field is applied to induce localised hyperthermia. This hyperthermia not only enhances the drug's effectiveness by increasing the permeability of the cancer cells but also induces direct cytotoxic effects on the tumour[128].
Lactoferrin-coated iron oxide nanoparticles (LF-IONPs) are investigated for their ability to target gastric cancer cells. These nanoparticles enhance cytotoxicity through hyperthermia, showing potential for controlled drug release in cancer treatment [128]. By integrating insulin with iron, the nanoparticles enable specific insulin receptor targeting while offering protection against reactive oxygen species[129]
But, the biocompatibility of magnetic nanoparticles is a significant concern. While iron oxide nanoparticles are generally considered to have lower toxicity than other nanoparticles, there are still risks associated with their use. Potential toxicity can arise from the accumulation of nanoparticles in organs, inflammation, and oxidative stress[130]. Long-term studies are needed to assess the chronic effects of magnetic nanoparticles on human health. However, Magnetic nanoparticles may trigger immune responses, leading to inflammation or allergic reactions[131]. The surface characteristics of nanoparticles, including coatings and functional groups, can influence their interaction with the immune system. Ensuring that nanoparticles are designed to minimise immune system activation is crucial for their safe application in medicine[131].

2.1.5 Drug Delivery through Liposomes

Drug delivery through liposome systems is a promising method in pharmaceutical technology for encapsulating and targeting therapeutic molecules. These spherical vesicles, made up of lipid bilayers, can contain various pharmacological compounds that include nucleic acids, proteins, peptides, and small molecules[132]. Liposomes represent a versatile and highly effective drug delivery system that improves solubility, stability, and targeted delivery. Their ability to encapsulate a wide range of therapeutic agents and release them in a controlled manner makes them a valuable tool in modern medicine, with ongoing research continually expanding their applications and optimising their performance for various therapeutic needs[6]. A notable application of liposomes is in the delivery of chemotherapeutic agents, as exemplified by liposomal doxorubicin (Doxil) and amphotericin B (Ambisome)[133,134]. These formulations have shown significant improvements in therapeutic index and reduced toxicity compared to their conventional counterparts. It is possible to modify the surface of

liposomes with ligands, such as peptides, antibodies, or aptamers, to target certain cells or tissues. This allows for increased accuracy while simultaneously reducing the likelihood of systemic adverse effects. It has been reported that the effectiveness of liposomes in gene therapy is leading to improved treatment outcomes[135].

Liposomes can also be engineered to release their contents in response to specific environmental stimuli, such as pH, temperature, or enzymatic activity. For instance, pH-sensitive liposomes release their drug cargo in the acidic microenvironment of tumours, ensuring higher local drug concentrations[136].

Apart from cancer treatment, liposomes have been explored for gene delivery. Cationic liposomes, which can encapsulate nucleic acids, protect them from degradation and facilitate their delivery into target cells. This application shows promise in treating genetic disorders and in cancer gene therapy[135,137].

Recent research has expanded the application of liposomes to antihelminthic therapy and demonstrated the potential of liposomal formulations in enhancing the efficacy of anthelminthic drugs. By encapsulating these drugs in liposomes, improved stability and targeted delivery to parasitic infection sites were achieved, leading to better therapeutic outcomes and reduced side effects. This research underscores the versatility of liposomes in addressing an extensive range of various medical conditions beyond traditional applications[111].

2.1.6 Microspheres

Microspheres, ranging from a few micrometres to a millimetre in diameter, are now recognised as versatile carriers in drug administration because of their capacity to encapsulate a diverse assortment of therapeutic agents with profiles of controlled release and targeted delivery capabilities. Recent advancements in microsphere technology have focused on improving fabrication methods, including emulsion-solvent evaporation, electrospinning, and spray drying, to achieve uniform size and enhanced drug stability[138]. Innovations in materials, including biodegradable polymers (e.g., PLA, PLGA), natural polymers (e.g., chitosan, alginate), and inorganic materials (e.g., silica, magnetic nanoparticles), have broadened their applications. Functionalisation strategies, such as targeted surface modifications and stimuli-responsive systems, have further enhanced their effectiveness[89,139,140]. Applications span various fields, including cancer therapy, vaccine delivery, and gene therapy. Despite their potential, scalability, regulatory compliance, and long-term stability must be addressed for successful clinical implementation[61].

2.1.7 Drug delivery through Micelles:

Micelle-based drug delivery systems are a potential technique for solubilising and delivering hydrophobic medicines, peptides, and nucleic acids. Micelles are self-assembled colloidal structures of amphiphilic molecules like surfactants and block copolymers that form spherical or rod-like structures in aqueous solutions[141]. The hydrophobic segments of amphiphilic molecules cluster in the micelle's centre, providing a hydrophobic environment for the encapsulation of weakly water-soluble medicines, while the hydrophilic segments form the outside shell, adding stability and biocompatibility to the micellar system[142].

Micelle-based drug delivery systems have the capacity to solubilise hydrophobic medicines at concentrations above their aqueous solubility limit, which improves medication bioavailability and therapeutic effectiveness. Moreover, medication delivery systems that are based on micelles have the ability to offer regulated and prolonged drug release[143]. The release rate of the encapsulated drug can be modulated by altering the composition and structure of the micelles, allowing for prolonged therapeutic effects and reduced dosing frequency. This controlled release mechanism has been shown to improve the therapeutic efficacy of various drugs, which developed thermosensitive micelles for the controlled release of chemotherapeutic agents[144].

Recent advancements in micelle-based drug delivery include the development of stimuli-responsive micelles that release their drug cargo in response to specific triggers such as pH, temperature, or enzymatic activity. For instance, pH-sensitive micelles have been designed to release their contents in the acidic environment of tumours, enhancing the localised delivery of anticancer drugs[145].

Researchers have used a new type of drug delivery vehicle, which is polymeric micelles[97]. Amphiphilic copolymers are the constituents of Liquid micelles[143,145]. Liquid micelles are nanostructured colloidal particles popular in delivering hydrophobic drugs to the body[97]. Recently, many micelle-based drugs got FDA approval and are used for delivering various drugs, including breast cancer drugs[145]. The micelle can be targeted just like a liposome to deliver drugs to the site of action by the conjugating antibody[141]. Despite many advantages, such as the pH-dependent controlled release of a drug molecule in the target tissue, the use of micelle has remained primarily limited to intravenous injection[34,142].

2.1.8 Virus-mediated drug delivery

Virus-mediated drug delivery leverages the unique ability of viruses to transfer genetic material into cells. By modifying viral vectors, researchers can design these systems to deliver therapeutic agents—including genes, proteins, and small molecules—with high specificity and efficiency[146]. Recent advancements have broadened the potential applications of viral vectors, addressed existing challenges, and explored novel strategies to enhance their efficacy and safety. Studies have focused on various types of viral vectors, each offering distinct advantages for drug delivery. Moreover, adding targeting ligands to the viral vector surface enables specific binding to target cells. For example, adenoviral vectors with ligands for selective delivery to cancer cells, improving therapeutic outcomes and reducing off-target effects. Modifying the viral genome to include therapeutic genes or proteins has been a common approach[146].

Recent advancements have focused on designing lentiviral vectors with inducible gene expression systems, allowing for the controlled release of therapeutic agents in response to specific stimuli[147,148].

Virus-mediated drug delivery has emerged as a transformative strategy in modern therapeutics, leveraging the inherent capabilities of viruses to introduce therapeutic agents into target cells. By harnessing the natural mechanisms of viral infection, researchers aim to achieve precise targeting and improved therapeutic outcomes[149].

In addition to gene delivery, virus-mediated systems are employed to deliver therapeutic proteins and drugs. For instance, oncolytic viruses, such as the vesicular stomatitis virus (VSV), engineered to express tumour necrosis factor-alpha (TNF-α), have been developed to selectively target and kill cancer cells. These engineered VSVs not only induce an anti-tumour immune response but also directly attack cancer cells, exemplifying a novel approach to cancer therapy[150].

The field has also seen significant advancements in developing viral vectors for delivering antibody-based drugs. For example, lentiviral vectors have been engineered to deliver genes encoding chimeric antigen receptor (CAR) proteins to T cells, which are used to treat various cancers. This technique, known as CAR-T cell therapy, has shown remarkable success in treating diseases such as acute lymphoblastic leukaemia (ALL) and large B-cell lymphoma[149,151].

Despite these advancements, several challenges remain. Immunogenicity is a significant concern, as the host immune system can initiate a response against viral vectors, potentially resulting in the rapid clearance of the therapeutic agent[135]. To address this issue, strategies such as incorporating immune evasion mechanisms or employing less immunogenic viral strains are being explored. Additionally, the production and scalability of viral vectors continue to present technical difficulties[152]. Innovations in biomanufacturing processes are essential to ensure the consistent and cost-effective production of these vectors for widespread clinical application. Looking ahead, the future of virus-mediated drug delivery holds exciting prospects. Personalised medicine approaches, which tailor viral vectors to individual patient profiles, could improve the specificity and efficacy of therapies. Integrating viral delivery systems with other targeted treatment modalities, such as chemotherapy or immunotherapy, may provide synergistic benefits in managing complex diseases. Additionally, advancements in targeting strategies, including the use of CRISPR/Cas9 technology to engineer viruses with enhanced targeting capabilities, are likely to expand the potential applications of this approach[153,154].

2.1.9 Genetically modified Bacteria-mediated drug delivery

Bacteria-mediated drug delivery uses genetically altered bacteria to deliver medicinal medicines, targeting specific tissues or cells and releasing therapeutic payloads on demand[155]. These bacteria can express complex biological agents and respond to environmental signals, allowing controlled drug release. However, challenges include precise control, safety, and immunogenicity concerns. Cationic nanoparticle-coated bacteria vectors for oral DNA vaccination in cancer immunotherapy improve infection efficiency by coating live attenuated bacteria with synthetic nanoparticles[66].

Genetically modified bacteria offer a cutting-edge approach to drug delivery by harnessing the natural capabilities of microorganisms and enhancing them through precise genetic modifications[70]. For instance, engineered *Salmonella typhimurium* has shown promise in targeting tumours, exploiting its natural tendency to accumulate in cancerous tissues to deliver cytotoxic agents directly where they are needed most. This approach has been exemplified by studies where *Salmonella* strains were modified to produce and release tumour necrosis factor-alpha (TNF-α), a potent anti-cancer agent, directly into the tumour microenvironment[66,156]. Another innovative application involves *Escherichia coli*, genetically altered to synthesise and secrete therapeutic proteins in response to specific environmental cues. This method was effectively used to address metabolic disorders like phenylketonuria (PKU), where the engineered bacteria produced essential enzymes to compensate for the genetic deficiency[157].

Recent advancements further illustrate the versatility of genetically modified bacteria. Engineered *E. coli* and other bacterial strains have been coated with nanoparticles to create oral DNA vaccines. These nanoparticle-coated bacteria can deliver genetic material directly to the mucosal surfaces of the gastrointestinal tract, providing a non-invasive method of vaccination that could revolutionise vaccine administration [158]. Additionally, *Lactococcus lactis* has been re-engineered to act as a vaccine delivery vehicle, showcasing its ability to produce antigenic proteins that can stimulate an immune response against diseases such as influenza[159].

Furthermore, targeted molecules, such as specific ligands or antibodies, can be engineered into bacterial surfaces to enhance their ability to bind to and penetrate specific tissues or cells. For example, bacterial strains can be modified to express ligands that specifically target cancer cells or inflamed tissues, thereby improving the precision and effectiveness of therapeutic delivery[7]. These advancements highlight the potential of genetically modified bacteria not only in targeted drug delivery but also in developing novel vaccination strategies and improving the specificity of therapeutic interventions[158].

Therapies in future will consist of engineered bacterial strains capable of diagnosing disease and producing and delivering Therapeutics[160]. Lactic acid bacteria (LAB) have a widely reported history of safe human consumption, including in food production, preservation, and probiotics. Some LAB species can survive through the gastrointestinal tract, playing a crucial role in the intestine. LABs have been developed as microbial cell factories for producing proteins and have been successfully used for treating various diseases like diabetes, cancer, and viral infections[161]. These LABs have a significant decrease in production costs due to their autonomous amplifying and production capabilities. Genetically modified bacteria create biocompatible isomeric microbeads for targeted delivery, containing drug and bacterial capsules, potentially targeting Salmonella typhimurium to avoid immune response in the human body[162]. The use of engineered oral bacteria for targeted drug delivery within the tumour microenvironment, emphasising controlled and sustained drug release for effective therapy[155]. Use of CRISPR-engineered bacteria for oral delivery of therapeutic genes[154]. Genetically modified yeasts for drug production have also been into the drug delivery systems[163]

2.1.10 Immunologically modified bacteria

A microbe-targeting magnetic micro-bead is a tool used to target and capture microbes or microbial matter, aiding in the diagnosis or treatment of infections. It consists of a carbohydrate recognition domain of mannose-binding lectin, a linker between the microbe surface-binding domain and the substrate-binding domain, and requires removal with a magnet or buffer solution. This method is sensitive to antigen antibodies and can be used through immunological modification of the bacteria[164].

2.1.11 Colloidosomes

Multi-stimuli Responsive Colloidosomes for Intelligent Transmembrane Transport focuses on developing multi-stimuli responsive colloidosomes, which can facilitate intelligent drug delivery and sustained release through responsive transmembrane transport mechanisms[90].

The recent development of nanotechnology has boomed in many aspects of science, including the pharmaceutical industry. A different formulation, such as solid-liquid emulsion, was also used by various researchers to improve the efficiency, solubility, and permeability of the system[61,165]. The nanostructured solid-liquid particles have many advantages, such as higher entrapment efficiency and controlled release of drugs over existing technology[166].

2.1.12 Market Available Sustain release formulations and target therapy

The work is carried out for more sustained or prolonged-release medications in the market[167]. These also make the drug more efficient by using targeted therapy, especially for tumours. For example, the

research discussed stabilising genetic structures to inhibit cancer growth, with implications for sustained drug release and targeted therapy[168]. There is also research on microbial therapy through colonic trans-endoscopic enteral tubing, which could offer sustained release and targeted delivery of therapeutic agents[169]. Sustained-release formulations and innovative Nano formulations facilitate the development of more efficient and customised therapies. Metformin Extended-Release (Glucophage XR) provides steady blood glucose control for type 2 diabetes, minimising the need for recurrent administration and gastrointestinal discomfort [38]. Similarly, Morphine Extended-Release offers prolonged pain relief, which is essential for managing chronic pain with less frequent administration[56]. In addition to these sustained-release options, nanoformulations are revolutionising the delivery of drugs using their capabilities to enhance bioavailability and target specific sites within the body. For example, nano vitamin D formulations utilise nanotechnology to improve the solubility and absorption of vitamin D, addressing deficiencies with higher efficacy and potentially allowing for lower dosages[142]. Nano-encapsulated drugs, such as Nano paclitaxel, offer targeted delivery to cancer cells, minimising side effects and enhancing therapeutic outcomes[170].

2.1.13 Limitations of existing formulations:

Various approaches have been devised to overcome the constraints associated with oral drug administration for low-permeability medications. Sustain-release formulations are used, but they extend the drug's presence in the GI tract and may enhance absorption by releasing it gradually over time[171]. Their ability to dramatically lengthen the half-life of drugs—the amount of time it takes for the concentration of the drug in the body to be cut in half—may be restricted, though[172]. In the harsh environment of the GI tract, liposomes, micelles, and other nanocarriers provide benefits in terms of enhanced drug stability and solubility[7,86,96,173]. It is still difficult to significantly prolong the medication half-life in the stomach and small intestine [6,7]. For low-permeability medications taken orally, there is a clear need for more efficient drug delivery techniques, even though current methods provide modest benefits. This is why they have a limited effect on Half-Life: they pose possible toxicity risks. Excessive dosages required to make up for inadequate bioavailability may raise the possibility of negative consequences on the body and the Environment. Medicines that are not absorbed can lead to antibiotic resistance and environmental damage that can occur due to the use of genetically modified microorganisms[174]. Researchers are currently investigating innovative drug delivery methods to overcome these restrictions and greatly enhance the oral delivery of low-permeability medications[175].

2.2 Research gaps:

From the above literature review, we have found the following Research gaps:

I. In spite of having sustained-release formulations using nanoparticles, they are not residing in the intestine for longer. They have a very short window for the drug delivery in the gut.

II. Bacteria like *Lactobacillus, Escherichia*, etc., live inside the gut. They are only used as genetically modified systems for drug delivery till now. There is no system to use them live as normal gut microflora for efficient oral drug delivery.

III. The complex nanoparticles and other formulations have not been stable over a long time period, and there is a need for a stable, sustainable oral drug delivery system that can be used universally.

Problem statement:

I. Short half-life in the GI tract and low permeability lead to the requirement of the fold of excess use of drugs through the oral route than the body requires

II. Excess drug causes unwanted toxicity

III. Bio-magnification

IV. Drug Resistance development and Economic loss

2.3 Proposed Hypothesis

Based on the below research questions, we proposed the hypothesis.

I. Drugs delivered through oral routes have relatively low efficiency of intestinal absorption because of a shorter half-life inside the gut. Therefore, can it be possible to establish a mechanism for the delivery of drugs that will increase the half-life of drugs inside the system and thereby allow increased intestinal absorption efficiency?

II. Bacteria like Lactobacillus, Escherichia, etc, live inside the gut. They anchor the intestinal surface by producing biofilms and propagate over there. Therefore, can it be possible to use natural gut microflora as a drug delivery tool through the oral route?

III. Because of inefficient absorption and short half-life in the intestine, many drugs need to be applied in excess through the GI tract, which causes undesirable toxicity. Hence, is it possible to reduce side effects using intestinal microbes-based drug delivery tools?

Based on the above research questions, we hypothesise that encapsulating intestinal microflora with mesoporous carbohydrate materials to develop BB will provide a more efficient and targeted approach for delivering low-permeability drugs, such as the anticancer drug 5-FU. We further hypothesise that utilising *Lactobacillus reuteri* bacteria as a carrier platform for drug delivery will lead to improved bioavailability and reduced toxicity compared to traditional formulations. Therefore, the efficacy of drug-loaded Bacterioboats will surpass that of existing 5-FU formulations, potentially addressing the challenges associated with oral administration of low-permeability drugs and offering a promising solution for enhanced patient therapeutic outcomes. A universal drug delivery system will utilise the natural ability of *L. reuteri* to adhere to the intestinal wall through biofilm formation [176,177]. Biofilm is a complex mixture of polysaccharides and proteins secreted by bacteria, allowing them to adhere to surfaces. This biofilm may help anchor the bacteria in the intestine for a longer duration, potentially increasing the drug's half-life and promoting sustained release of the therapeutic ag[63]. By enhancing drug targeting and potentially minimising high dosing requirements, our system will have the potential

to reduce the risk of side effects associated with conventional oral drug delivery. High doses often lead to a greater burden on the body's natural detoxification pathways, potentially causing adverse effects in various organs[81,178].

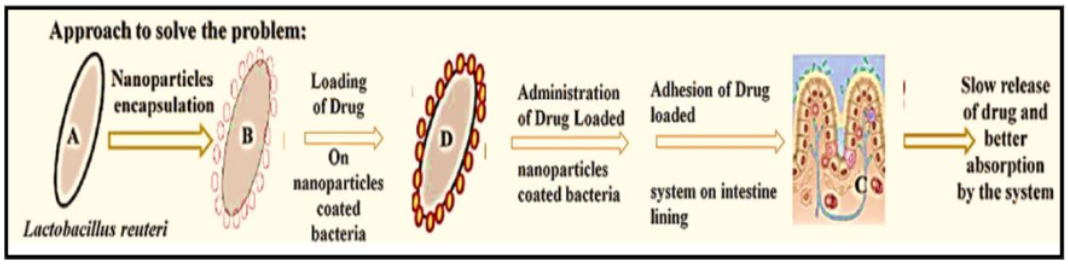


Figure 4: Schematic representation of the hypothesis to generate a universal drug delivery system

2.4 Research Objectives

The primary objective of this research was to develop and evaluate a novel drug delivery system, **Bacterioboat (BB)**, for enhanced oral delivery of low-permeability drugs. This study focused on utilising *L. reuteri* bacteria as a carrier platform for surface-encapsulated mesoporous nanoparticles loaded with the drug, as depicted in Figure 4. The research objectives include

I. Encapsulation of intestinal microflora with mesoporous carbohydrate materials to develop a novel microbial drug delivery system.

II. Loading and evaluation of Anticancer drug 5-FU in Bacterioboat.

III. Evaluation of drug-loaded Bacterioboat efficacy and comparison with existing 5-FU formulations.

2.5 Introduction of Bacterioboat (BB) System: A Novel Drug Delivery System and its advantages

This research introduces Bacterioboat (BB), a ground-breaking drug delivery system designed to address the challenges associated with the oral administration of low-permeability drugs. BB leverages the unique properties of *Lactobacillus reuteri* bacteria as a carrier distributi of drugs in a specific manner inside the GI tract[179]. *L. reuteri* is a Gram-positive bacterium classified as Generally Recognized as Safe (GRAS) by the U.S. Food and Drug Administration (FDA)[176]. This designation indicates its safety for human consumption.

2.5.1 Advantages of BB:

Bacterioboat offers several advantages over existing drug delivery systems for low-permeability drugs:

- **Targeted Delivery**

BB utilises *L. reuteri*, a **naturally occurring probiotic bacteria** in the human gut. The bacteria allows targeted drug delivery to the intestinal lining, potentially improving absorption compared to free drugs[180].

- **Enhanced Bioavailability**

BB employs **surface-encapsulated mesoporous nanoparticles** that efficiently carry drugs. These nanoparticles can potentially release the drug in a controlled manner, facilitating better absorption and maximising the therapeutic effect [1]

2.5.2 Proof-of-Concept Study with 5-Fluorouracil (5-FU)

To evaluate the potential of BB as a drug delivery system, a proof-of-concept study was conducted using 5-FU as a drug model. 5-FU is a chemotherapeutic agent commonly used to treat various cancers, but its oral bioavailability is limited due to low permeability[1]. This study aimed to investigate the efficacy of BB-encapsulated 5-FU compared to conventional oral administration of the drug. We compared the following aspects:

- Drug loading capacity and encapsulation efficiency of BB with 5-FU.
- In vitro release profile of 5-FU from BB under simulated digestive conditions.
- Ex-vivo studies (optional) to assess the interaction of BB with intestinal tissue.
- In vivo efficacy of BB-encapsulated 5-FU in a tumour-bearing mouse model. This evaluation included comparing tumour growth, drug potency with BB and existing formulations, and side effects with various treatment groups, including free 5-FU and unloaded BB.

2.5.3 Significance of the Research

The development of BB as a novel drug delivery system holds significant promise for improving the oral delivery of low-permeability drugs. Here's how this research can contribute to advancements in the field:

I. **Enhanced Therapeutic Efficacy:** BB can potentially improve the bioavailability and therapeutic effect of orally administered drugs, leading to better patient outcomes.

II. **Reduced Dosing Requirements:** By facilitating targeted delivery and sustained release, BB may allow for lower drug doses, potentially minimising side effects and improving patient compliance.

III. **Improved Patient Comfort:** Oral administration is generally preferred by patients due to its convenience and non-invasiveness. BB can potentially enhance the effectiveness of oral drug delivery, reducing the need for injectable or other less favourable routes of administration.

IV. **Broader Applicability:** BB can be potentially adapted for delivering various drugs with low oral bioavailability, offering a versatile platform for improved medication delivery.

2.6 Conclusion

This Literature review has provided an overview of the key challenges associated with the oral delivery of drugs that exhibit low permeability, a common issue in many therapeutic treatments. One of the primary difficulties with such drugs is their limited absorption in the gastrointestinal tract, which often leads to reduced bioavailability and, consequently, diminished therapeutic efficacy[117,181]. Existing drug delivery systems, though offering some solutions, have significant limitations in overcoming these barriers, especially for drugs that undergo rapid degradation or poor absorption in the gastrointestinal environment.

Furthermore, while various nanoparticles and genetically modified (GM) bacteria have emerged as promising alternatives for oral drug delivery, they also present challenges. Nanoparticles, despite their ability to enhance drug solubility and stability, can face issues related to inconsistent release profiles and potential toxicity. Additionally, the fabrication processes involved in developing nanoparticles may introduce complexities that complicate their clinical application. Similarly, using GM bacteria for drug delivery raises concerns regarding safety, regulatory hurdles, and the potential for unintended interactions within the gut microbiome, which may compromise therapeutic effectiveness[182].

In response to these challenges, this thesis introduces Bacterioboat (BB), a novel and innovative drug delivery system that utilises the probiotic bacterium *Lactobacillus reuteri*. This bacterial system is selected for its inherent advantages, including its ability to survive the harsh gastrointestinal environment and its potential to form biofilms, which enhance drug stability and localised delivery. The *Bacterioboat* system leverages these probiotic properties to serve as a protective carrier for oral drug delivery, specifically for low-permeability drugs. By incorporating *L. reuteri*, the system aims to improve drug bioavailability and enable more effective therapeutic outcomes compared to conventional delivery methods.

The research objective of this thesis is to demonstrate a proof-of-concept for this novel delivery system using 5-Fluorouracil (5-FU), a chemotherapeutic agent known for its poor oral bioavailability due to extensive first-pass metabolism and low permeability. By encapsulating 5-FU within *L. reuteri* in the *Bacterioboat* system, the aim is to explore whether this approach can improve the stability, absorption, and therapeutic efficacy of the drug when administered orally. This study holds significant potential for the broader application of probiotic-based drug delivery systems, particularly in overcoming the limitations associated with the oral administration of other low-permeability drugs.

The subsequent chapters of this thesis will delve into the detailed methodology employed to develop and evaluate the *Bacterioboat* system. A comprehensive analysis of the results from the proof-of-

concept study using 5-FU will also be presented, followed by an in-depth discussion of the implications of these findings. Ultimately, this research seeks to provide valuable insights into the viability of using probiotic bacteria, such as *L. reuteri*, as an effective drug delivery platform, addressing critical challenges in oral drug administration and paving the way for future innovations in this field.

Chapter 3: Materials and Methodology

This section details the specific methods and techniques used to develop and evaluate the Bacterioboat (BB) system. Here's a breakdown of the critical points you can elaborate on:

1.1 Materials

Chitosan, pepsin, potassium monophosphate monobasic, dialysis membrane, CuSO4, sulfuric acid, glacial acetic acid, and ethyl acetate were bought from HiMedia, India. Sodium tripolyphosphate, pancreatin, 5-FU, orthophosphoric acid, glacial acetic acid, trichloroacetic acid, and Rhodamine were purchased from Merck (previously Sigma-Aldrich) in the United States. Formic acid, acetonitrile, and p-hydroxydiphenyl were purchased from Loba Chemie. All the reagents used were of analytical grade. Dulbecco's modified Eagle's medium, 5% povidone-iodine solution, glutaraldehyde, ethanol, glacial acetic acid, and xylene purchased from Sigma-Aldrich (Merk).

1.2 Culturing and maintenance of *L. reuteri*

The *Lactobacillus reuteri* strain UBLRu87 was a generous gift provided by Unique Biotech Company. To initiate the bacterial culture, Lactobacillus reuteri was introduced into a one-times diluted MRS (de Man, Rogosa, and Sharpe) medium. The culture environment was carefully maintained at a pH of 6.5 and a temperature of 37 degrees Celsius, as illustrated in Fig. 5 A. These conditions are optimal for the growth of L. reuteri, facilitating robust metabolic activity and proliferation of bacteria shown in Fig 5B.

Following the initial growth phase, individual colonies were isolated on MRS agar plates, specifically designed to support the growth of lactic acid bacteria. A single colony was meticulously selected and subsequently inoculated into a fresh MRS medium, ensuring that the inoculation conditions remained consistent for 48 hours. Throughout this incubation period, the optical density (OD) of the culture was monitored at 600 nm using a spectrophotometer. This measurement provided quantitative data on the bacterial growth, allowing for the assessment of the culture's viability and density.

After the 48-hour incubation, a portion of the freshly cultured *L. reuteri* (0.5 millilitres) was combined with an equal volume of 40% glycerol within a cryogenic vial. The glycerol serves as a cryoprotectant, safeguarding the bacterial cells from damage during the freezing process. Following thorough mixing through gentle agitation and vortexing, the resulting mixture was subjected to deep freezing at -80 degrees Celsius. This procedure is critical for establishing a long-term bacterial stock, ensuring the preservation of *L. reuteri* culture viability for future experimental applications. By employing these methods, we can effectively maintain a stable culture of *L. reuteri*, essential for subsequent high-throughput screening and further microbiological analyses.

Figure 5: (A) MRS broth for the culture of bacteria (B) Cultured *L. reuteri*

3.3 Biofilm production

Effect of the biofilm production in the presence of different chitosan concentrations

Biofilm, a mixture of secretory protein and polysaccharide, infers intestinal anchorage ability to the bacteria; therefore, by altering biofilm production in the presence of chitosan, surface encapsulation was measured following the method described earlier with certain modifications [183].

The experiment aimed to examine the effect of chitosan on biofilm formation by Lactobacillus reuteri. Chitosan is known to possess mild antibacterial properties, which could potentially influence the growth and biofilm formation of bacteria. However, for *L. reuteri* to be effectively used in applications involving chitosan, it is crucial to demonstrate that chitosan does not adversely affect its ability to form biofilms.

Biofilm, a mixture of secretory protein and polysaccharide, confers intestinal anchorage ability to the bacteria. Therefore, by altering biofilm production in the presence of chitosan, surface encapsulation was measured following the method described earlier with certain modifications. To conduct this experiment, *L. reuteri* was grown in MRS (de Man, Rogosa, and Sharpe) media, specifically designed to support the growth of lactic acid bacteria.

In the experimental setup, 200 μl of MRS broth containing freshly prepared *L. reuteri* culture (OD 0.6) was seeded into each well of 96-well plates along with a medium blank. Different concentrations of chitosan (0.00, 0.02, 0.04, 0.05, 0.06, and 0.08 mg/ml) were added to each well and incubated statically for 24 and 48 hours at 37°C, pH 6.5. Following incubation, the supernatants were carefully discarded without disrupting the biofilm, and 10 μl of 0.5% crystal violet solution was added to each well and incubated for 10 minutes at room temperature. The excess crystal violet solution was removed by

washing it with distilled water twice. Subsequently, 200 µl of 100% ethanol was added to each well, and optical density (OD) was measured at 570 nm. The results were plotted as a percentage of biofilm production per unit weight of bacteria against the concentrations of chitosan.

The experiment aims to:

1. **Evaluate Compatibility:** Assess whether chitosan at various concentrations inhibits or supports biofilm formation by *L. reuteri*.

2. **Optimize Conditions:** Determine the optimal concentration of chitosan that allows *L. reuteri* to maintain or enhance its biofilm production, ensuring that the antibacterial properties of chitosan do not negatively impact the bacteria's biofilm-forming capabilities.

3. **Ensure Efficacy:** Confirm that chitosan can be used alongside *L. reuteri* without compromising its beneficial properties, which is crucial for applications where both chitosan's antimicrobial and the probiotic or functional properties of *L. reuteri* are desired.

The results from this experiment help validate that chitosan does not hinder the biofilm production of *L. reuteri*, thereby supporting its potential use in various formulations and applications where both biofilm formation and antimicrobial properties are beneficial.

3.4 Synthesis of Bacterioboat (BB)

Synthesis of BBs Mesoporous carbohydrate nanoparticles were synthesised on *L. reuteri* after harvesting from the medium and thoroughly washed. At first, we prepared 1% chitosan stock solution by dissolving crystalline chitosan in 85% o-phosphoric acid solution at pH 6.0 under constant stirring at 120 rpm for 2 h at 50°C temperature. Then, freshly grown and harvested *L. reuteri* cells were washed thrice using phosphate buffer (pH 7.0) to remove residual medium and other debris. Cleaned cells ($\sim 1 \times 10^8$ cells/ml) [optical density (OD) 2.0] were then suspended in 0.05% chitosan solution (pH 6) and incubated for 30 min at room temperature. After incubation, 1 mg/ml of STPP (40 ml for each 100 ml) as cross-linker was added to the solution dropwise with constant stirring at 60 rpm at room temperature. The mixture was stirred for 2 h to complete the reaction. After the response, the microbial cells were harvested and cleaned using phosphate-buffered saline (PBS) buffer at pH 7.0 (two times) before further processing. Processed and cleaned cells (BBs) were pelleted down by centrifugation at 1500 rpm and then were lyophilised for further use. The scheme of the synthesis of BBs is presented in Fig. 6 A and 6 B[184].

3.4.1 *Ex-situ* development of BB

For this work, 5-FU, an anticancerous drug, has been used as a standard drug to demonstrate the delivery system's efficiency. 5-FU has a half-life of 8 to 20 min in the GI tract [185]and is in medical use for treating various cancers like colorectal, breast, oesophagal, gastric, neuroendocrine, pancreatic, and ovarian through the I.V. route of drug administration. Two mutually independent strategies were taken to achieve drug loading, namely, in situ and ex-situ loading. For ex-situ drug loading, lyophilised BBs

were suspended and incubated in 5-FU (26 mg/ml) solution for 2 h at room temperature. The drug-loaded BBs were again lyophilised after two times washing with the physiological buffer or can be directly used for immediate use. Lyophilisation makes it store for a longer lapse of time, as shown in Fig 6A.

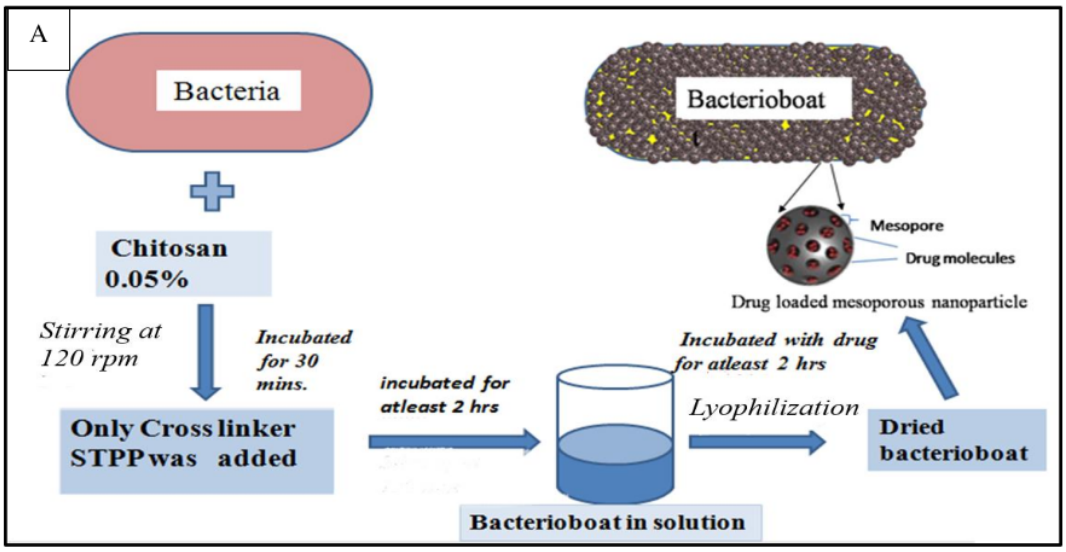


Figure 6(A): Bacterioboat synthesis ex-situ Schematic representation of the synthesis of Bacterioboat with in situ loading protocol for drugs of choice

3.4.2 In situ development of BB

For in situ loading, the saturated solution of the drug for 5-FU (26 mg/ml) along with STPP (1 mg/ml) was added dropwise to the chitosan pre-incubated *L. reuteri* (5×10^8 cells/ml), and the solution was kept under constant stirring (at 60 rpm) for 2 h to form drug-loaded BBs. After incubation, the drug-loaded BBs were harvested by spinning (1500 rpm), quickly washed twice with 1× PBS to remove excess materials, and lyophilised for storage for further use. The drug encapsulation efficiency (EE, %) of drugs that were entrapped into the nanoparticles was calculated using Eq. 1, and the drug loading capacity (LC), the ability of nanoparticles to in-trap drugs by nanocarrier dry weight, was determined by Eq. 2 as stated in Equations at 3.6.2.

In the two methods, the encapsulation efficiency of the loaded drug was different.

The entrapment of the drug was higher in the Ex-situ method of encapsulation of the drug in BB.

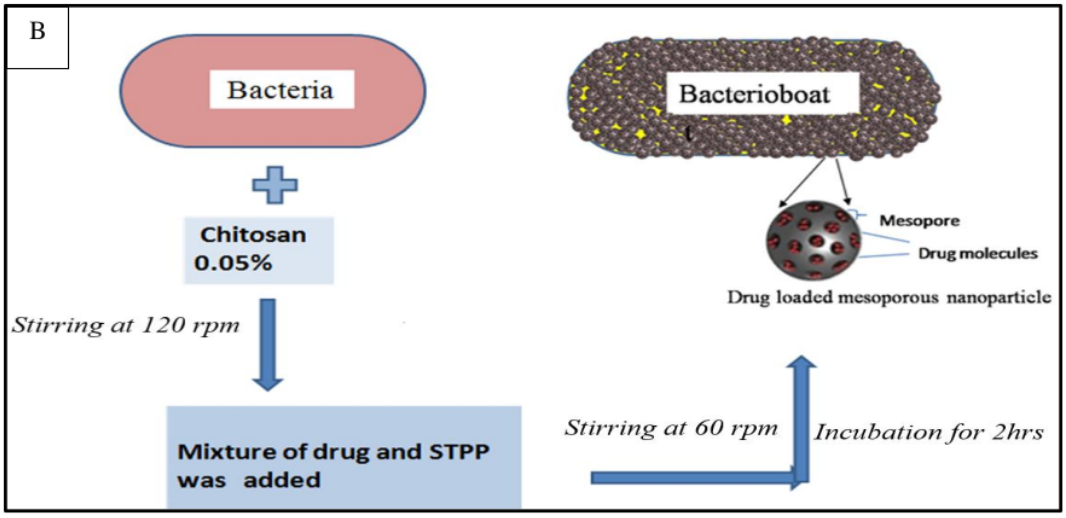


Figure 6(B): Bacterioboat synthesis in situ Schematic representation of the synthesis of Bacterioboat with in situ loading protocol for drugs of choice.

3.4.3 Preparation of SIF (simulated intestinal fluid and SGF (Simulated gastric fluid)

Simulated intestinal fluid is a synthetic fluid used to analyse pharmaceuticals in lab conditions. It tells us the extent to which the drug to be analysed is dissolved and released in the intestine. The formulation of SGF consisted of 0.03 M NaCl, 0.2 M HCl, and 3.2 g of pure porcine pepsin per litre of buffer at pH 2.0[186]. Additionally, two SIF preparations were created, one at pH 6.4 and the other at pH 7.4. Both had 0.05 M monobasic potassium phosphate, 0.015 M sodium hydroxide, and 1 g of purified porcine pancreatin (w/w) per litre buffer[88].

3.4.4 The production ability of biofilm by BB in SGF and SIF

Fresh *L. reuteri* culture was taken, and BBs were synthesised using the earlier protocol. BBs (1 × 106) were then seeded in 96-well plates in the presence of 300 µl of 1× MRS medium at SGF (pH 2.0) or SIF (pH 6.4 and 7.4) in the presence or absence of pepsin and pancreatin for SGF and SIF, respectively. After that, the plates were incubated for 24, 48, and 72 hours at 37°C in static conditions. After incubation, plates were processed as before, and calculation was done using OD at 570 as the percentage of biofilm production per unit weight of BBs[187]. Stability and structural integrity of BB in SGF and SIF To determine the structural stability of the mesoporous nanoparticles on the bacterial surface, FUBBs were incubated for 72 hours at 37°C with constant stirring at 60 rpm in SGF (pH 2.0) and SIF (pH 6.4 and pH 7.4) in the presence of pepsin (SGF) or pancreatin (SIF). Sampling was done at different time points (0, 12, 24, 48, and 72hour intervals) and was monitored under FE-SEM following the same protocol as before

3.5 Characterization of BB:

Characterisation of BBs Morphological characteristics of thus formed BBs were elicited using FE-SEM. Freshly prepared *L. reuteri* cells and BB samples (1×10^5 cells) were centrifuged at 5000 g for 10 min, and the pellet was then suspended in 100 mM PBS (1.0 ml) buffer (pH 7.0). The cells were then fixed using 3% glutaraldehyde solution in PBS for 3 h at 4 °C. After fixation, cells were harvested by centrifugation at 5000 g for 10 min, and thus, obtained pellets were washed with 50 mM buffer twice before re-suspending them in the same buffer (1.0 ml). Ten microliters of the suspension from each tube were placed separately on cleaned silicon wafers (grids) and incubated for 45 min at room temperature in the humidified chamber. The samples were dehydrated using 50, 70, 95, and 100% ethanol gradients.

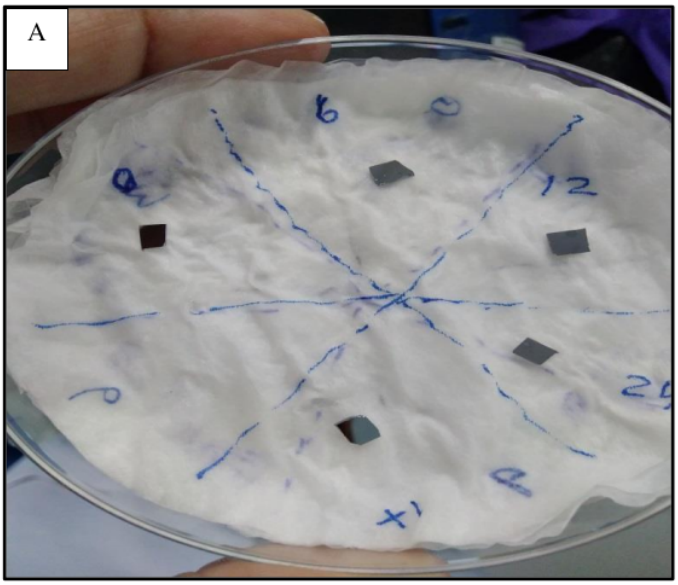


Figure 7(A): Silicon Grid Sample Silicon Grids carrying samples were placed for dehydration before gold coating for FE-SEM studies.

After dehydration, the sample grids were coated with 10-nm gold using electro-sputtering. Last, the size, shape, mean diameter, and pore diameter of nanoparticles and the size distribution and morphology of *L. reuteri* and BBs were analysed using SEM (Nova Nano SEM-450, FEI). The clear markation from the morphology was seen under the FE-SEM. The Comparison of the morphology shows the differences in the coating, showing the encapsulation of *L.reuteri* with the chitosan nanoparticles.

3.5.1 Metabolic characteristics of BBs in drug-unloaded and drug-loaded conditions

To study the effect of BBs on the lively activity of *L. reuteri*, further characterisations were done. Effect of BB formation in the bacterial growth curve to see the effect of surface encapsulation of *L.*

reuteri on the metabolic activity, bacterial growth curve analysis followed by the doubling time analysis was done for *L. reuteri*, EBBs, FUBB, and 5-FU + *L. reuteri*. Systems were incubated for 168 hours at 37°C at 120 rpm, and OD was collected from time to time at 600 nm. Initial inoculum for *L. reuteri*/BBs was taken (100 mg), and 5-FU (0.1 mg) was loaded on BBs or incubated with *L. reuteri*.

3.5.2 Effect of BB formation on lactic acid production

The name Lactobacillus is given because of the ability to produce secretory lactic acid by the genus of bacteria[188]. Therefore, lactic acid production is chosen as their signature metabolic activity parameter. It was studied to check any metabolic activity alteration from BB formation or drug loading. To culture, *L. reuteri* or BB whey was used as the medium. While boiling, whey was prepared from skimmed milk with glacial acetic acid (0.3 ml/litre). After cooling, whey was separated by centrifugation at 8000g for 5 min to remove the remaining proteins, and pH was adjusted to 6.0. An inoculum of L. reuteri, EBBs, and FUBB was respectively added to sterile whey medium (25 ml) and cultured at 37°C for 168 hours at 120 rpm. The inoculum included about 1.6 × 106 cells per ml. Samples were collected at various intervals and analysed using a modified version of Barker and Summerson's technique[189]. Subsequently, following the centrifugation of cells at 16,000g for 5 minutes, 100 μl of trichloroacetic acid was added per 1 ml of the clear supernatant and incubated on ice for 30 minutes. Then, the precipitate was removed by centrifugation at 16,000g for 5 min and the clear solution was diluted using a 2% (w/v) CuSO4 solution. After that, 1 g of Ca (OH)2 was added per 10 ml of the sample solution. In this condition, the mixture was incubated at 37°C for 30 minutes, followed by precipitation removal by centrifugation at 16,000g for 5 minutes. The sample was incubated in a boiling water bath for 5 minutes after adding 50 μl of 4% (w/v) CuSO4 and 6 ml of concentrated H2SO4 per millilitre of solution. Subsequent to cooling, 0.1 ml of p-hydroxy diphenyl (0.2 M, pH 10) was included per millilitre of solution A and incubated at 30°C for 30 minutes. The absorbance was measured at 570 nm.

3.6 Drug release study from BB

For this work, 5-FU, a pre-pro-drug of 5-FU, has been used as a standard drug to demonstrate the delivery system's efficiency. 5-FU has a half-life of 8 to 20 min in the GI tract, which is very small. Two mutually independent strategies were taken to achieve drug loading, namely, in situ and ex-situ loading. For in situ loading, the saturated solution of the drug for 5-FU (26 mg/ml) along with STPP (1 mg/ml) was added dropwise to the chitosan preincubated *L. reuteri* (5×10^8 cells/ml), and the solution was kept under constant stirring (at 60 rpm) for 2 hours to form drug-loaded BBs. After incubation, the drug-loaded BBs were harvested by spinning (1500 rpm), quickly washed twice with 1× PBS to remove excess materials, and lyophilised for storage for further use. For ex-situ drug loading, lyophilised BBs (5×10^8 cells/ ml) were suspended and incubated in 5-FU (26 mg/ml) solution for 2 hours at room temperature. The drug-loaded BBs were processed as before. The drug encapsulation efficiency (EE, %) of drugs that were entrapped into the nanoparticles was calculated using Eq. 1, and the drug loading capacity (LC), the ability of nanoparticles to in-trap drugs by nanocarrier dry weight, was determined by Eq. 2 [88,184]

3.6.1 *in vitro* drug release studies

Drug release study in vitro from drug-loaded surface-encapsulated nanoparticles. In vitro release experiments of the medication (5-FU) were conducted in simulated gastric fluid (SGF) and simulated intestinal fluid (SIF). The formulation of SGF consisted of 0.03 M NaCl, 0.2 M HCl, and 3.2 g of pure porcine pepsin per litre of buffer at pH 2.0[186]. Additionally, two SIF formulations were made, one at pH 6.4 and the other at pH 7.4. Both had 0.05 M monobasic potassium phosphate, 0.015 M sodium hydroxide, and 1 g of purified porcine pancreatin (w/w) per litre of the buffer[88]. Drug-loaded lyophilised BBs (200 mg) were suspended in 1 ml of SIF or SGF and loaded in dialysis bags (12 kDa cutoff), and the samples were placed in 10 ml of the same buffer as before and incubated at 37°C in a constant stirring condition at 60 rpm for 15 hours. The samples were collected at different time points until 15 hours in each interval. The data obtained were plotted and calculated against the standard curve made using known concentrations of drugs in the respective buffer following its characteristic ultraviolet-visible absorption peak at 241 nm[190] and were collected at different time points until 15 h in each interval. The data obtained were plotted and calculated against the standard curve made using known concentrations of drugs in the respective buffer following its characteristic ultraviolet-visible absorption peak at 241 nm[190] using a Spectrophotometer.

3.6.2 Equations

Encapsulation Efficiency and Loading Capacity

The drug encapsulation efficiency (EE, %) of drugs that were entrapped into the nanoparticles was calculated using Eq. 1, and the drug loading capacity (LC), the ability of nanoparticles to in-trap drugs by nanocarrier dry weight, was determined by Eq. 2[88,184]

EE%= [(Total drug − Free drug)/Total drug]*10 …….. Eq. 1

LC%= [(Total drug − Free drug)/Nano-carrier dry weight] *100 …….. Eq. 2

3.6.3 Data Analysis Using Statistical Tools

Statistical analysis

All data were analysed using Student's t-test and one-way and two-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. The Kaplan-Meier survival analysis and the log-rank test were conducted for survival analysis. Post-treatment survival benefits of tumour-bearing mice were obtained from Kaplan-Meier analysis of data on animal survival at the end of the study. The median survival values were listed as 15.5, 16.5, 19, 23.5, 21, and 25 for control, EBB, FU-25, FUBB-25, FU-50, and FUBB-50 groups, respectively (log-rank P = 0.0343, $\chi 2 = 12.04$).

3.7 *Ex vivo* studies

Animal experimentation A genetically homogeneous breed of Swiss albino mice, Mus musculus, with an initial weight of 30 ± 5 g, was raised and housed at the animal facility of the Chittaranjan National Cancer Institute (CNCI), Kolkata. The animal facility was maintained in a clean, sanitary, and disinfected condition in accordance with the requirements set out by the Animal Ethics Committee,

Government of India. The animals were kept at an ambient temperature of 24 ± 1°C under 12-hour light and 12-hour dark cycles. Mice were provided with standardised pelleted food and clean, uncontaminated drinking water ad libitum. The Institutional Animal Ethics Committee has sanctioned the protocol (no. IAEC-1774/BB-6/2020/6).

3.7.1 Adsorption on BBs on the GI tract of mouse ex vivo - FE-SEM study

Three healthy adult male Swiss albino mice were euthanised, and the small stomach and intestinal regions were collected. After that, the collected intestinal regions were washed thoroughly with sterile PBS to clean any faecal residuals, cut into small pieces, and incubated in 5% povidone-iodine solution for 15 min for disinfection. Samples of this intestine were used for the FESEM and the fluorescent study in 96 well plates and confocal microscopy using Rhodamine dye. The tissue was then prepared for the FESEM study. For FESEM, after thorough washing, the tissue section was fixed using 4% glutaraldehyde solution for 2 hours at 4°C. Then, dehydration of tissues was done using an ethanol gradient (50, 70, 75, 90, 95, and 100%) treatment, 20 min each. After gold coating, the samples were processed for FE-SEM, as mentioned above.

3.7.2 Adsorption on BBs on the GI tract of mouse ex vivo - Fluorescence study

Protocol:

In the study, three healthy adult male Swiss albino mice were euthanised, and their stomachs and intestinal regions were collected for analysis. The small intestinal sections were cut and rinsed with sterile PBS to remove residual faecal matter. Subsequently, the cleaned sections were immersed in a 5% povidone-iodine solution for 10 minutes to disinfect. After disinfection, the sections were cut open to expose the internal microvilli and then sliced into thin, approximately 5 mm by 5 mm pieces using a sterile blade. These tissue slices were placed in a 6-well plate containing DMEM media and incubated at 37°C for 3 hours. For control purposes, only the intestine and *L. reuteri* were used. Rhodamine B dye, a fluorescent marker, was employed to visualise the bacteria or bacterioboat on the intestinal surface. The tissue was washed thoroughly with sterile PBS and floated on DMEM with 10% serum in a 96-well plate. A 25 μl aliquot of each sample, including *L. reuteri* (1 × 10^7 cells/ml), bacterioboats (BBs), and BBs loaded with Rhodamine B dye (0.1 mg in 100 mg BB concentration), was applied to the intestinal tissue. After a 30-minute incubation, the tissue samples were washed extensively with sterile PBS—10 times—before being processed for confocal microscopy. The Rhodamine B dye was also incubated with the empty bacterioboat and *L. reuteri*, followed by ten washes to remove any unbound dye. The treated bacteria and bacterioboat were then incubated with the intestinal tissue for 30 minutes, and the slides were prepared using glycerol. These slides were examined under a confocal microscope using the TRITC channel to visualise the fluorescently labelled bacteria and bacterioboats. The images show the attachment of BB on the microvillar lining of the intestine of mice, as shown in Figure 8.

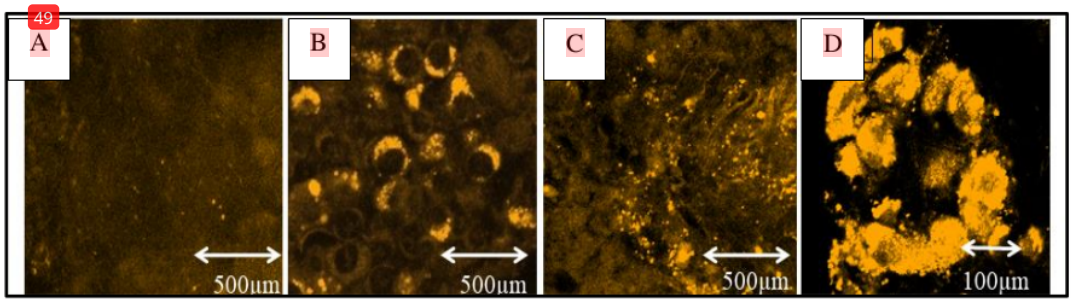


Figure 8: Anchorage of BB on the intestinal surface (*ex vivo*): Confocal images for a BB anchorage on the intestinal surface. (**A**) Rd treated mice intestine (**B**) Rd loaded Bacterioboats anchored around the microvillar regions of mice intestine (**C**) *L. reuteri* treated with Rhodamine on intestine (**D**) Bacterioboat treated with Rhodamine attachment on intestine (Magnified view)

Fluorescence analysis was done in 96-well fluorescence plates, one section in each suitable format, and data were collected after excitation at 485 nm[111].

3.7.3 Liquid chromatography-electrospray ionisation–mass spectrometry analysis for detecting 5-FU [M-H] + abundance from peak intensity

Sample preparation

The sample was prepared using the protocol described previously, where 100 µl of the murine plasma sample was mixed with 1 ml of ethyl acetate and 10 µl of glacial acetic acid[191]. The resultant mixture went through vortex for 10 minutes and then centrifuged for 5 minutes at 13,000g at room temperature. The supernatant was aliquoted into 1.5-ml Eppendorf tubes and evaporated to dryness using a SpeedVac vacuum concentrator (Thermo Fisher Scientific). After being desiccated, the residues were reconstituted in 100 μl of acetonitrile/water/formic acid (97:3:0.1, v/v/v), vortexed for 10 s, and subsequently transferred to autosampler containers. Vials were inserted into the autosampler, which was maintained at 4°C, and 10 μl of the contents of each vial was injected. The samples were prepared in independent replicates.

The liquid chromatography (LC) system (Xevo G2-XS QToF, Waters, Milford, MA, USA) consisted of an autosampler with a binary pump, a C18 BEH column 1.7 µm, 1 mm by 100 mm dimension, and an isocratic flow of isoamyl alcohol. The mobile phase solvents A and B comprised 0.1% (v/v) formic acid in water and 0.1% (v/v) formic acid in acetonitrile, respectively. The initial composition of the mobile phase consisted of 95% solvent A and 5% solvent B, delivered at a flow rate of 40 μl/min for a duration of 7 minutes. Between 7 and 8 minutes, solvent A decreased from 95 to 65%, and solvent B increased from 5 to 30%, with the same flow rate. From 8 to 10 min, solvent A was decreased to 15%, and solvent B was kept at 85%. The duration was 10 minutes, with a flow rate of 40 µl/min. Mass spectrometric (MS) detection was carried out using a Waters Xevo G2-XS QToF mass spectrometer connected to the ACQUITY UPLC H-Class System (Waters, Milford, MA, USA) via ZSpray dual orthogonal spray source. Electrospray ionisation (ESI) was operated in positive-ion, MS-MS (MSE) mode. The mass spectrometer settings were kept as follows: capillary voltage, 3 kV; cone voltage, 30 V; source temperature, 100°C; and desolvation temperature, 250°C. The cone and

desolvation gas flows were kept at 50 and 300 litres/hour, respectively. The ramp collision voltage ranged from 18 to 40 volts. The instrument was calibrated with sodium formate to achieve mass accuracies of <0.5 mDa. Internal calibration was performed with reference (lock mass), where leucine enkephalin was used. Its mass of 556.2771 was calibrated within 10-ppm (parts per million) error to ensure mass accuracies and reproducibility of the optimised MS conditions. The liquid chromatography apparatus and mass spectrometer were operated using Waters MassLynx software (version 4.0) and the 5-FU [M-H+] peak intensity at 131 (molecular weight of [M-H+]) was collected using the same software. The relative abundance of 5-FU [M-H+] was plotted as means ± SE at different time points concerning the untreated murine plasma, which served as a control.

3.7.4 qPCR analysis

Oral gavaging of vancomycin + neomycin + ampicillin + metronidazole + gentamicin at 200 µl of 1.0 g/liter each for 3 days was applied to deplete intestinal microbiota. One group of animals received BB by oral gavaging. Another group remains untreated. After 8 hours, intestinal tissue samples from both groups of animals were collected and homogenised for qPCR analysis. gDNA was extracted from the homogenised murine intestinal samples (control with antibiotic treated and antibiotic + BB treated) using the Takara NucleoSpin Tissue Kit, following the manufacturer's protocol. The concentration of the extracted gDNA was measured using a NanoDrop spectrophotometer. To check the presence of BB attached to the intestine wall, *L. reuteri* specific gene primers were designed using PREMIER Biosoft software and tested for efficacy by performing normal PCR. Quantitative PCR was used to calculate the relative abundance of *L. reuteri* . qPCR analysis was carried out with Takara TB Green Premix Ex Taq II (Tli RNaseH Plus) using the CFx96 Touch Real-Time PCR machine (Bio-Rad). The following program was performed: 3 min at 98°C, 40 cycles of 10 s at 95°C, and 15 s at 50°C. The CFX Maestro software automatically determined the Cq (quantification cycle) values after 40 cycles, followed by melt curve analysis. The Cq values obtained for *L. reuteri* –specific genes were normalised against the abundance of the 16S rDNA gene, which remains conserved for different lactobacillus species present in the murine intestine. The relative abundance of *L. reuteri* in the intestine was calculated using the Livak 2−ΔΔCT method, using 16S rDNA as the reference gene. For each gene, the analysis was carried out for at least three replicates. Primers used for qPCR analysis are as follows: *L. reuteri* specific, 5′-TGATGATGAAGTGCGGC-3′ (forward) and 5′-CGTCATTAAACAAGCTGAGAAT-3′ (reverse); Lactobacillus 16S rDNA, 5′-CGCCTGGGGAGTACGA-3′ (forward) and 5′-CTGGTAAGGTTCTTCGC-3′ (reverse).

3.7.5 Mouse model of tumour and treatments

S180 cells were injected into the peritoneal cavity of the mice and proliferated to produce ascites. The cell colonies were maintained by weekly transplantation of the tumour cells from the ascitic fluid into the peritoneal cavity of another mouse. S180 cells were isolated from the ascitic fluid and suspended in PBS. 36 Swiss albino mice aged 5 to 6 weeks were injected with 5 × 106 cells (in 100 µl of PBS) into the right leg flank. After 10 days, when the palpable tumour was observed, all mice were divided into six groups (six mice in each group) as follows: (i) vehicle control (saline-treated), (ii) EBB, (iii) FU-25, (iv) FUBB-25, (v) FU-50, and (vi) FUBB-50. The solution was centrifuged in two separate

vials at 1000 rpm for 2 to 3 min, and the supernatant was discarded. FUBB-25 and FUBB-50 solutions were prepared by resuspending 5-FU with the pellet to get the effective concentration of 25 mg/kg BW and 50 mg/kg BW, respectively, at the time of oral gavaging. For survival analysis, another 36 Swiss albino mice (6 mice in each group) were injected with 5×10^6 cells (in 100 µl of PBS) into the peritoneal cavity for ascites tumour. All groups of animals (both solid and ascites tumours) were orally administrated with drugs on days 1, 4, 7, and 10 and observed for 30 days.

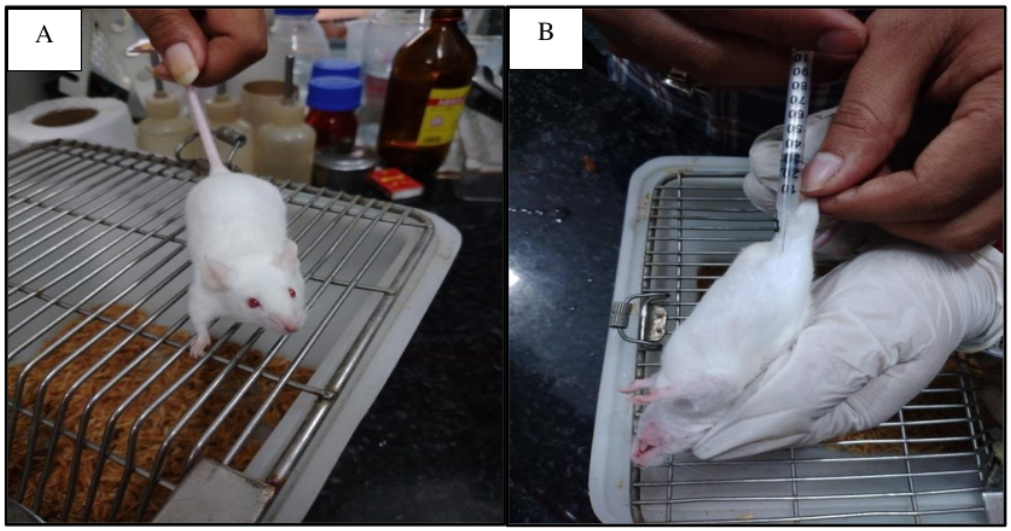


Figure 9: **Swiss albino mice for the in vivo studies** (A) Healthy Swiss albino mice (B)Injecting S180 cells in the peritoneal cavity of healthy mice

3.7.6 In vivo Tumour Regression and Survivability Study

Tumour volumes were measured for 29 days from the initiation of treatment. The tumours were measured using a Vernier calliper over three days to calculate the minimum diameter (A) and the maximum diameter (B). The volume was computed using V = (A2 B)/2. Data were analysed for statistical significance using Student's t-test. A P value of < 0.05 was deemed statistically significant. Mice were euthanised after 29 days post-tumour transplantation and tumours and organs were harvested for histological examination. The relative tumour volume (RTV) on day n was computed using the formula RTV = TVn/TV0, where TVn represents the tumour volume on day n, and TV0 denotes the tumour volume on day 0. The following formula calculated tumour growth inhibition rate (TIR): TIR = {(1 − (mean volume of treated tumours)/mean volume of control tumours)} × 100%[48]. Kaplan-Meier survival analysis was used to calculate the statistical differences in survival between different groups of tumour-bearing mice.

3.8 Histopathological analysis

All the specimens (tumour, liver, and kidney tissues) were washed carefully in PBS and fixed in 10% neutral buffered formalin within 30 min after resection. After 24 hours, tissues were immersed in

increasing alcohol concentration, i.e., alcohol gradation as 50, 70, 90, and 100% ethanol for 30 min each. Then, the specimens are placed into xylene for 15 to 20 min to displace the alcohol from the tissue. Tissues were placed into liquid wax (65°C) for 4 hours in the oven to maintain a constant temperature. Tissue blocks were prepared, and 5-µm sections were cut and placed onto poly-lysin-coated slides. For staining, slides containing sections were placed in xylene to remove wax. Slides were then placed into decreasing alcohol concentration (100, 90, 70, and 50% ethanol) for 10 min each. Then, a haematoxylin stain was applied for 3 minutes. Slides were placed in 70 and 90% ethanol for 5 min each. After that, the eosin stain was applied for 1 min. Slides were then passed through increasing alcohol concentration (90 and 100% ethanol). Slides were then kept in xylene for 10 minutes, mounted with DPX mountant (a mixture of distyrene, a plasticiser, and xylene), and observed in different magnifications under a bright-field microscope. Evaluation of hepatic and renal biochemical parameters to understand the effect on hepatic and renal biochemical parameters, if any, by BB alone or combined with different 5-FU doses, blood from all groups of animals was collected on the 29th day. Blood was placed in a centrifuge tube for 30 minutes, then centrifuged at 3000 rpm for 10 minutes, and serum was collected. Then, cholesterol, triglycerides, SGOT, and SGPT were estimated from the serum sample using the fully automatic analyser (Cobas Integra 400, Roche) method. To estimate total cholesterol, the analyser measured the formation of red quinone imine dye at 540/600 nm. A decrease in absorbance at 340 nm was measured for SGOT and SGPT levels in the blood[192]. For serum albumin analysis, the bromocresol green method was adopted. The automatic analyser also measured serum bilirubin, globulin, creatinine, and alkaline phosphatase.

3.8.1 Measurement of host immune response against BB-mediated drug delivery

To investigate the immune response associated with BB-mediated drug delivery, this study focused on measuring blood cell counts and the expression levels of the cytokines IL-6 and IL-10 in mice after a two-week treatment period. Eighteen Swiss albino mice were utilised, divided into three groups: two treatment groups (FU-50 and FUBB-50) and one control group, with six mice in each category. The treatment involved administering 5-fluorouracil (5-FU) at 50 mg/kg body weight. The FU-50 group received 5-FU alone, while the FUBB-50 group received the same dosage delivered via the BB carrier. The drug was administered on the 1st, 4th, 7th, and 10th days of the treatment regimen. On the 15th day, blood samples were collected from all mice using EDTA-coated vials, essential for preserving the samples and preventing coagulation. The quantification of haematological parameters—including erythrocyte, leukocyte, and platelet counts—was performed using an automated blood analyser, which provides precise measurements of these blood components. Additionally, to evaluate the serum levels of the cytokines IL-6 and IL-10, enzyme-linked immunosorbent assay (ELISA) kits specifically designed for mice were utilised, sourced from Invitrogen and R&D Systems. This comprehensive analysis aims to elucidate the effects of BB-mediated drug delivery on immune modulation, thereby contributing valuable insights into the interactions between drug delivery systems and immune responses in the context of cancer therapy. The findings from this study are anticipated to enhance our understanding of how novel drug carriers like BB may influence treatment outcomes and immune activity during cancer treatment.

4.1 Development and Characterisation of Bacterioboat (BB)

Synthesis and ultramicroscopic study of BBs A schematic illustration of the synthesis procedure of BB is depicted in Fig. 6A and 6B. BB drug loading can be achieved using both in-situ and ex-situ methods. BB was prepared using chitosan by surface encapsulation with mesoporous nanoparticles on *L. reuteri*. Field-emission scanning electron microscopy (FE-SEM) studies showed smooth cell surface organisation of freshly cultured control *L. reuteri* Fig.10A, whereas surface encapsulation of the bacteria with nanoparticles was observed in BBs Fig 10B.

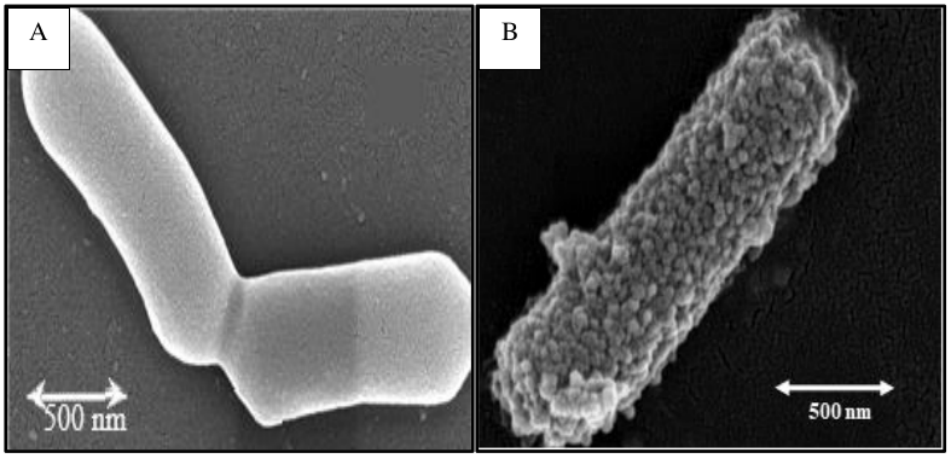


Figure 10: BB morphology (A) FE-SEM image of *L. reuteri* cells. (B) FE-SEM image of freshly prepared BB showing chitosan nanoparticles on the cell surface.

4.2 Effect of BB formation on the growth of *L. reuteri*

Growth curve analyses were done to understand the effect of surface encapsulation on the cellular metabolism of *L. reuteri*. At the initial stage, a phase delay (elongation of lag phase) was prominent until 9 hours in BB, whereas in control, it was between 3 and 6 hours. However, after 48 hours, a complete retrieval of the growth of BB was established. Effect of BB formation on lactic acid metabolism No significant changes in lactic acid production were observed compared to control *L. reuteri* upon surface encapsulation with mesoporous carbohydrate nanoparticles.

4.3 Preliminary growth test -Culture BB and *L. reuteri* on MRS agar plates

Fig 11A shows the BB inoculum colonies, and Fig 11B shows the colonies of *L. reuteri* after streaking with respective inoculums for 24 hours of incubation. The plates show no effect of chitosan nanoparticle coating on the bacteria in the culture plates containing BB and *Lactobacillus reuteri,*

suggesting that the specific formulation and concentration of the nanoparticles used do not exert significant antimicrobial activity against *L. reuteri*. This could be due to the inherent resilience of *L. reuteri*, known for its robustness and ability to thrive in various conditions, including those containing antimicrobial agents. Additionally, chitosan's antimicrobial effectiveness can be influenced by its concentration, molecular weight, and degree of deacetylation. In this experiment, the conditions may not have favoured the antimicrobial action of the chitosan nanoparticles.

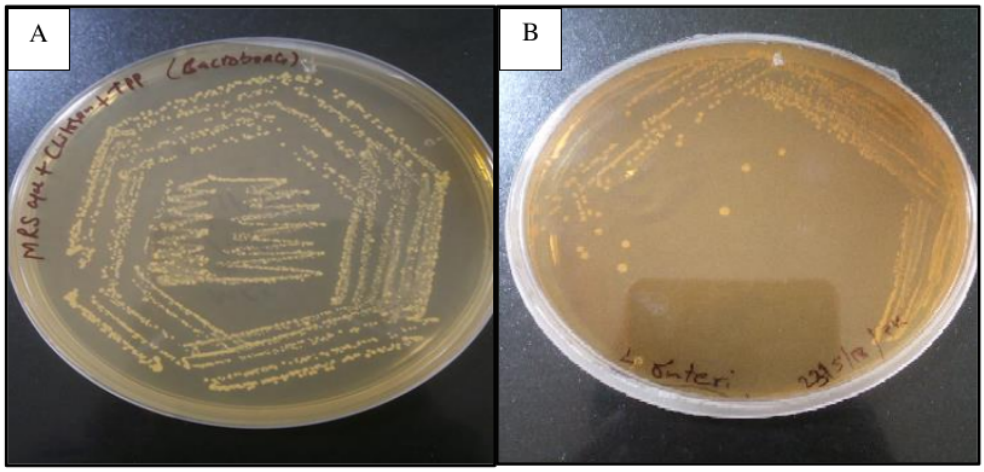


Figure 11: MRS agar plates for the preliminary Growth Test for the EBB:(**A**) Culture plate containing chitosan-coated BB, demonstrating no significant impact on bacterial growth, indicating that the chitosan coating does not adversely affect the bacteria. (**B**) Culture plate with only *Lactobacillus reuteri*, showing normal bacterial growth, serving as a control to compare the effect of chitosan-coated BB.

4.4 The surface potential of BBs

L. reuteri develops negative potential at low pH, and chitosan develops a positive one; therefore, at low pH, chitosan will interact with the bacterial cell wall, and applying STPP as a crosslinker can improve the stabilisation of the system. Therefore, the zeta potential of the bacteria before and after the formation of BBs was determined using Zetasizer (Nano Series Nano-ZS, model ZEN 3600, Malvern Instruments Limited). A zeta potential of −30.3 ± 3.57 mV of *L. reuteri* was turned into +5.39 ± 0.54 due to the formation of EBBs. The zeta potential of 5-FU–loaded (ex-situ) BBs further changed into +17.62 ± 0.59, −0.74 ± 0.63, and − 5.8 ± 1.2 for pH 2.0, 6.4, and 7.4, respectively.

4.5 Drug Binding Constant Study using Isothermal Calorimetry

In an isothermal calorimetry study to evaluate the binding characteristics of 5-Fluorouracil (5-FU) with a chitosan-based bacterioboat, the interaction between the drug and carrier was investigated to understand the binding affinity and release kinetics. The experiment was conducted at 25°C to determine the binding constant of 5-FU with the chitosan bacterioboat. The isothermal titration

calorimetry (ITC) results indicated a strong binding affinity, with an enthalpy change (▲H) of -581.7 cal/mol, suggesting a highly exothermic interaction. The calculated Gibbs free energy (▲G) of -194.2 cal/mol further confirmed the spontaneity of the binding process, reinforcing that the interaction between 5-FU and the bacterioboat is thermodynamically favourable. This efficient binding ensures a controlled and sustained delivery of 5-FU from the bacterioboat, establishing an ideal system for targeted and sustained medication administration, as shown in Figure 12.

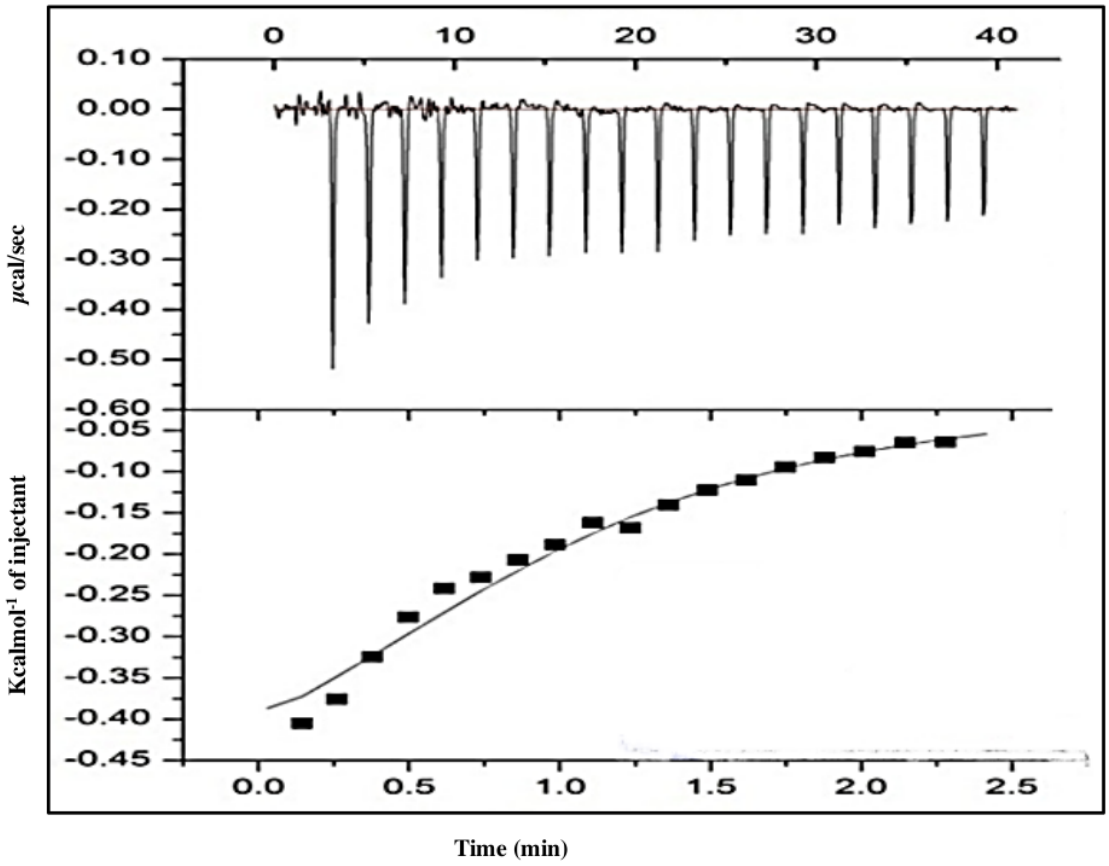


Figure 12: Isothermal Calorimetry (ITC) Analysis of 5-Fluorouracil (5-FU) Binding to Chitosan Bacterioboats. ITC binding curve showing the heat change (ΔH) as a function of the molar ratio of 5-FU to chitosan bacterioboats, demonstrating the exothermic nature of the interaction. The graph provides insight into the binding affinity and interaction kinetics between the drug and carrier.

4.6 Metabolic Characteristics of BB through FE-SEM study of morphology (surface and biofilm production)

The biofilm formation of *L. reuteri* was analysed at different pH levels in MRS (De Man, Rogosa, and Sharpe agar) medium only and with enzymes present in the simulated gastric fluid (SGF) and simulated intestinal fluid (SIF). FE-SEM images show the morphological comparison of the structure of *L.*

reuteri and BB. The freshly cultured *L.reuter* in a log phase of growth showed no biofilm formation Fig. 13A, whereas 48-hour aged *L. reuteri* showed the production of biofilm Fig. 13B. The coating of mesoporous nanoparticles on the freshly cultured *L. reuteri* showed no biofilm formation Fig. 13C. However, 48-hour ageing of BB showed the formation of biofilm: BB with biofilm at pH 2.0, 6.4, and 7.4 at 48 hours after encapsulation ageing Fig. 13, D to F

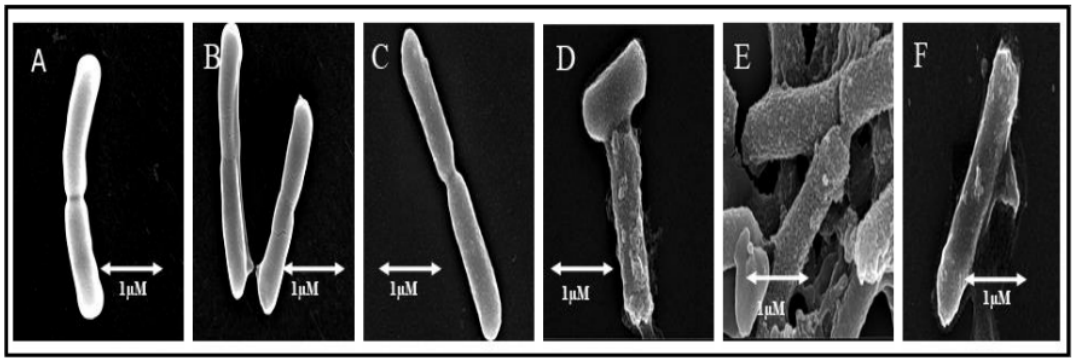


Figure 13: Stability of BB with Time(A) freshly cultured *L. reuteri* in log phase, (B) *L. reuteri* in 48-h outraged culture (stationary phase), (C) freshly prepared BBs, (D) 48-h SGF (pH 2.0) incubated BB, (E) 48-h SIF (pH 6.4) incubated BB, and (F) 48-h SIF (pH 7.4) incubated BB.

4.7 Comparison of the metabolic activity of *L. reuteri* Bacteria and BB

4.7.1 Effect of BB formation on the growth of *L. reuteri*

To understand the effect of surface encapsulation on the cellular metabolism of *L. reuteri*, growth curve analyses were done. At the initial stage, a phase delay (elongation of lag phase) was prominent until 9 hours in BB, whereas in control, it was between 3 and 6 hours. However, after 48 hours, a complete retrieval of the growth of BB was established in Fig. 14A.

4.7.2 Effect of BB Formation on Lactic Acid Metabolism

No significant changes in lactic acid production were observed compared to control *L. reuteri* upon surface encapsulation with mesoporous carbohydrate nanoparticles Fig. 14B.

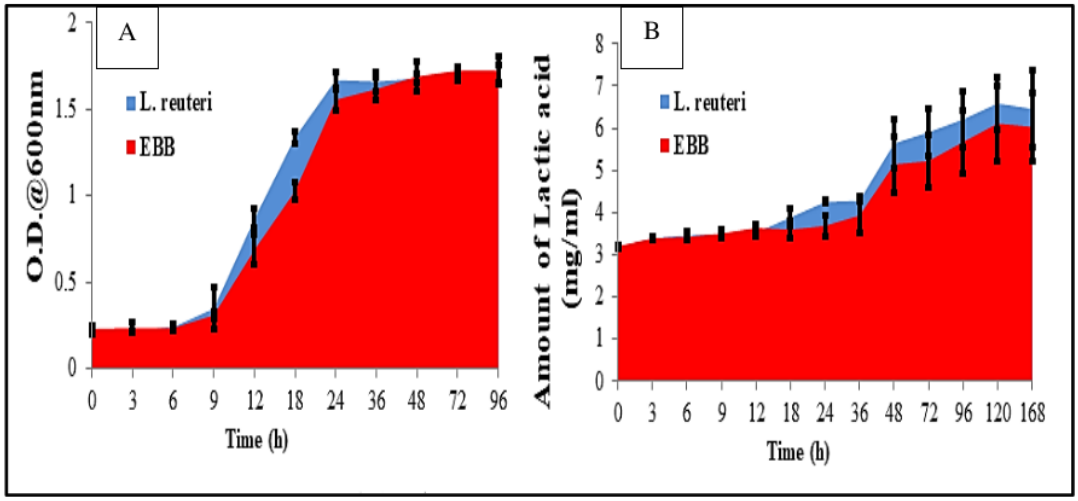


Figure 14: Comparison of the metabolic activity of *L. reuteri* Bacteria and BB
(A) Comparative cellular growth of *L. reuteri* (blue) and EBB (red). Because of cell wall encapsulation with mesoporous particles, the lag phase was extended. (B) Comparative lactic acid production

4.8 Metabolic characteristic of BB

4.8.1 Biofilm producibility at different chitosan concentrations

Biofilm, a mixture of secretory protein and polysaccharide, infers intestinal anchorage ability to the bacteria; therefore, by altering biofilm production in the presence of chitosan, surface encapsulation was measured following the method described earlier with certain modifications [183].
The study analysed the biofilm formation of *L. reuteri* in an MRS medium in different chitosan concentrations to see the effect of chitosan concentration on the biofilm formation. The study examined the influence of different chitosan concentrations as 0.02,0.04,0.05,0.06,0.08 mg/ml on biofilm production for 72 hrs[183,187]. It was shown that no significant effect was shown up to the range of 0.04 to 0.08 mg/ml in Fig 15A.

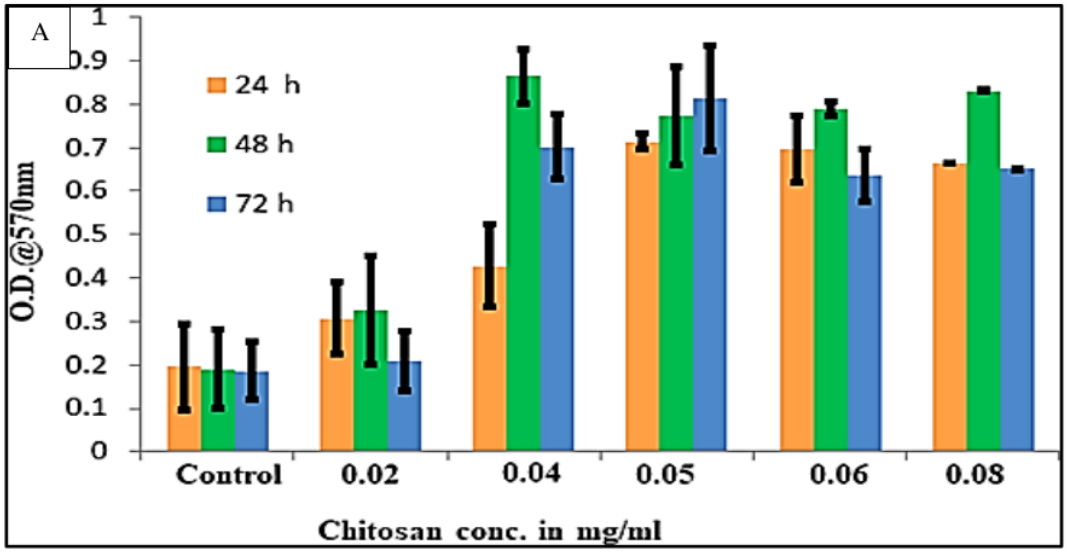


Figure 15A: Biofilm production at different chitosan concentrations: *L. reuteri* was treated with various concentrations of chitosan (0 to 0.8 mg/ml) for up to 72 h. And the production of the efficiency of biofilm by the cells was determined, as mentioned before. Orange, Green, and blue represent 24, 48, and 72 h biofilm production.

Analysis of Biofilm Production at Varying Chitosan Concentrations: Statistical Significance using Dunnett's Multiple comparisons was done as shown in Table -1

4.8.2 Table-1 Dunnett's Multiple Comparison Test for the significance Biofilm with different chitosan concentrations

Dunnett's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant	Summary	Adjusted P Value
24 hrs					
0.0 vs. 0.02	-0.111	-0.2243 to 0.002369	No	ns	0.0571
0.0 vs. 0.04	-0.232	-0.3453 to -0.1186	Yes	****	<0.0001
0.0 vs.0.05	-0.5191	-0.6325 to -0.4058	Yes	****	<0.0001
0.0 vs. 0.06	-0.5001	-0.6135 to -0.3868	Yes	****	<0.0001
0.0 vs. 0.08	-0.4697	-0.5830 to -0.3563	Yes	****	<0.0001
48 hrs					
0.0 vs. 0.02	-0.1363	-0.2496 to -0	Yes	*	0.0123
0.0 vs. 0.04	-0.6739	-(Molar Ratio)6	Yes	****	<0.0001
0.0 vs.0.05	-0.5818	-0.6951 to -0.4685	Yes	****	<0.0001
0.0 vs. 0.06	-0.6003	-0.7136 to -0.4869	Yes	****	<0.0001

0.0 vs. 0.08	-0.641	-0.7543 to -0.5276	Yes	****	<0.0001
72 hrs					
0.0 vs. 0.02	-0.02293	-0.1363 to 0.09041	No	ns	0.9801
0.0 vs. 0.04	-0.5167	-0.6300 to -0.4033	Yes	****	<0.0001
0.0 vs.0.05	-0.6272	-0.7406 to -0.5139	Yes	****	<0.0001
0.0 vs. 0.06	-0.4497	-0.5630 to -0.3364	Yes	****	<0.0001
0.0 vs. 0.08	-0.4654	-0.5787 to -0.3520	Yes	****	<0.0001

4.8.3 Biofilm producibility of BBs at different physiological pH.

The biofilm formation of *L. reuteri* was analysed at different pH levels in MRS (De Man, Rogosa, and Sharpe agar) medium only and with enzymes present in the simulated gastric fluid (SGF) and simulated intestinal fluid (SIF). The increase in the biofilm formation in all the samples containing *L. reuteri* and BB with increasing time intervals is shown.

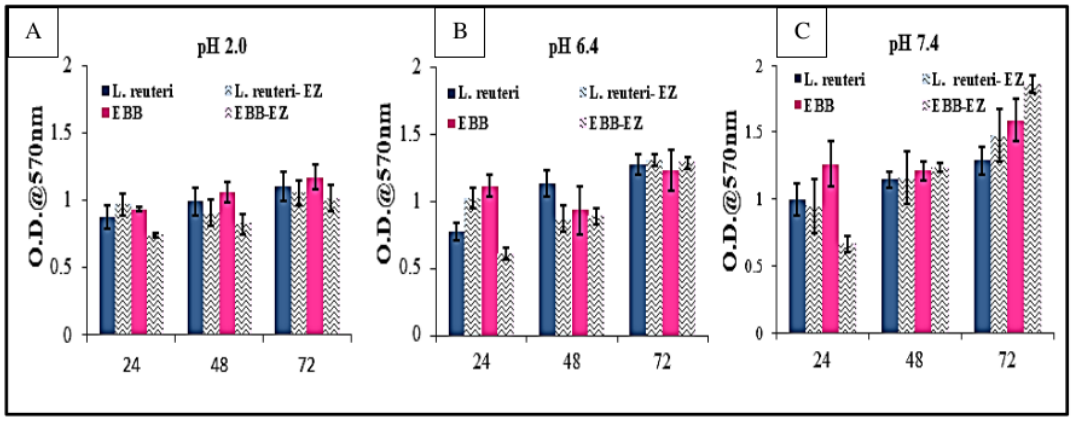


Figure 16: Biofilm producibility of BBs at different pH conditions (A) Comparative time depending on the study of biofilm production at pH 2.0 by BB and L. reuteri in SGF and SIF in the presence or absence of digestive enzymes. (B) Comparative time depends on the study of biofilm production at pH 6.4 by L. reuteri and BB in SGF and SIF in the presence or absence of digestive enzymes. (C) Comparative time depends on a study of biofilm production at pH 7.4 by L. reuteri and BB in SGF and SIF in the presence or absence of digestive enzymes.

The biofilm production by *Lactobacillus reuteri* and BB under various conditions is shown in Fig. 16. It has been found that in the absence of a digestive enzyme, biofilm production gradually increases up to 72 hours. However, in the presence of a digestive enzyme (especially pancreatin), biofilm was reduced after the initial increase at 24 hours. This may have happened because of the amylase activity of the enzymes in SIF**.** Across all panels, biofilm production was observed to increase over time in both the presence and absence of enzymes, demonstrating that *L. reuteri* and BB can form biofilms under a range of pH conditions and that enzymatic presence does not inhibit this process.

4.8.4 Table -2 Tukey's Multiple Comparison Test for the Significance of the Biofilm Production at pH 2.0, 6.4, 7.4. within EBB without Enzyme

Tukey's multiple comparisons test EBB @570	Mean Diff.	95.00% CI of diff.	Significant	Summary	Adjusted P-Value
Row 1					
EBB2.0 vs. EBB6.4	-0.0134	-0.3034 to 0.2766	No	ns	0.9927
EBB2.0 vs. EBB7.4	-0.03003	-0.3200 to 0.2600	No	ns	0.9639
EBB 6.4 vs. EBB7.4	-0.01663	-0.3066 to 0.2734	No	ns	0.9888
Row 2					
EBB 2.0 vs. EBB6.4	-0.1823	-0.4723 to 0.1077	No	ns	0.2776
EBB 2.0 vs. EBB7.4	-0.3317	-0.6216 to -0.0416	Yes	*	0.0228
EBB 6.4 vs. EBB7.4	-0.1493	-0.4393 to 0.1407	No	ns	0.4165
Row 3					
EBB 2.0 vs. EBB6.4	0.126	-0.1640 to 0.4160	No	ns	0.5322
EBB 2.0 vs. EBB7.4	-0.1482	-0.4382 to 0.1418	No	ns	0.4218
EBB 6.4 vs. EBB7.4	-0.2742	-0.5642 to 0.01578	No	ns	0.0663
Row 4					
EBB 2.0 vs. EBB6.4	-0.059	-0.3490 to 0.2310	No	ns	0.8681
EBB 2.0 vs. EBB7.4	-0.4197	-0.7096 to -0.1297	Yes	**	0.0038
EBB 6.4 vs. EBB7.4	-0.3607	-0.6506 to -0.07068	Yes	*	0.0129

4.8.5 Table-3 Tukey's Multiple Comparison Test for the significance of the biofilm production at pH 2.0, 6.4, 7.4. in EBB with enzyme

Tukey's multiple comparisons test BBE 570	Mean Diff.	95.00% CI of diff.	Significant	Summary	Adjusted P-Value
Row 1					
EBB-EZ 2.0 vs. EBB-EZ 6.4	0.05933	-0.07655 to 0.1952	No	ns	0.529
EBB-EZ 2.0 vs. EBB-EZ 7.4	-0.03467	-0.1705 to 0.1012	No	ns	0.8012

EBB-EZ 6.4 vs. EBB-EZ 7.4	-0.094	-0.2299 to 0.04188	No	ns	0.2157
Row 2					
EBB-EZ 2.0 vs. EBB-EZ 6.4	0.131	-0.004879 to 0.2669	No	ns	0.0603
EBB-EZ 2.0 vs. EBB-EZ 7.4	0.077	-0.05888 to 0.2129	No	ns	0.3492
EBB-EZ 6.4 vs. EBB-EZ 7.4	-0.054	-0.1899 to 0.08188	No	ns	0.5886
Row 3					
EBB-EZ 2.0 vs. EBB-EZ 6.4	-0.0617	-0.1976 to 0.07418	No	ns	0.5031
EBB-EZ 2.0 vs. EBB-EZ 7.4	-0.4117	-0.5476 to -0.2758	Yes	****	<0.0001
EBB-EZ 6.4 vs. EBB-EZ 7.4	-0.35	-0.4859 to -0.2141	Yes	****	<0.0001
Row 4					
EBB-EZ 2.0 vs. EBB-EZ 6.4	-0.273	-0.4089 to -0.1371	Yes	***	0.0001
EBB-EZ 2.0 vs. EBB-EZ 7.4	-0.8423	-0.9782 to -0.7065	Yes	****	<0.0001
EBB-EZ 6.4 vs. EBB-EZ 7.4	-0.5693	-0.7052 to -0.4335	Yes	****	<0.0001

4.8.6 Table-4 Tukey's Multiple Comparison Test for the significance of the Biofilm production at pH 2.0 in EBB with *L. reuteri* with and without enzyme

Comparison of EBB with *L. reuteri* Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant	Summary	Adjusted P-Value
Row 1					
***L. reuteri* 2.0 vs. *L. reuteri* - EZ 2.0**	-0.0934	-0.3651 to 0.1783	No	ns	0.7793
***L. reuteri* 2.0 vs. EBB 2.0**	-0.05737	-0.3290 to 0.2143	No	ns	0.9364
***L. reuteri* 2.0 vs. EBB-EZ 2.0**	0.1316	-0.1400 to 0.4033	No	ns	0.5495
***L. reuteri* - EZ 2.0 vs. EBB 2.0**	0.03603	-0.2356 to 0.3077	No	ns	0.9829
***L. reuteri* - EZ 2.0 vs. EBB-EZ 2.0**	0.225	-0.04662 to 0.4967	No	ns	0.1297
EBB 2.0 vs. EBB-EZ 2.0	0.189	-0.08265 to 0.4607	No	ns	0.2468
Row 2					
***L. reuteri* 2.0 vs. *L. reuteri* - EZ 2.0**	0.0857	-0.1860 to 0.3574	No	ns	0.82

***L. reuteri* 2.0 vs. EBB 2.0**	-0.073	-0.3447 to 0.1987	No	ns	0.8794
***L. reuteri* 2.0 vs. EBB-EZ 2.0**	0.164	-0.1077 to 0.4357	No	ns	0.3631
***L. reuteri* - EZ 2.0 vs. EBB 2.0**	-0.1587	-0.4304 to 0.1130	No	ns	0.3913
***L. reuteri* - EZ 2.0 vs. EBB-EZ 2.0**	0.0783	-0.1934 to 0.3500	No	ns	0.8559
EBB 2.0 vs. EBB-EZ 2.0	0.237	-0.03465to 0.5087	No	ns	0.1028
Row 3					
***L. reuteri* 2.0 vs. *L. reuteri* - EZ 2.0**	0.05	-0.2217 to 0.3217	No	ns	0.9564
***L. reuteri* 2.0 vs. EBB 2.0**	-0.06634	-0.3380 to 0.2053	No	ns	0.906
***L. reuteri* 2.0 vs. EBB-EZ 2.0**	0.08933	-0.1823 to 0.3610	No	ns	0.8012
***L. reuteri* - EZ 2.0 vs. EBB 2.0**	-0.1163	-0.3880 to 0.1553	No	ns	0.644
***L. reuteri* - EZ 2.0 vs. EBB-EZ 2.0**	0.03933	-0.2323 to 0.3110	No	ns	0.9779
EBB 2.0 vs. EBB-EZ 2.0	0.1557	-0.1160 to 0.4273	No	ns	0.4079

4.8.7 Table-5 Tukey's Multiple Comparison Test for the significance of the biofilm production at pH 6.4 in EBB with *L. reuteri* with and without enzyme

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant	Summary	Adjusted P-Value
Row 1					
***L. reuteri* 6.4 vs. *L. reuteri* - EZ 6.4**	-0.2463	-0.4577 to -0.03496	Yes	*	0.0181
***L. reuteri* 6.4 vs. EBB 6.4**	-0.337	-0.5484 to -0.1256	Yes	**	0.001
***L. reuteri* 6.4 vs. EBB-EZ 6.4**	0.1653	-0.04604 to 0.3767	No	ns	0.164
***L. reuteri* - EZ 6.4 vs. EBB 6.4**	-0.09067	-0.3020 to 0.1207	No	ns	0.6429
***L. reuteri* - EZ 6.4 vs. EBB-EZ 6.4**	0.4117	0.2003 to 0.6230	Yes	****	<0.0001
EBB 6.4 vs. EBB-EZ 6.4	0.5023	0.2910 to 0.7137	Yes	****	<0.0001
Row 2					
***L. reuteri* 6.4 vs. *L. reuteri* - EZ 6.4**	0.2623	0.05096 to 0.4737	Yes	*	0.0111
***L. reuteri* 6.4 vs. EBB 6.4**	0.197	-0.01437 to 0.4084	No	ns	0.074

L. reuteri 6.4 vs. EBB-EZ 6.4	0.2463	0.03493 to 0.4577	Yes	*	0.0182
L. reuteri - EZ 6.4 vs. EBB 6.4	-0.06533	-0.2767 to 0.1460	No	ns	0.8288
L. reuteri - EZ 6.4 vs. EBB-EZ 6.4	-0.01603	-0.2274 to 0.1953	No	ns	0.9967
EBB 6.4 vs. EBB-EZ 6.4	0.0493	-0.1621 to 0.2607	No	ns	0.9168
Row 3					
L. reuteri 6.4 vs. *L. reuteri* - EZ 6.4	-0.0283	-0.2397 to 0.1831	No	ns	0.9824
L. reuteri 6.4 vs. EBB 6.4	0.04703	-0.1643 to 0.2584	No	ns	0.9267
L. reuteri 6.4 vs. EBB-EZ 6.4	-0.0113	-0.2227 to 0.2001	No	ns	0.9988
L. reuteri - EZ 6.4 vs. EBB 6.4	0.07533	-0.1360 to 0.2867	No	ns	0.7602
L. reuteri - EZ 6.4 vs. EBB-EZ 6.4	0.017	-0.1944 to 0.2284	No	ns	0.996
EBB 6.4 vs. EBB-EZ 6.4	-0.05833	-0.2697 to 0.1530	No	ns	0.8709

4.8.8 Table-6 Tukey's Multiple Comparison Test for the significance of the biofilm production at pH 7.4 in EBB with *L. reuteri* with and without enzyme

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant	Summary	Adjusted P-Value
Row 1					
L. reuteri 7.4 vs. *L. reuteri* - EZ 7.4	0.0497	-0.1607 to 0.2601	No	ns	0.9139
L. reuteri 7.4 vs. EBB 7.4	-0.2656	-0.4760 to -0.05525	Yes	**	0.0097
L. reuteri 7.4 vs. EBB-EZ 7.4	0.332	0.1216 to 0.5424	Yes	**	0.0012
L. reuteri - EZ 7.4 vs. EBB 7.4	-0.3153	-0.5257 to -0.1049	Yes	**	0.002
L. reuteri - EZ 7.4 vs. EBB-EZ 7.4	0.2823	0.07195 to 0.4927	Yes	**	0.0057
EBB 7.4 vs. EBB-EZ 7.4	0.5977	0.3873 to 0.8080	Yes	****	<0.0001
Row 2					
L. reuteri 7.4 vs. *L. reuteri* - EZ 7.4	-0.0134	-0.2238 to 0.1970	No	ns	0.998
L. reuteri 7.4 vs. EBB 7.4	-0.0669	-0.2773 to 0.1435	No	ns	0.8165
L. reuteri 7.4 vs. EBB-EZ 7.4	-0.0934	-0.3038 to 0.1170	No	ns	0.6177

***L. reuteri* - EZ 7.4 vs. EBB 7.4**	-0.0535	-0.2639 to 0.1569	No	ns	0.8955
***L. reuteri* - EZ 7.4 vs. EBB-EZ 7.4**	-0.08	-0.2904 to 0.1304	No	ns	0.7228
EBB 7.4 vs. EBB-EZ 7.4	-0.0265	-0.2369 to 0.1839	No	ns	0.9852
Row 3					
***L. reuteri* 7.4 vs. *L. reuteri* - EZ 7.4**	-0.1903	-0.4007 to 0.02005	No	ns	0.0862
***L. reuteri* 7.4 vs. EBB 7.4**	-0.3033	-0.5137 to -0.09295	Yes	**	0.0029
***L. reuteri* 7.4 vs. EBB-EZ 7.4**	-0.5703	-0.7807 to -0.3599	Yes	****	<0.0001
***L. reuteri* - EZ 7.4 vs. EBB 7.4**	-0.113	-0.3234 to 0.09738	No	ns	0.4636
***L. reuteri* - EZ 7.4 vs. EBB-EZ 7.4**	-0.38	-0.5904 to -0.1696	Yes	***	0.0002
EBB 7.4 vs. EBB-EZ 7.4	-0.267	-0.4774 to -0.05662	Yes	**	0.0093

4.8.9 Effect of drug loading on bacterial growth and metabolism as Lactic Acid production

Effect of drug loading on bacterial growth and metabolism 5-FU is known for its growth-inhibitory effect on bacteria[140]; therefore, the effect of 5-FU loading on BB was essential to understand. *L. reuteri* showed a prolonged lag phase of up to 18 hours, and then, a slow recovery of the growth rate in the presence of 15 mg/100 mg (w/w) of the dry weight of *L. reuteri* was observed, although it could achieve only half of its maximum growth up to 96 hours, yet 5-FU– loaded BB (FUBB) [15 mg/100 mg (w/w)] resulted in a reduction of system toxicity in the mouse model, which may be due to sustained release of the drug with reduction of the lag phase of *L. reuteri* up to 12 hours, followed by a faster recovery of the growth rate of *L. reuteri* . To understand the effect of surface encapsulation, the study investigates the impact of 5-FU loading on bacterial growth and metabolism in *L. reuteri*. The bacteria showed a prolonged lag phase. Growth curve analyses showed a phase delay until 9 h in BB, but after 48 h, complete growth retrieval was established, as shown in Fig17 A.

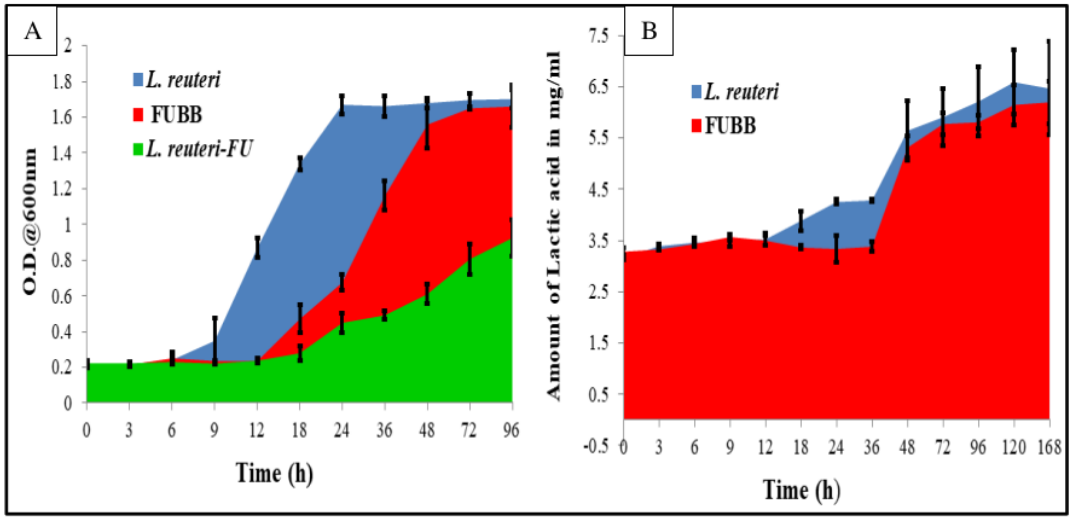


Figure 17: Growth rate and Metabolic rate comparison of BB due to drug loading (A) Effect of growth on BBs due to loading of 5-FU. The drug prevented the growth of BB. FUBB (red) showed up to 12 hours of prolonged lag phase, whereas in EBB (blue) it was around 6 hours. 5-FU also caused a reduction of growth of *L. reuteri* (green), and the extent was higher than that of BB. (B) Lactic acid production, although it did not alter much due to 5-FU loading.

Effect of drug loading on lactic acid production

Lactic acid production was studied as a metabolic characteristic of the live bacteria in the drug-loaded BB. Around a 10% decrease in lactic acid production was observed due to the loading of 5-FU in BB compared to EBB Fig.17 B

4.8.10 Table-7 Comparison of the growth curve by comparing the area ratio of *L. reuteri* with EBB and FUBB.

Time (h)	O.D. @600nm *L. reuteri*	O.D. @600nm EBB	O.D. @600nm FUBB	Area ratio EBB with *L. reuteri*	Area ratio FUBB with *L. reuteri*
0	3.168027	3.202721	3.282993	-0.03629	-3.62895
3	3.390476	3.390476	3.327211	0.01866	1.865971
6	3.460544	3.421769	3.434694	0.00747	0.747002
9	3.47619	3.506122	3.570748	-0.0272	-2.72016
12	3.52517	3.637415	3.50068	0.006947	0.694712
18	3.887075	3.595238	3.364626	0.134407	13.44067
24	4.253061	3.687075	3.336735	0.215451	21.54511
36	4.283673	3.932653	3.382993	0.210259	21.02589
48	5.641497	5.144898	5.314286	0.058001	5.800072
72	5.906122	5.22517	5.772109	0.022691	2.269062

96	6.211565	5.67415	5.808163	0.064944	6.49436

4.8.11 Table-8 Comparison of the percentage growth rate ratio of *L. reuteri* with EBB and FUBB

Time (h)	O.D. @ 600 *L. reuteri*	O.D. @ 600 EBB	O.D. @ 600 FUBB	%growth rate *L. reuteri* /EBB	%growth rate *L. reuteri* /FUBB
0	0.200333	0.231	0.200667	-15.3078	-0.00166
3	0.217333	0.213333	0.214667	1.840491	0.01227
6	0.267	0.229	0.251	14.23221	0.059925
9	0.415667	0.274667	0.235667	33.92141	0.433039
12	0.8675	0.6217	0.235	28.33429	0.729107
18	1.339333	1.028333	0.470333	23.22051	0.64883
24	1.670033	1.455667	0.6754	12.83607	0.595577
36	1.661633	1.584333	1.160933	4.652049	0.30133
48	1.678959	1.690933	1.558547	-0.71321	0.071718
72	1.696284	1.724633	1.649627	-1.67124	0.027505
96	1.701667	1.724667	1.659767	-1.35162	0.024623

5.1 Ultramicroscopic study of BBs

Field-emission scanning electron microscopy (FE-SEM) studies showed surface encapsulation of the bacteria with nanoparticles Fig 18A. Magnification of surface nanostructures revealed the formation of sponge ball-shaped nanoparticles of 15 to 25 nm diameter with mesopores of 2 to 3 nm diameter on the surface of BBs in lyophilised conditions Fig. 18B.

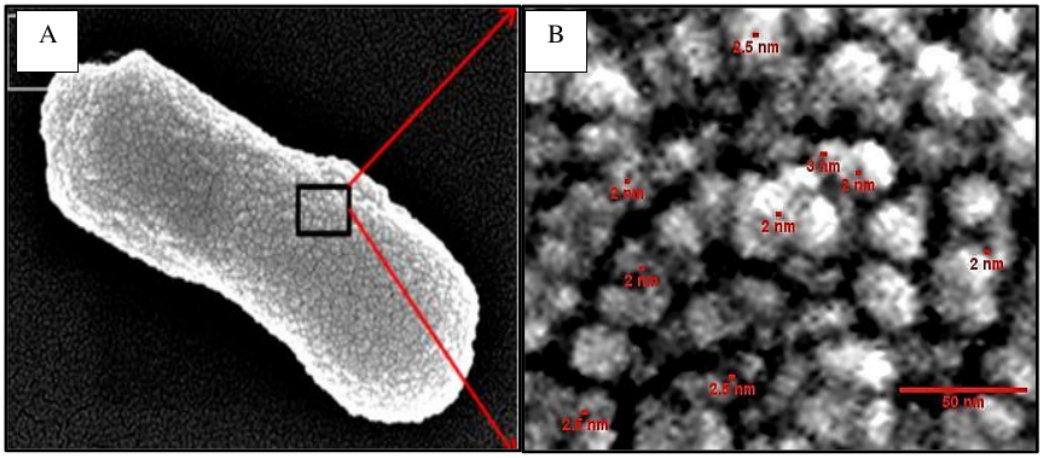


Figure 18: FE-SEM image showing surface morphology of BB (A) FE-SEM image of freshly prepared BB showing chitosan nanoparticles on the cell surface. (B) An enlarged view of the black box area of (A) illustrates the sponge ball-shaped nanoparticles of 15 to 25 nm in size with a pore diameter of around 2 to 3 nm.

5.2 Calculation Method of Drug Loading Efficiency

Test S. No.	BB Dry weight (lyophilised)	Drug loaded	% age drug loaded
Test 1 (*ex-situ* loading)	100 mg	334 μl of 50mg/ml of 5FU was loaded (direct loading, no washout in ex-situ)	16.7 mg of drug in 100 mg of BB (16.7%)
Loading capacity (*ex-situ* loading)	100 mg	16.7-0.0/16.7*100	100%
Test 2 (*in situ* loading)	100 mg	EE% = [(Total drug- free drug) / total drug] * 100 EE% = (100-85)/100 x100	15%
Loading capacity (*ex-situ* loading)	100 mg	LC%=[500–425.8169) /]*150	49.45%

Tabulated results of 3 independent experiments of in situ and ex-situ drug encapsulation efficiency.

(*Ex situ* loading)	Sample 1	Sample 2	Sample 3	Std Dev
EE %	16.4	16.5	16.8	0.0288
LC%	100	100	100	0
(*In situ* loading)	**Sample 1**	**Sample 2**	**Sample 3**	**Std dev**
EE %	12.5	11.9	13.00513	0.2041
LC%	49.46%	51.40%	50.02%	0.0082

For ex-situ and in-situ, the sample was lyophilised before and after the drug loading. For ex-situ, the BB sample was lyophilised, and for 100 mg, it was incubated with the drug and then again lyophilised. The final drug-loaded BB was weighed and was found to be 116.7 ±1 mg. So, the drug-loaded was around 16±1%, similar to the in-situ loading. Two samples were taken, one as a reference and one without a drug, and the in-situ drug loading was done. The samples were lyophilised to check the weight difference between the two and to calculate the loading efficiency.

5.3 Drug Release Study

5.3.1 *In vitro* drug release studies

The study found that chitosan nanoparticles (Bacterioboat, BB) demonstrated higher efficiency in ex-situ drug loading conditions (16.7 ± 1.6%) compared to in situ conditions (12.3 ± 2.4%). The nano-encapsulated surface exhibited sustained drug release patterns in both simulated gastric fluid (SGF) and simulated intestinal fluid (SIF). In SGF at pH 2.0, 98.43 ± 0.82% of the drug was released within 8.5 hours. Similarly, in SIF at pH 6.4 and pH 7.4, 97.6 ± 1.5% of the drug was released within the same time frame. This data highlights the efficiency and reliability of the BB system is controlled drug release under various physiological conditions.

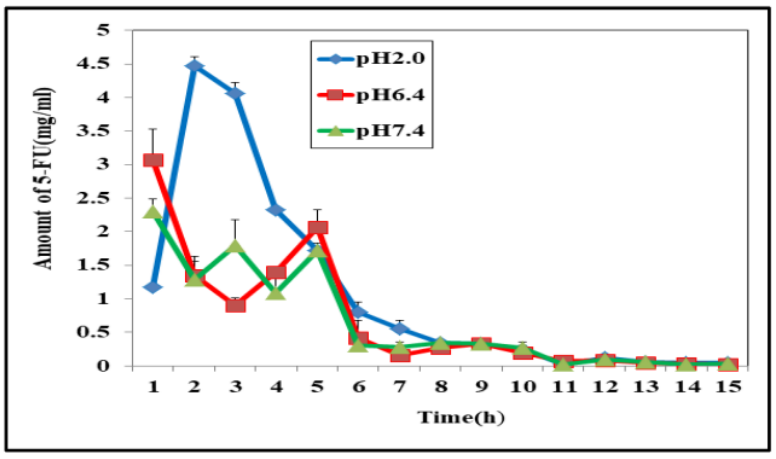


Figure 19: Drug release study of 5FU from FUB at different pH Release study of 5-FU after loading on BB in SGF at pH 2.0 (red), SIF at pH 6.4 (green), and SIF at pH 7.4 (blue). After the initial blast in SIF delayed blast in SGF (between 30 min and 1 hour), a sustained-release pattern was observed in all formulations.

5.3.2 Stability of BBs during drug release

The stability of surface encapsulation is of utmost importance inside the intestinal environment to achieve increased half-life and sustained release. Therefore, the stability of BB was determined in SGF and SIF for up to 72 hours. Freshly prepared BB showed even the formation and distribution of surface nanoparticles Fig. 20, (A to C). Upon incubation for 24 hours in SGF or SIF, no noteworthy changes in microscopically observable stability were found in Fig. 20 (D to F). Secretory biofilm was observed in BB incubated in SIFs for 24 hours at both pHs, with more in pH 7.4 Fig. 20, (E and F). Subsequently, BB, after 48 hours of incubation in SGF, showed stability in the nanoparticle-encapsulated surface and a moderate increase in biofilm production Fig. 20(G). Whereas an extensive increase in biofilm formation and little change in structural integrity was observed in Fig. 20(H), BB was incubated with SIF at pH 6.4. However, BB, after incubation in SIF at pH 7.4, showed a change in surface morphology Fig. 20(I), with a huge enhancement of biofilm production. An overwhelming biofilm production completely covered the nanoparticle's encapsulated surface Fig. 20(I). After 72, the partial disruption of the structure of the Nano encapsulated surface, along with enhanced biofilm production, was observed in SGF and SIF at pH 6.4 Fig. 20 (J and K). In SIF at pH 7.4, a complete disruption of the surface and swelling of the overall microbial structures were observed in Fig. 20L

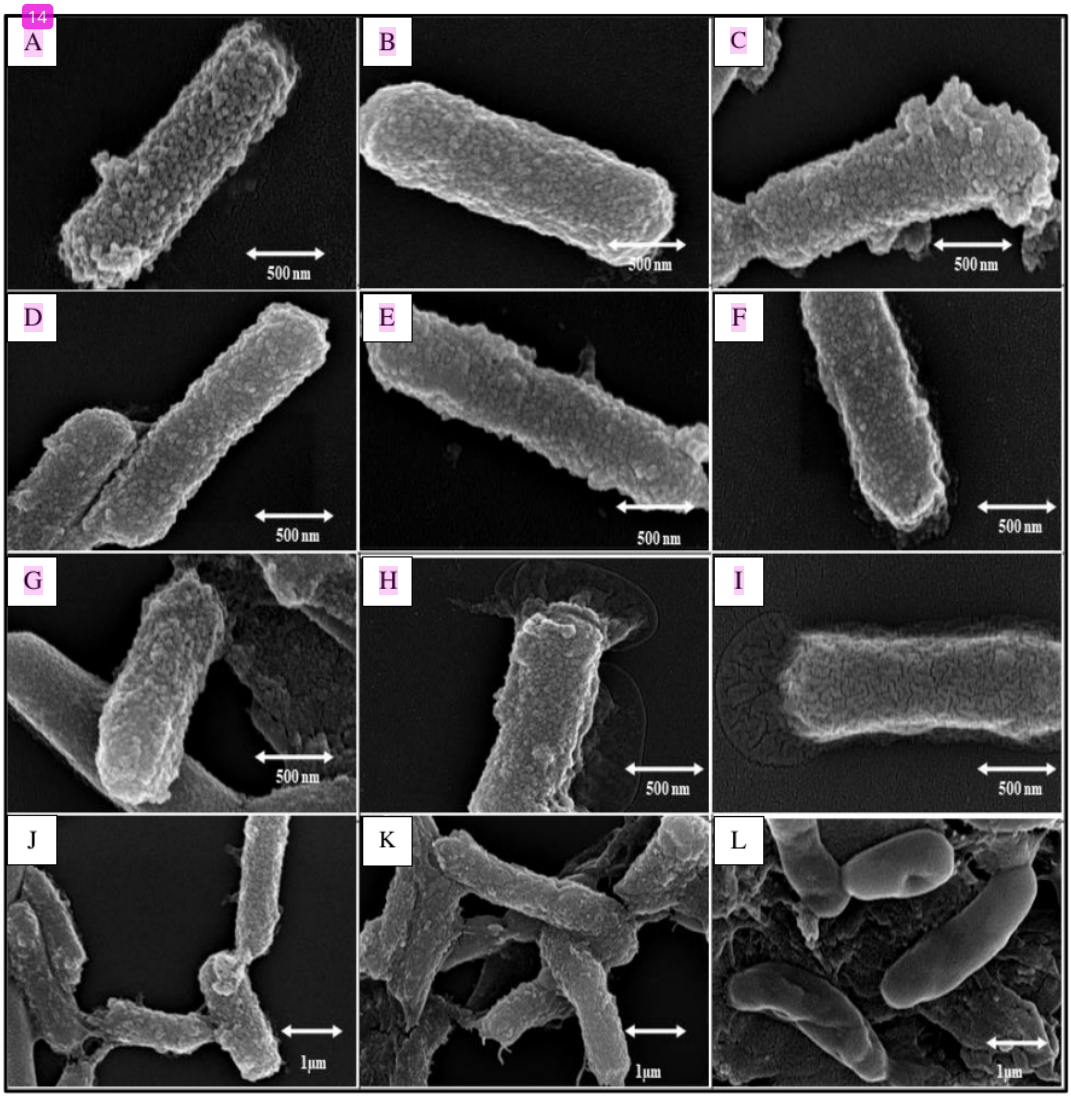


Figure 20: Stability of the nanoparticle-encapsulated cell surface. FE-SEM images show the surface stability of BBs in SGF and SIF during drug release in the presence of digestive enzymes: (A) 0 hour in SGF at pH 2.0, (B) 0 hour in SIF at pH 6.4, (C) 0 hour in SIF at pH 7.4, (D) 24 hours in SGF at pH 2.0, (E) 24 hours in SIF at pH 6.4, (F) 24 hours in SIF at pH 7.4, (G) 48 hours in SGF at pH 2.0, (H) 48 hours in SIF at pH 6.4, (I) 48 hours in SIF at pH 7.4, (J) 72 hours in SGF at pH 2.0, (K) 72 hours in SIF at pH 6.4), and (L) 72 hours in SIF at pH 7.4.

5.3.3 Ex-Vivo studies

The study investigated the intestinal anchorage of a biofilm (BB) on the small intestinal sections of Swiss albino mice using confocal microscopy. The BB was loaded with Rhodamine 123, and the

intestine was observed to be localised around the alveoli of the intestine. A fluorescence intensity comparison assay showed that fluorescence intensity was maximum in Rd-loaded BB on the intestine, indicating good anchorage on the intestinal microvilli region and reasonable dye deportation and release.

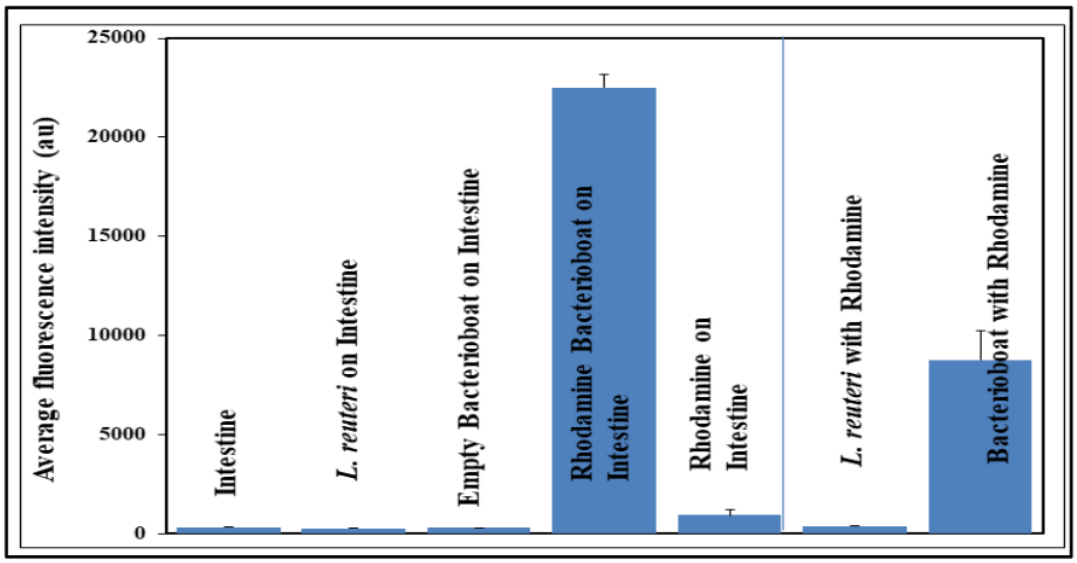


Figure 21: Comparative fluorescence intensity detection using a 96-well plate by BB loaded with fluorescent dye Rd on mouse intestinal tissue shows maximum fluorescence with the Rd-loaded BB on the intestine after Rd-loaded BB directly on the polystyrene plate well, while the other shows the least fluorescence. Au, arbitrary units

5.3.4 Table-9 Tukey's Multiple Comparison Test for the Significance of Fluorescent Intensity Graph

Tukey's multiple comparisons test Fluorescent Intensity Graph	Mean Diff.	95.00% CI of diff.	Significant	Summary	Adjusted P-Value
Intestine vs. *L. reuteri*	47	-1667 to 1761	No	ns	>0.9999
Intestine vs. EBB	34.67	-1679 to 1748	No	ns	>0.9999
Intestine vs. Rh-BB on intestine	-22182	-23896 to -20469	Yes	****	<0.0001
Intestine vs. Rh on Intestine	-624.7	-2338 to 1089	No	ns	0.8651
Intestine vs. *L. reuteri* -Rh on intestine	-60.66	-1774 to 1653	No	ns	>0.9999
Intestine vs. Rh-BB	-8443	-10156 to -6729	Yes	****	<0.0001

L. reuteri vs. EBB	-12.33	-1726 to 1701	No	ns	>0.9999
L. reuteri vs. Rh-BB on intestine	-22229	-23943 to -20516	Yes	****	<0.0001
L. reuteri vs. Rh on Intestine	-671.7	-2385 to 1042	No	ns	0.8235
L. reuteri vs. *L. reuteri* -Rh on intestine	-107.7	-1821 to 1606	No	ns	>0.9999
L. reuteri vs. Rh-BB	-8490	-10203 to -6776	Yes	****	<0.0001
EBB vs. Rh-BB on intestine	-22217	-23931 to -20503	Yes	****	<0.0001
EBB vs. Rh on Intestine	-659.3	-2373 to 1054	No	ns	0.8349
EBB vs. *L. reuteri* -Rh on the intestine	-95.33	-1809 to 1618	No	ns	>0.9999
EBB vs. Rh-BB	-8477	-10191 to -6764	Yes	****	<0.0001
Rh-BB on intestine vs. Rh on Intestine	21558	19844 to 23271	Yes	****	<0.0001
Rh-BB on intestine vs. *L. reuteri* -Rh on intestine	22122	20408 to 23835	Yes	****	<0.0001
Rh-BB on intestine vs. Rh-BB	13740	12026 to 15453	Yes	****	<0.0001
Rh on Intestine vs. *L. reuteri* -Rh on intestine	564	-1150 to 2278	No	ns	0.9103
Rh on Intestine vs. Rh-BB	-7818	-9532 to -6104	Yes	****	<0.0001
L. reuteri -Rh on intestine vs. Rh-BB	-8382	-10096 to -6668	Yes	****	<0.0001

5.3.5 Intestinal Anchorage- FE-SEM Imaging (Ex-Vivo)

The intestinal anchorage of BB on the mouse small intestinal sections ex vivo was monitored using FE-SEM. The FE-SEM images of the mouse intestine, as shown in Fig.22A and BB anchored on the intestinal lining of mice in Fig. 22B, portray the anchorage of BB on the intestinal lining.

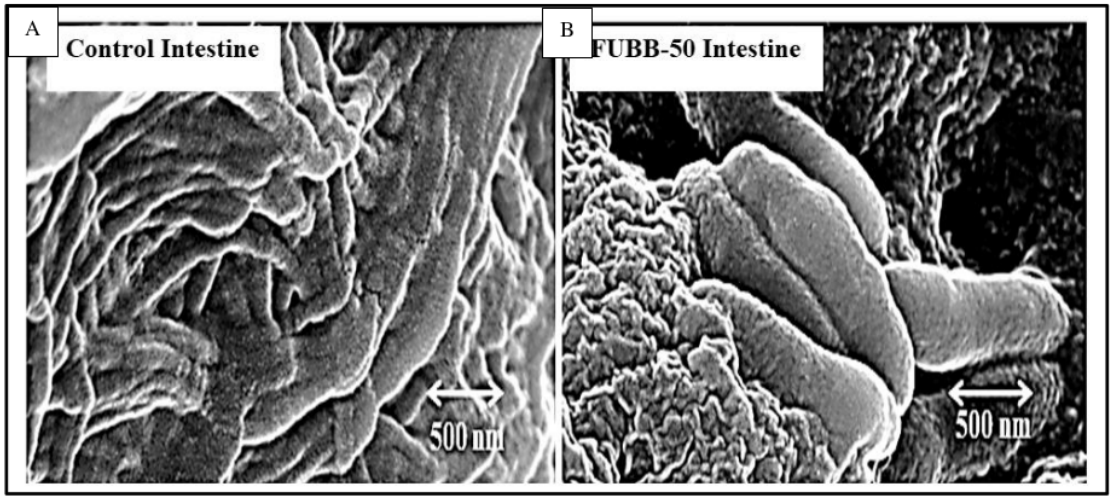


Figure 22: Anchorage of BB on the intestinal surface (ex vivo) (A)FE-SEM image of cleaned mouse intestine showing clear projections of the mouse intestine. (B) FE-SEM image of BB anchored on the mouse intestinal lining.

5.4 *In vivo* studies

5.4.1 Liquid chromatography-electrospray ionisation–mass spectrometry of BB attachment on mice intestine

BB can attach to the wall of the murine intestine, and anchorage of FUBB leads to sustained release of the drug compared to direct 5-FU treatment in mice studied through liquid chromatography-electrospray ionisation–mass spectrometry To check the presence of *L. reuteri* getting attached to the mouse's intestine treated with FUBB, quantitative polymerase chain reaction qPCR) analysis was performed using genomic DNA (gDNA) extracted from the murine intestine samples. All the mice used in these experiments were orally gavaged with antibiotics to reduce a load of nonspecific bacteria commonly found in their intestine. Because *L. reuteri* is a typical intestinal flora, the CT (cycle threshold) values for *L. reuteri*-specific genes were normalised against the universal 16S ribosomal DNA (rDNA) used as a reference. Our study shows that *L. reuteri* BB is efficiently attached to the wall of the murine intestine. In addition, a comparative analysis of the presence of *L. reuteri*, BB, and FUBB (50 mg/kg) has been from the faeces samples of treated mice for up to 24 hours. In all cases, a comparable amount of *L. reuteri* was found in the stool samples of mice. Fig. 23A shows the comparative expression of 16S rDNA of *L. reuteri* in the faeces of all three groups of mice at different time points (6, 12, and 24 hours) with *L. reuteri* –-specific genes in the treated mice that were normalised against the universal 16S rDNA as a reference. In Fig. 23B, qPCR analysis with *L. reuteri* –specific primers revealed that the abundance of the bacteria was around 10-fold higher in the case of the treated murine intestine sample compared to the untreated murine intestine 24 hours after oral gavage. This observation proves that FUBB acts as a better drug carrier, enabling the slow and sustained release of drugs compared to direct 5-FU treatment.

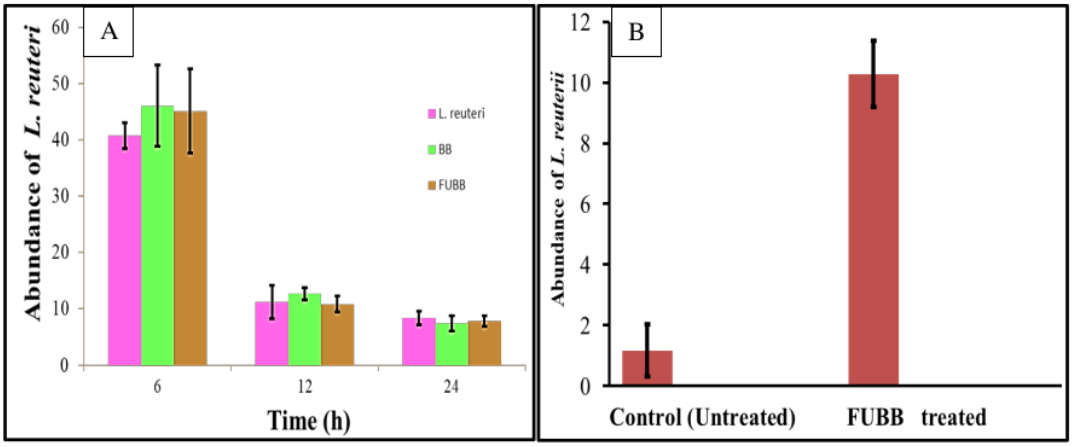


Figure 23: Stability of BB in the mice in vivo using RT-PCR (A) Comparative expression of 16S rDNA of *L. reuteri* in the mice Feces of all three groups of mice at different time points (6h, 12h and 24 h) was collected to compare the 16S rDNA of *L. reuteri*. (B) RT-PCR data show the presence of *L. reuteri* in the mouse intestine, as the signal for the FUBB-treated mice is 10 times that of the control, even 24 hours after administration.

5.4.2 Bioavailability of 5-FU in the plasma of treated mice using LC-ESI-MSAB

In the case of normal 5-FU treatment, Fig. 24A shows that the 5-FU drug persisted in the murine plasma sample for 5 hours, with the maximum abundance around 3 hours. After 8 hours, 5-FU drug abundance was found to decrease gradually. However, in the case of FUBB treatment, as shown in Fig 24B, 5-FU drug abundance in the murine plasma sample was seen for almost 24 hours, although the [M-H]+ abundance for 5-FU was less compared to direct 5-FU–treated murine plasma samples. This observation proves that FUBB acts as a better drug carrier, enabling the slow and sustained release of drugs compared to direct 5-FU treatment[191]

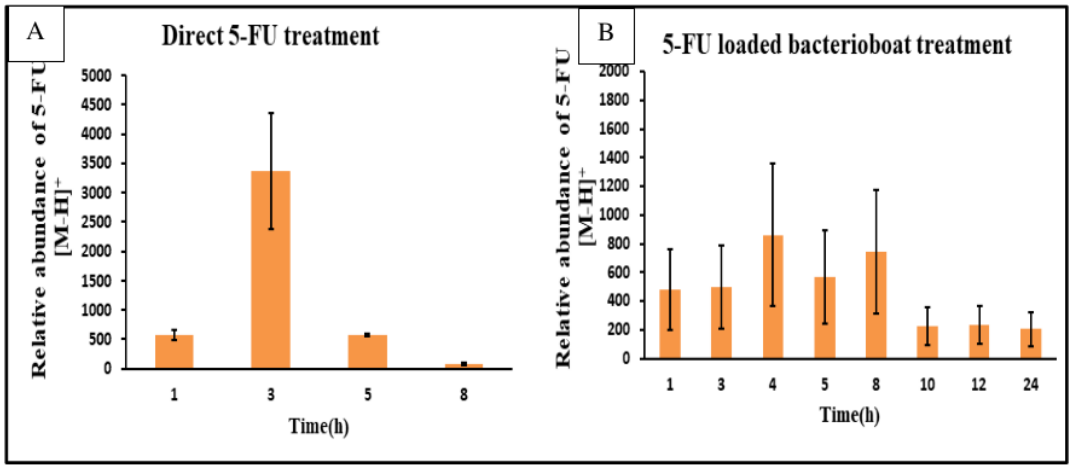


Figure 24: Bioavailability of 5-FU in the plasma of treated mice using LC-ESI-MSAB. The relative abundance of 5-FU [M-H+] was plotted as means ± SE at different time points concerning the untreated murine plasma, which served as a control (A)Detectable amount of the 5-FU drug in the mouse plasma up to 8 hours when applied alone using the oral delivery route (B) Detectable amount of 5-FU delivered orally using FUBB up to 24 hours (**P < 0.01 and ***P < 0.001).

5.4.3 Table-10 Sidak's Multiple Comparison Test for the significance of the LC-ESI-MS comparison of FU-50 and FUBB-50

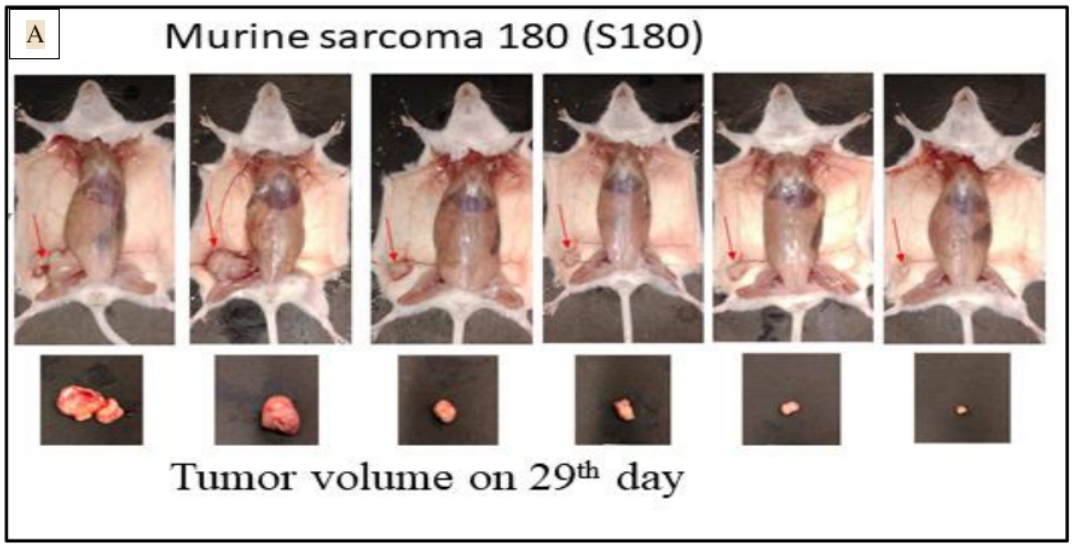
Sidak's multiple comparisons test FU-50 VS FUBB-50	Mean Diff.	95.00% CI of diff.	Significant	Summary	Adjusted P Value
Row 2	67.33	-1270 to 1404	No	ns	>0.9999
Row 3	1860	523.2 to 3197	Yes	**	0.0027
Row 4	-370	-1707 to 966.8	No	ns	0.9805
Row 5	-2413	-3750 to -1076	Yes	***	0.0001
Row 6	-717.3	-2054 to 619.5	No	ns	0.6281
Row 7	-740.7	-2078 to 596.2	No	ns	0.5918
Row 8	-484	-1821 to 852.8	No	ns	0.9207

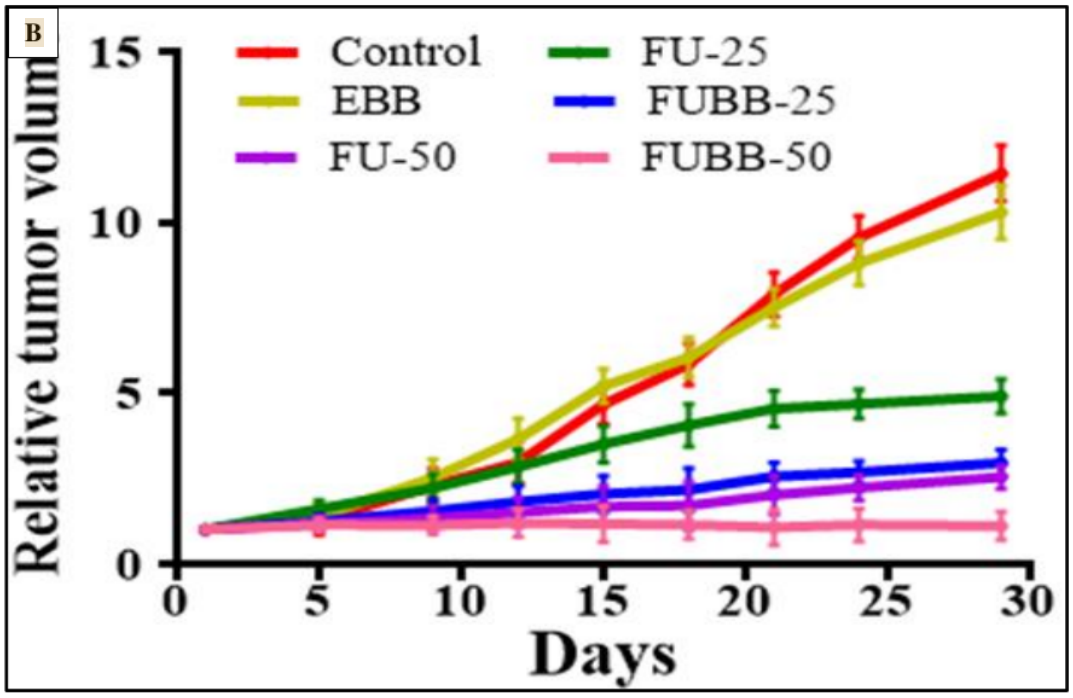
5.5 The effect of 5-FU with or without BB in tumour regression *in vivo* and survivability of mice

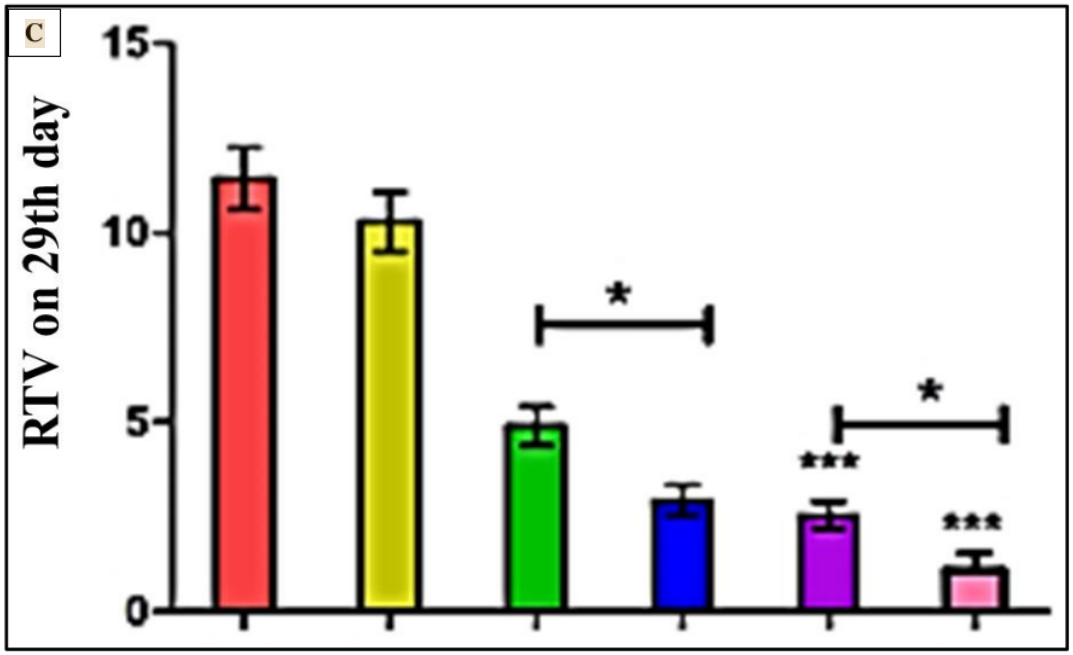
Tumour volume was measured at 3-day intervals for each treatment group up to 29 days from the day of treatment onset, and data were graphically represented from three independent experiments Fig. 25, A to D. Relative tumour volume on the 29th day was compared, and anti-tumour growth inhibition (TGI) was quantified as a percentage reduction from the control group. EBB-treated groups showed negligible TGI (9.98%) compared to the control group, and different doses of 5-FU treatment inhibited tumour growth differently [FU-25 (5-FU, 25 mg/kg BW) by 57.13% and FU-50 (5-FU, 50 mg/kg BW) by 77.69%]. FUBB-25 (FUBB, 25 mg/kg BW) treatment inhibited tumour growth by 74.19%, whereas FUBB-50 (FUBB, 50 mg/kg BW) treatment showed the highest TGI (90.29%, i.e., 12% higher treatment benefit from the FU-50–treated group) than the control group. BB strategy achieved treatment benefits for the FUBB-25 and FUBB-50 groups in terms of higher efficacy than the FU-25 and FU-50 groups. Tumour reduction was almost equivalent for FU-50– and FUBB-25–treated groups, inferring that 5-FU at a 50% low dose with BB is as effective as 5-FU at a conventional dose. Fig. 25 shows S180 (sarcoma 180) solid tumours in all groups on the 29th day of treatment. Fig. 25 B shows relative tumour volume over the treatment period. The data show a distinguishable difference between the relative tumour volume of the control 5–FU–treated tumour versus the FUBB-treated tumour. In the case of a newly developed system, better tumour reduction was observed. Fig. 25 C shows a graphical representation of the relative tumour volume on the 29th day of treatment, implying the treatment's effectiveness with BB.

Survivability of tumour-bearing mice

Posttreatment survival benefits of tumour-bearing mice were obtained from Kaplan-Meier analysis of data on animal survival at the end of the study. The median survival values were listed as 15.5, 16.5, 19, 23.5, 21, and 25 for control, EBB, FU-25, FUBB-25, FU-50 and FUBB-50 groups, respectively (log-rank P = 0.0343, $\chi 2 = 12.04$) Fig. 25 D.







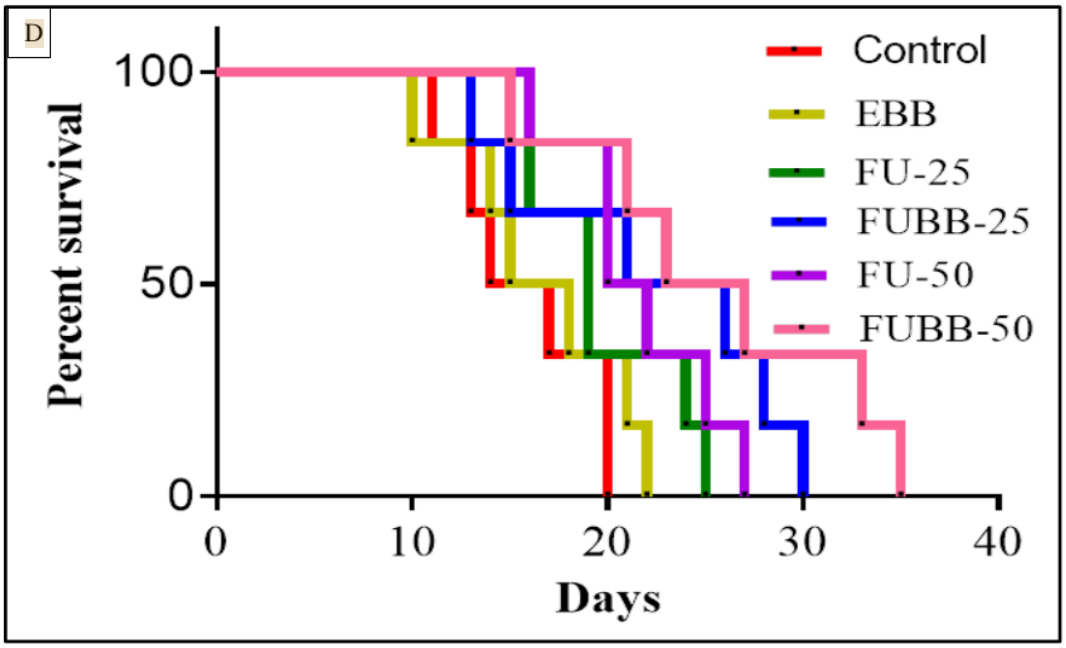


Fig.25. In vivo validation of the efficacy of BBs. (A) S180 solid tumours in all groups were photographed immediately after euthanisation (the 29th day of treatment). The red colour scale bar shows 1 cm of the length of the S180 tumour shown in the euthanised mice. (B) The relative tumour

volume of mice in different treatment groups is graphically represented. (C) Relative tumour volume on the 29th day. Values indicate means ± SD (n = 6). *P < 0.05, **P < 0.01, and ***P < 0.001. (D) Survival benefits among different groups are represented following Kaplan-Meier analysis of S180 ascites tumour-bearing mice

5.5.1 Table-11 Tukey's Multiple Comparison Test for the significance of RTV on 29th day

Tukey's Multiple Comparison Test RTV 29th day	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
Con vs EBB	1.14	3.45	No	ns	-0.4312 to 2.711
Con vs FU-25	6.53	19.8	Yes	***	4.959 to 8.101
Con vs FUBB-25	8.48	25.6	Yes	***	6.909 to 10.05
Con vs FU-50	8.88	26.9	Yes	***	7.309 to 10.45
Con vs FUBB-50	10.32	31.2	Yes	***	8.749 to 11.89
EBB vs FU-25	5.39	16.3	Yes	***	3.819 to 6.961
EBB vs FUBB-25	7.34	22.2	Yes	***	5.769 to 8.911
EBB vs FU-50	7.74	23.4	Yes	***	6.169 to 9.311
EBB vs FUBB-50	9.18	27.8	Yes	***	7.609 to 10.75
FU-25 vs FUBB-25	1.95	5.9	Yes	*	0.3788 to 3.521
FU-25 vs FU-50	2.35	7.11	Yes	**	0.7788 to 3.921
FU-25 vs FUBB-50	3.79	11.5	Yes	***	2.219 to 5.361
FUBB-25 vs FU-50	0.4	1.21	No	ns	-1.171 to 1.971
FUBB-25 vs FUBB-50	1.84	5.56	Yes	*	0.2688 to 3.411
RTFU-50 vs FUBB-50	1.44	4.35	No	ns	-0.1312 to 3.011

5.6 Histopathological analysis of tumour and other vital organs following treatment

Tissue architecture changes were obtained from the histopathological analysis by haematoxylin and eosin (H&E) staining Fig. 26. Haematoxylin is a DNA binding dye converting nuclei into blue or purple colour, while cytoplasm or stromal parts remain pink by eosin. Tumour tissue morphology was in control, and EBB was characterised by well-organized and high-density nuclei and no visible

necrosis. Noticeable tumour necrosis and reduced nuclei density were observed in the FU-25 group. FUBB-25 and FU-50 groups showed higher tumour necrotic areas and decreased tumour nuclei than the FU-25 group, but both have shown nearly similar results. The FUBB-50 group has shown substantially higher necrotic areas and nuclei density among all groups. Histopathological analysis of liver and kidney sections was also carried out in all groups to understand any toxicity-related changes due to treatment. EBB, FU-25, and FUBB-25 groups showed normal hepatocyte structure and prominent central vein without any sign of cytoplasmic degeneration and necrotic foci, but FU-50 and FUBB-50 have shown sinusoidal destruction. In kidney sections, all groups showed normal glomerular architecture with abundant tubule and capsular space, and no toxicity features like necrotic foci, leukocyte infiltration, and distorted glomeruli were observed.

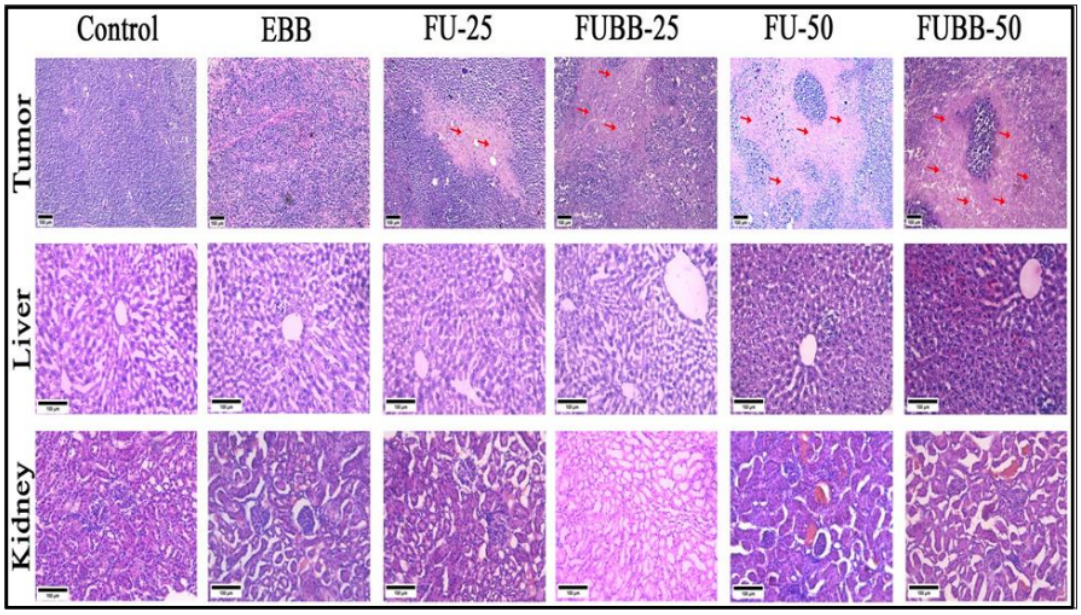


Figure 26: Photomicrograph of S180 tumour, liver and kidney (H&E-stained). H&E staining of sections from S180 solid tumour (×100 magnification), liver (×200 magnification), and kidney (×200 magnification) shows tumour architecture in different treatment groups and the effect of treatment in vital organs. Red arrows in tumour histology indicate necrotic areas.

5.7 Biochemical parameters

Blood serum analysis on the 29th day from each group for liver enzymes [serum glutamic oxaloacetic transaminase (SGOT) and serum glutamic pyruvic transaminase (SGPT)] and other biochemical parameters (albumin, alkaline phosphatase, bilirubin, cholesterol, triglycerides, globulin, creatinine, liver weight, and BW of test mice) was graphically represented. Elevated values of almost all parameters were observed with the increase of 5-FU doses (FU-25 and FU-50). Treatment with FUBB-50 reduced these, whereas treatment with the FUBB-25 dose significantly decreased these parameter

values. For all parameters, EBB treatment showed a negligible change in values compared to the control groups.

5.7.1 Liver Toxicity Parameters

Liver parameters

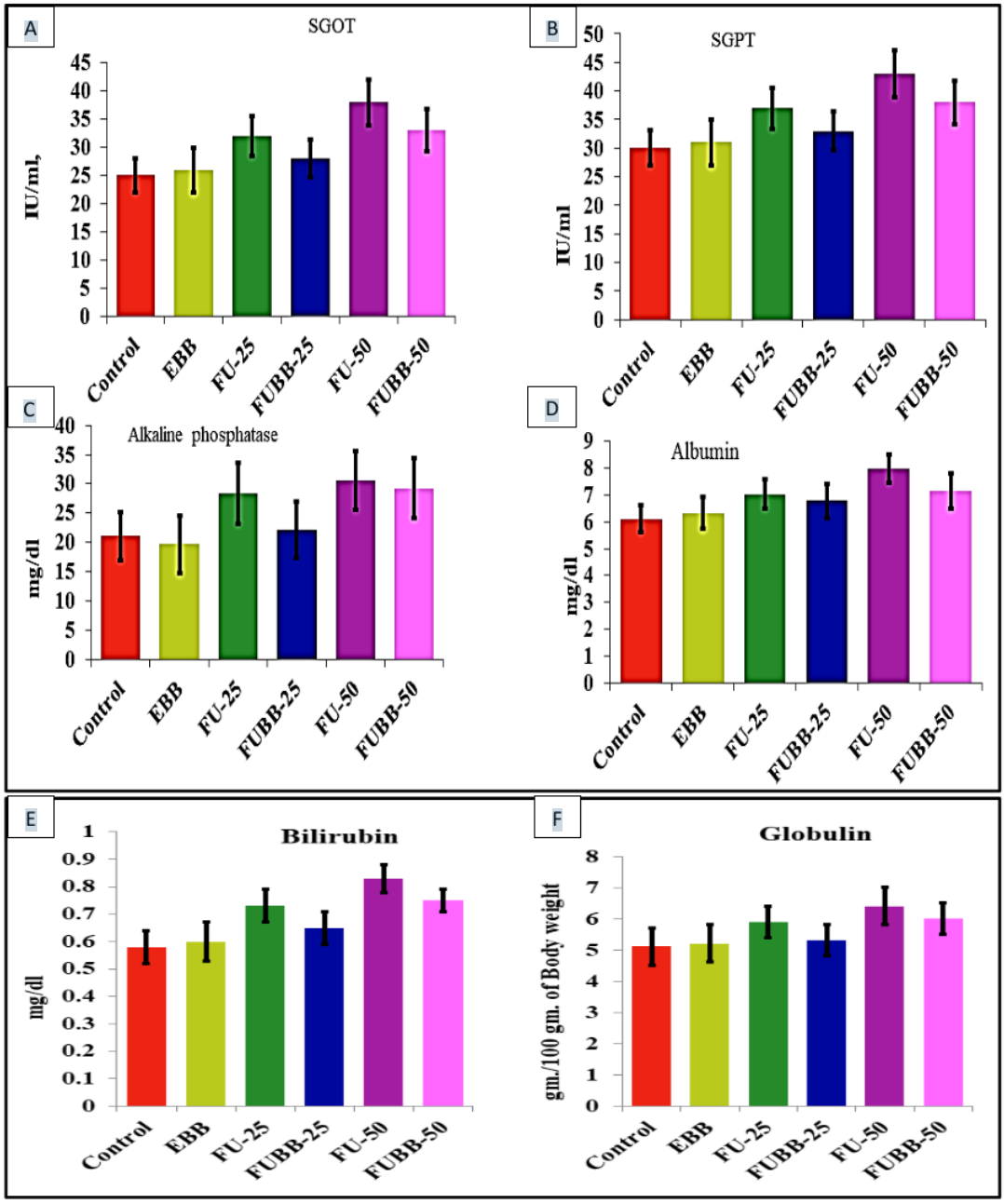
In vivo drug trials involving mice, assessing liver toxicity parameters is essential to ensure the safety and efficacy of the drug being tested[193]. Key liver toxicity parameters include serum glutamic-oxaloacetic transaminase (SGOT), serum glutamic-pyruvic transaminase (SGPT), alkaline phosphatase (ALP), bilirubin, albumin, and globulin[194].

SGOT (AST) and SGPT (ALT) are enzymes that are released into the bloodstream when liver cells are damaged. Elevated levels of these enzymes indicate hepatocellular injury, making them crucial markers for detecting liver toxicity early in drug trials. ALP is another enzyme that, when elevated, can signal bile duct obstruction or liver damage, providing additional insights into hepatic health. Bilirubin, a by-product of red blood cell breakdown, is processed by the liver and excreted in bile. Increased bilirubin levels in the blood suggest impaired liver function or bile flow, highlighting potential issues with drug metabolism or liver clearance. Monitoring bilirubin is vital for understanding the extent of liver impairment[195].

Albumin and globulin are proteins synthesised by the liver. Albumin helps maintain osmotic pressure and transports various substances, while globulins play a role in the immune response. Abnormal levels of these proteins can indicate liver dysfunction, reduce synthetic capacity, or alter immune status. By evaluating albumin and globulin levels, researchers can gain insights into the liver's ability to perform its essential functions.

Together, these parameters provide a comprehensive picture of liver health, guiding dose adjustments, identifying potential hepatotoxicity, and ensuring the drug's safety profile before progressing to human trials. Assessing these markers helps researchers anticipate adverse effects, optimise dosing regimens, and comply with regulatory standards, ultimately contributing to developing safer and more effective pharmaceuticals.

Analysis on the 29th day from each group for liver enzymes [serum glutamic oxaloacetic transaminase (SGOT) and serum glutamic pyruvic transaminase (SGPT)] and other biochemical parameters (albumin, alkaline phosphatase, bilirubin, Globulin) was done. Elevated values of almost all parameters were observed with the increase of 5-FU doses (FU-25 and FU-50). Treatment with FUBB-50 reduced these, whereas treatment with the FUBB-25 dose significantly decreased these parameter values. For all parameters, E.B treatment showed a negligible change of values compared to the control group, as shown in Fig.27.


A
SGOT
IU/ml,
45
40
35
30
25
20
15
10
5
0
Control
EBB
FU-25
FUBB-25
FU-50
FUBB-50
B
SGPT
IU/ml
50
45
40
35
30
25
20
15
10
5
0
Control
EBB
FU-25
FUBB-25
FU-50
FUBB-50
C
Alkaline phosphatase
mg/dl
40
35
30
25
20
15
10
5
0
Control
EBB
FU-25
FUBB-25
FU-50
FUBB-50
D
Albumin
mg/dl
9
8
7
6
5
4
3
2
1
0
Control
EBB
FU-25
FUBB-25
FU-50
FUBB-50
E
Bilirubin
mg/dl
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Control
EBB
FU-25
FUBB-25
FU-50
FUBB-50
F
Globulin
gm./100 gm. of Body weight
8
7
6
5
4
3
2
1
0
Control
EBB
FU-25
FUBB-25
FU-50
FUBB-50

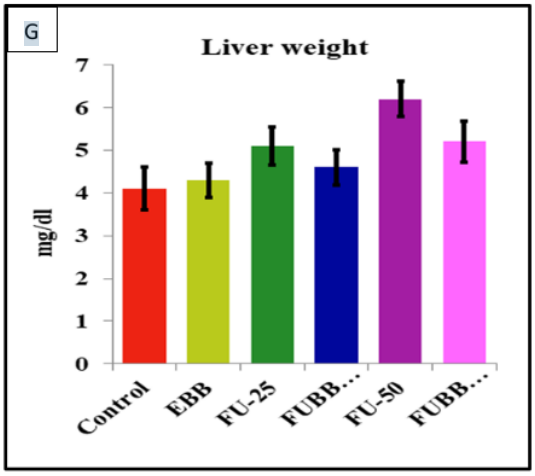


Figure 27: Liver Toxicity due to administration of 5-FU using BB To determine the Liver toxicity profile, serum samples were taken from different treated groups for (A) SGOT, (B) SGPT, (C) alkaline phosphatase, (D) albumin, (E) bilirubin (F) Globulin (G) Liver weight.

5.7.2 Table-12 Tukey's Multiple Comparison Test for the significance of biochemical parameters for Globulin

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant	Summary	Adjusted P Value
Globulin					
Control vs. EBB	-0.2	-14.31 to 13.91	No	ns	>0.9999
Control vs. FU-25	-1	-15.11 to 13.11	No	ns	>0.9999
Control vs. FUBB-25	-0.5	-14.61 to 13.61	No	ns	>0.9999
Control vs. FU-50	-2.1	-16.21 to 12.01	No	ns	0.9981
Control vs. FUBB-50	-1.1	-15.21 to 13.01	No	ns	>0.9999
EBB vs. FU-25	-0.8	-14.91 to 13.31	No	ns	>0.9999
EBB vs. FUBB-25	-0.3	-14.41 to 13.81	No	ns	>0.9999
EBB vs. FU-50	-1.9	-16.01 to 12.21	No	ns	0.9988
EBB vs. FUBB-50	-0.9	-15.01 to 13.21	No	ns	>0.9999
FU-25 vs. FUBB-25	0.5	-13.61 to 14.61	No	ns	>0.9999
FU-25 vs. FU-50	-1.1	-15.21 to 13.01	No	ns	>0.9999
FU-25 vs. FUBB-50	-0.1	-14.21 to 14.01	No	ns	>0.9999
FUBB-25 vs. FU-50	-1.6	-15.71 to 12.51	No	ns	0.9995
FUBB-25 vs. FUBB-50	-0.6	-14.71 to 13.51	No	ns	>0.9999
FU-50 vs. FUBB-50	1	-13.11 to 15.11	No	ns	>0.9999

5.7.3 Table-13 Tukey's Multiple Comparison Test for the significance of biochemical parameters for liver weight

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant	Summary	Adjusted P Value
Liver weight					
Control vs. EBB	-0.1	-14.21 to 14.01	No	ns	>0.9999
Control vs. FU-25	-0.8	-14.91 to 13.31	No	ns	>0.9999
Control vs. FUBB-25	-0.2	-14.31 to 13.91	No	ns	>0.9999
Control vs. FU-50	-1.3	-15.41 to 12.81	No	ns	0.9998
Control vs. FUBB-50	-0.9	-15.01 to 13.21	No	ns	>0.9999
EBB vs. FU-25	-0.7	-14.81 to 13.41	No	ns	>0.9999
EBB vs. FUBB-25	-0.1	-14.21 to 14.01	No	ns	>0.9999
EBB vs. FU-50	-1.2	-15.31 to 12.91	No	ns	0.9999
EBB vs. FUBB-50	-0.8	-14.91 to 13.31	No	ns	>0.9999
FU-25 vs. FUBB-25	0.6	-13.51 to 14.71	No	ns	>0.9999
FU-25 vs. FU-50	-0.5	-14.61 to 13.61	No	ns	>0.9999
FU-25 vs. FUBB-50	-0.1	-14.21 to 14.01	No	ns	>0.9999
FUBB-25 vs. FU-50	-1.1	-15.21 to 13.01	No	ns	>0.9999
FUBB-25 vs. FUBB-50	-0.7	-14.81 to 13.41	No	ns	>0.9999
FU-50 vs. FUBB-50	0.4	-13.71 to 14.51	No	ns	>0.9999

5.7.4 Table 14 Comparative changes in biochemical parameters in mice for system toxicity

Serum component (unit)	Control	EBB	FU-25	FUBB-25	FU-50	FUBB-50
SGOT (IU/ml)	25±3.1	26±4.0	32±3.6	28±3.4	38±4.1	**33±3.8**
SGPT (IU/ml)	30±3.8	31±4.3	37±3.2	33±3.6	43±3.5	**38±3.7**
Albumin (g/dL)	6.12±0.52	6.24±0.61	7.04±0.55	6.78±0.63	7.98±0.51	**7.14±0.65**
Bilirubin (mg/dL)	0.58±0.06	0.6±0.07	0.73±0.06	0.65±0.06	0.83±0.05	**0.75±0.04**
Cholesterol (mg/dL)	92.25±11.52	94.38±10.12	104.52±9.63	96.84±9.83	115.53±9.15	**107.63±10.01**
Triglyceride (mg/dL)	106.8±15.3	105.3±13.2	120.6±14.3	109.6±15.1	128.6±13.3	**121.3±14.1**
Globulin (g/dL)	4.1±0.5	4.3±0.4	5.1±0.45	4.6±0.42	6.2±0.41	**5.2±0.48**
Creatinine (mg/dL)	0.73±0.04	0.75±0.04	0.81±0.05	0.76±0.04	0.89±0.05	**0.84±0.04**

Alkaline phosphatase (mg/dL)	**21.05±4.15**	**19.65±5.01**	**28.35±5.23**	**22.15±4.73**	**30.62±4.98**	**29.25±5.11**

5.7.5 Table-15 Tukey's Multiple Comparison Test for the significance of biochemical parameters for SGOT

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
SGOT					
Control vs. EBB	-1	-15.11 to 13.11	No	ns	>0.9999
Control vs. FU-25	-7	-21.11 to 7.109	No	ns	0.7045
Control vs. FUBB-25	-3	-17.11 to 11.11	No	ns	0.9897
Control vs. FU-50	-13	-27.11 to 1.109	No	ns	0.0893
Control vs. FUBB-50	-8	-22.11 to 6.109	No	ns	0.5724
EBB vs. FU-25	-6	-20.11 to 8.109	No	ns	0.8205
EBB vs. FUBB-25	-2	-16.11 to 12.11	No	ns	0.9985
EBB vs. FU-50	-12	-26.11 to 2.109	No	ns	0.1435
EBB vs. FUBB-50	-7	-21.11 to 7.109	No	ns	0.7045
FU-25 vs. FUBB-25	4	-10.11 to 18.11	No	ns	0.9631
FU-25 vs. FU-50	-6	-20.11 to 8.109	No	ns	0.8205
FU-25 vs. FUBB-50	-1	-15.11 to 13.11	No	ns	>0.9999
FUBB-25 vs. FU-50	-10	-24.11 to 4.109	No	ns	0.3193
FUBB-25 vs. FUBB-50	-5	-19.11 to 9.109	No	ns	0.9082
FU-50 vs. FUBB-50	5	-9.109 to 19.11	No	ns	0.9082

5.7.6 Table-16 Tukey's Multiple Comparison Test for the significance of biochemical parameters for SGPT

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant	Summary	Adjusted P Value
SGPT					
Control vs. EBB	-1	-15.11 to 13.11	No	ns	>0.9999
Control vs. FU-25	-7	-21.11 to 7.109	No	ns	0.7045
Control vs. FUBB-25	-3	-17.11 to 11.11	No	ns	0.9897
Control vs. FU-50	-13	-27.11 to 1.109	No	ns	0.0893
Control vs. FUBB-50	-8	-22.11 to 6.109	No	ns	0.5724
EBB vs. FU-25	-6	-20.11 to 8.109	No	ns	0.8205
EBB vs. FUBB-25	-2	-16.11 to 12.11	No	ns	0.9985
EBB vs. FU-50	-12	-26.11 to 2.109	No	ns	0.1435
EBB vs. FUBB-50	-7	-21.11 to 7.109	No	ns	0.7045

FU-25 vs. FUBB-25	4	-10.11 to 18.11	No	ns	0.9631
FU-25 vs. FU-50	-6	-20.11 to 8.109	No	ns	0.8205
FU-25 vs. FUBB-50	-1	-15.11 to 13.11	No	ns	>0.9999
FUBB-25 vs. FU-50	-10	-24.11 to 4.109	No	ns	0.3193
FUBB-25 vs. FUBB-50	-5	-19.11 to 9.109	No	ns	0.9082
FU-50 vs. FUBB-50	5	-9.109 to 19.11	No	ns	0.9082

5.7.7 Table-17 Tukey's Multiple Comparison Test for the significance of biochemical parameters for Albumin

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant	Summary	Adjusted P Value
Albumin					
Control vs. EBB	-0.22	-14.33 to 13.89	No	ns	>0.9999
Control vs. FU-25	-0.92	-15.03 to 13.19	No	ns	>0.9999
Control vs. FUBB-25	-0.66	-14.77 to 13.45	No	ns	>0.9999
Control vs. FU-50	-1.86	-15.97 to 12.25	No	ns	0.9989
Control vs. FUBB-50	-1.02	-15.13 to 13.09	No	ns	>0.9999
EBB vs. FU-25	-0.7	-14.81 to 13.41	No	ns	>0.9999
EBB vs. FUBB-25	-0.44	-14.55 to 13.67	No	ns	>0.9999
EBB vs. FU-50	-1.64	-15.75 to 12.47	No	ns	0.9994
EBB vs. FUBB-50	-0.8	-14.91 to 13.31	No	ns	>0.9999
FU-25 vs. FUBB-25	0.26	-13.85 to 14.37	No	ns	>0.9999
FU-25 vs. FU-50	-0.94	-15.05 to 13.17	No	ns	>0.9999
FU-25 vs. FUBB-50	-0.1	-14.21 to 14.01	No	ns	>0.9999
FUBB-25 vs. FU-50	-1.2	-15.31 to 12.91	No	ns	0.9999
FUBB-25 vs. FUBB-50	-0.36	-14.47 to 13.75	No	ns	>0.9999
FU-50 vs. FUBB-50	0.84	-13.27 to 14.95	No	ns	>0.9999

5.7.8 Table-18 Tukey's Multiple Comparison Test for the significance of biochemical parameters for Alkaline phosphatase

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant	Summary	Adjusted P Value
Alkaline Phosphatase					
Control vs. EBB	1.4	-12.71 to 15.51	No	ns	0.9997
Control vs. FU-25	-7.3	-21.41 to 6.809	No	ns	0.6659
Control vs. FUBB-25	-1.1	-15.21 to 13.01	No	ns	>0.9999
Control vs. FU-50	-9.57	-23.68 to 4.539	No	ns	0.3688
Control vs. FUBB-50	-8.2	-22.31 to 5.909	No	ns	0.5455
EBB vs. FU-25	-8.7	-22.81 to 5.409	No	ns	0.4787

EBB vs. FUBB-25	-2.5	-16.61 to 11.61	No	ns	0.9956
EBB vs. FU-50	-10.97	-25.08 to 3.139	No	ns	0.2223
EBB vs. FUBB-50	-9.6	-23.71 to 4.509	No	ns	0.3653
FU-25 vs. FUBB-25	6.2	-7.909 to 20.31	No	ns	0.7992
FU-25 vs. FU-50	-2.27	-16.38 to 11.84	No	ns	0.9972
FU-25 vs. FUBB-50	-0.9	-15.01 to 13.21	No	ns	>0.9999
FUBB-25 vs. FU-50	-8.47	-22.58 to 5.639	No	ns	0.5092
FUBB-25 vs. FUBB-50	-7.1	-21.21 to 7.009	No	ns	0.6917
FU-50 vs. FUBB-50	1.37	-12.74 to 15.48	No	ns	0.9998

5.7.9 Table-19 Tukey's Multiple Comparison Test for the significance of biochemical parameters for Bilirubin

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant	Summary	Adjusted P Value
Bilirubin					
Control vs. EBB	-0.02	-14.13 to 14.09	No	ns	>0.9999
Control vs. FU-25	-0.15	-14.26 to 13.96	No	ns	>0.9999
Control vs. FUBB-25	-0.07	-14.18 to 14.04	No	ns	>0.9999
Control vs. FU-50	-0.25	-14.36 to 13.86	No	ns	>0.9999
Control vs. FUBB-50	-0.17	-14.28 to 13.94	No	ns	>0.9999
EBB vs. FU-25	-0.13	-14.24 to 13.98	No	ns	>0.9999
EBB vs. FUBB-25	-0.05	-14.16 to 14.06	No	ns	>0.9999
EBB vs. FU-50	-0.23	-14.34 to 13.88	No	ns	>0.9999
EBB vs. FUBB-50	-0.15	-14.26 to 13.96	No	ns	>0.9999
FU-25 vs. FUBB-25	0.08	-14.03 to 14.19	No	ns	>0.9999
FU-25 vs. FU-50	-0.1	-14.21 to 14.01	No	ns	>0.9999
FU-25 vs. FUBB-50	-0.02	-14.13 to 14.09	No	ns	>0.9999
FUBB-25 vs. FU-50	-0.18	-14.29 to 13.93	No	ns	>0.9999
FUBB-25 vs. FUBB-50	-0.1	-14.21 to 14.01	No	ns	>0.9999
FU-50 vs. FUBB-50	0.08	-14.03 to 14.19	No	ns	>0.9999

5.7.10 Immunology parameters and Body weight of mice during the experiment

Host immune respon against BB-mediated drug delivery. The blood cell count from serum significantly changes the treatment group compared to the control. However, no significant differences were observed between the FU-50 and FUBB-50 groups. The figure represe the count of leukocytes, red blood cells, and platelets, respectively. Further, pro-inflammatory interleukin-6 (IL-6) level analysis by enzyme-linked immunosorbent assay (ELISA) revealed that, due to the treatment, a considerable change took place in the IL-6 level in comparison with control when treated with 5-FU. Still, no significant difference was observed between the FU-50 and FUBB-50 groups. For anti-inflammatory response (IL-10), a slight increase was observed in the treatment groups compared to

the control, as shown in Fig 25. However, the difference was not significant enough for any set at 5 days after treatment.

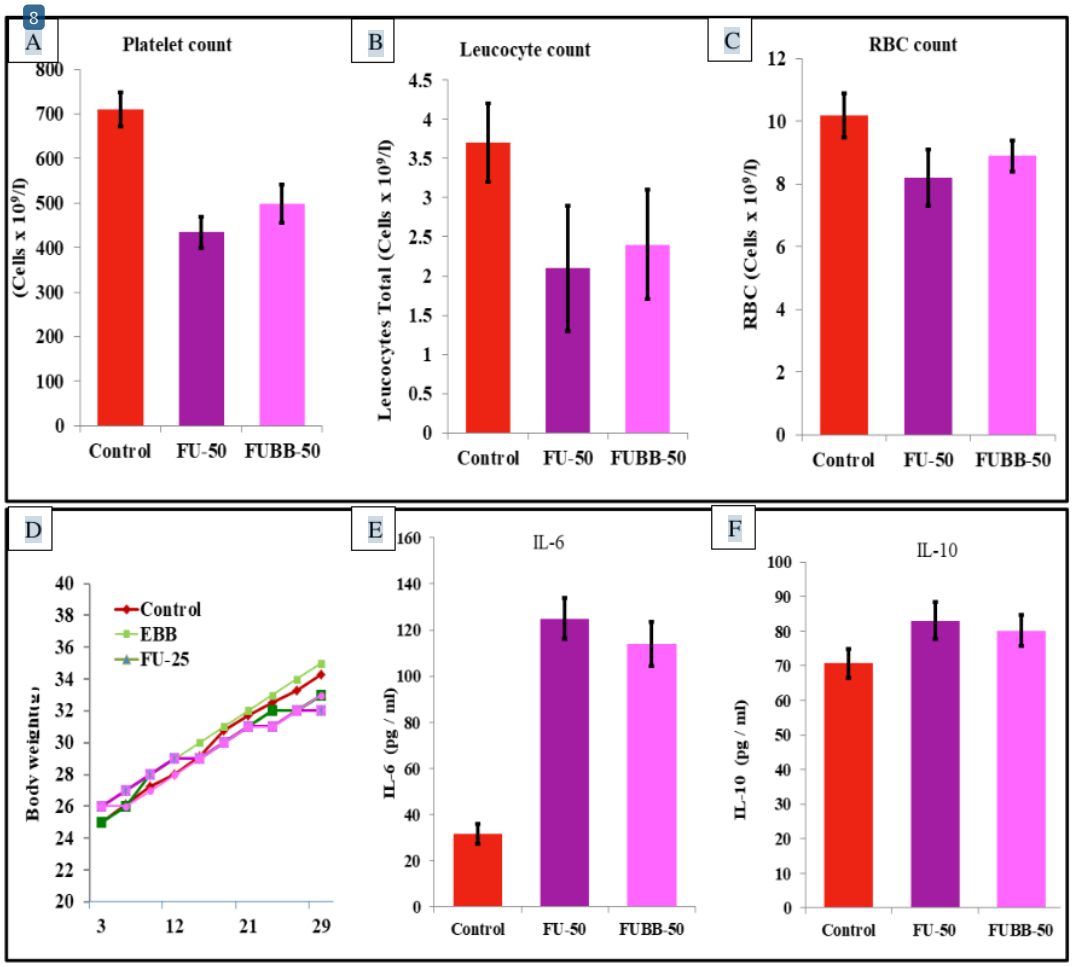


Figure 28: Immunology parameters and Body weight of mice due to administration of 5FU as anti-cancerous drug (A) Platelet count (B) Leucocyte (C) RBC (D) Body Weight (E) IL-6 (F) IL-10

5.7.11 Lipid Profile

In vivo studies involving the use of 5-fluorouracil (5-FU) as an anticancer agent, monitoring the lipid profile, specifically triglycerides and cholesterol, is crucial. The lipid profile serves as an important indicator of the metabolic impact of 5-FU treatment. Triglycerides and cholesterol are central to various biological processes, including cell membrane integrity, energy storage, and hormone production[196]. Aberrations in these lipid parameters can provide insights into the metabolic side effects of 5-FU.

Elevated triglyceride levels in mice treated with 5-FU may indicate disruptions in lipid metabolism, potentially caused by the drug's impact on the liver's ability to process and store fats. This can lead to hypertriglyceridemia, which is associated with an increased risk of cardiovascular diseases and could suggest hepatic toxicity or other metabolic disturbances induced by the treatment.
Similarly, monitoring cholesterol levels is essential, as alterations can reflect changes in lipid biosynthesis or degradation pathways.

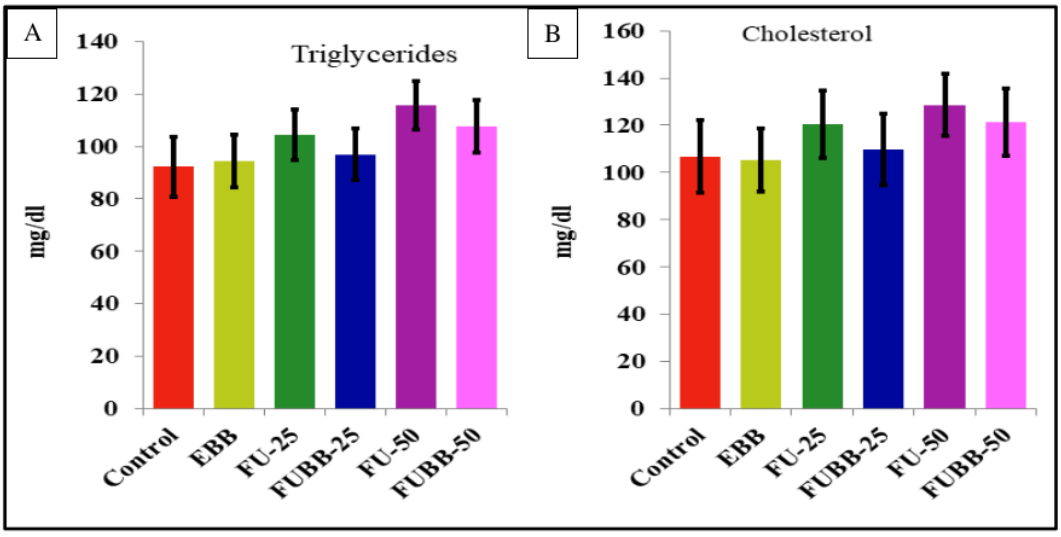


Figure 29: Lipid profile of mice after administration of 5FU as anti-cancerous drug (A) Triglycerides levels (B) Cholesterol

As the doses of 5-FU increased to FU-25 and FU-50, there was a substantial elevation observed in triglyceride and cholesterol level values. However, when treated with FUBB-50, it helped reduce these elevated levels, while the FUBB-25 dose resulted in a substantial reduction in both triglyceride and cholesterol serum levels. In comparison, treatment with EBB. showed negligible changes in these parameters when compared to the control groups.

5.7.12 Table-20 Tukey's Multiple Comparison Test for the significance of biochemical parameters for Cholesterol levels

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant	Summary	Adjusted P Value
Cholesterol					
Control vs. EBB	-2.13	-16.24 to 11.98	No	ns	0.9979
Control vs. FU-25	-12.27	-26.38 to 1.839	No	ns	0.1269

Control vs. FUBB-25	-4.59	-18.70 to 9.519	No	ns	0.9346
Control vs. FU-50	-23.28	-37.39 to -9.171	Yes	****	<0.0001
Control vs. FUBB-50	-15.38	-29.49 to -1.271	Yes	*	0.0241
EBB vs. FU-25	-10.14	-24.25 to 3.969	No	ns	0.304
EBB vs. FUBB-25	-2.46	-16.57 to 11.65	No	ns	0.9959
EBB vs. FU-50	-21.15	-35.26 to -7.041	Yes	***	0.0004
EBB vs. FUBB-50	-13.25	-27.36 to 0.8590	No	ns	0.0787
FU-25 vs. FUBB-25	7.68	-6.429 to 21.79	No	ns	0.6155
FU-25 vs. FU-50	-11.01	-25.12 to 3.099	No	ns	0.2188
FU-25 vs. FUBB-50	-3.11	-17.22 to 11.00	No	ns	0.9879
FUBB-25 vs. FU-50	-18.69	-32.80 to -4.581	Yes	**	0.0027
FUBB-25 vs. FUBB-50	-10.79	-24.90 to 3.319	No	ns	0.2387
FU-50 vs. FUBB-50	7.9	-6.209 to 22.01	No	ns	0.5859

5.7.13 Table-21 Tukey's Multiple Comparison Test for the significance of biochemical parameters for Triglycerides

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
Triglycerides					
Control vs. EBB	1.5	-12.61 to 15.61	No	ns	0.9996
Control vs. FU-25	-13.8	-27.91 to 0.3090	No	ns	0.0591
Control vs. FUBB-25	-2.8	-16.91 to 11.31	No	ns	0.9925
Control vs. FU-50	-21.8	-35.91 to -7.691	Yes	***	0.0002
Control vs. FUBB-50	-14.5	-28.61 to -0.3910	Yes	*	0.0402
EBB vs. FU-25	-15.3	-29.41 to -1.191	Yes	*	0.0253
EBB vs. FUBB-25	-4.3	-18.41 to 9.809	No	ns	0.95

EBB vs. FU-50	-23.3	-37.41 to -9.191	Yes	****	<0.0001
EBB vs. FUBB-50	-16	-30.11 to -1.891	Yes	*	0.0165
FU-25 vs. FUBB-25	11	-3.109 to 25.11	No	ns	0.2197
FU-25 vs. FU-50	-8	-22.11 to 6.109	No	ns	0.5724
FU-25 vs. FUBB-50	-0.7	-14.81 to 13.41	No	ns	>0.9999
FUBB-25 vs. FU-50	-19	-33.11 to -4.891	Yes	**	0.0022
FUBB-25 vs. FUBB-50	-11.7	-25.81 to 2.409	No	ns	0.1639
FU-50 vs. FUBB-50	7.3	-6.809 to 21.41	No	ns	0.6659

5.7.14 Kidney Toxicity

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Creatinine is a waste product resulting from the normal metabolism of muscle cells, specifically from the breakdown of creatine phosphate[197]. Under normal circumstances, the kidneys filter creatinine out of the bloodstream and excrete it in the urine. The efficiency of this filtration process is measured by the creatinine clearance rate, which serves as a direct indicator of kidney health. Since the rate at which creatinine is produced remains fairly constant and its primary elimination route is through the kidneys, fluctuations in blood creatinine levels can signal changes in kidney function.

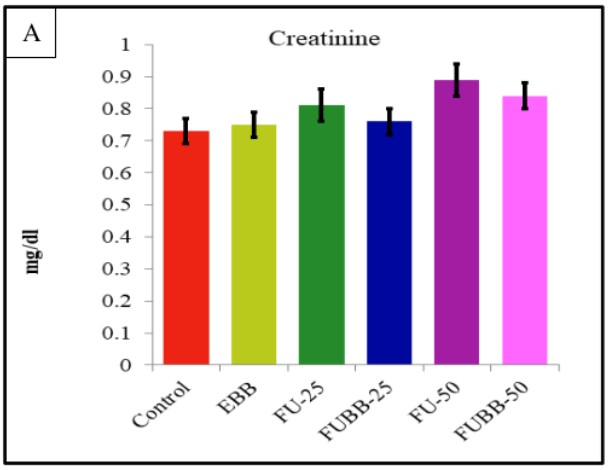

Figure 30 A: Kidney Toxicity profile of mice after administration of 5FU as anticancerous drug
Fig showing Creatinine levels in mice as kidney toxicity profile

With increasing doses of 5-FU (FU-25 and FU-50), there was a noticeable rise in nearly all measured parameters, including creatinine levels. However, treatment with FUBB-50 led to a reduction in these

elevated values, and the FUBB-25 dose significantly lowered the parameter values even further. In contrast, treatment with E.B. resulted in negligible changes in creatinine levels compared to the control groups.

5.7.15 Table-22 Tukey's Multiple Comparison Test for the significance of biochemical parameters for Creatinine

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
Creatinine					
Control vs. EBB	-0.02	-14.13 to 14.09	No	ns	>0.9999
Control vs. FU-25	-0.08	-14.19 to 14.03	No	ns	>0.9999
Control vs. FUBB-25	-0.03	-14.14 to 14.08	No	ns	>0.9999
Control vs. FU-50	-0.16	-14.27 to 13.95	No	ns	>0.9999
Control vs. FUBB-50	-0.11	-14.22 to 14.00	No	ns	>0.9999
EBB vs. FU-25	-0.06	-14.17 to 14.05	No	ns	>0.9999
EBB vs. FUBB-25	-0.01	-14.12 to 14.10	No	ns	>0.9999
EBB vs. FU-50	-0.14	-14.25 to 13.97	No	ns	>0.9999
EBB vs. FUBB-50	-0.09	-14.20 to 14.02	No	ns	>0.9999
FU-25 vs. FUBB-25	0.05	-14.06 to 14.16	No	ns	>0.9999
FU-25 vs. FU-50	-0.08	-14.19 to 14.03	No	ns	>0.9999
FU-25 vs. FUBB-50	-0.03	-14.14 to 14.08	No	ns	>0.9999
FUBB-25 vs. FU-50	-0.13	-14.24 to 13.98	No	ns	>0.9999
FUBB-25 vs. FUBB-50	-0.08	-14.19 to 14.03	No	ns	>0.9999
FU-50 vs. FUBB-50	0.05	-14.06 to 14.16	No	ns	>0.9999

6.1 Discussion of results

The study aims to develop a universal drug delivery system that can deliver many drugs through the oral route with an increased half-life in the intestinal region. The researchers used metabolically active *L. reuteri*, which secretes biofilm in the gastric and intestinal conditions, to achieve sustained cargo release in the gastrointestinal tract. The BB system was used for this purpose, allowing for efficient intestinal absorption of drugs and increased bioavailability in vivo[97].

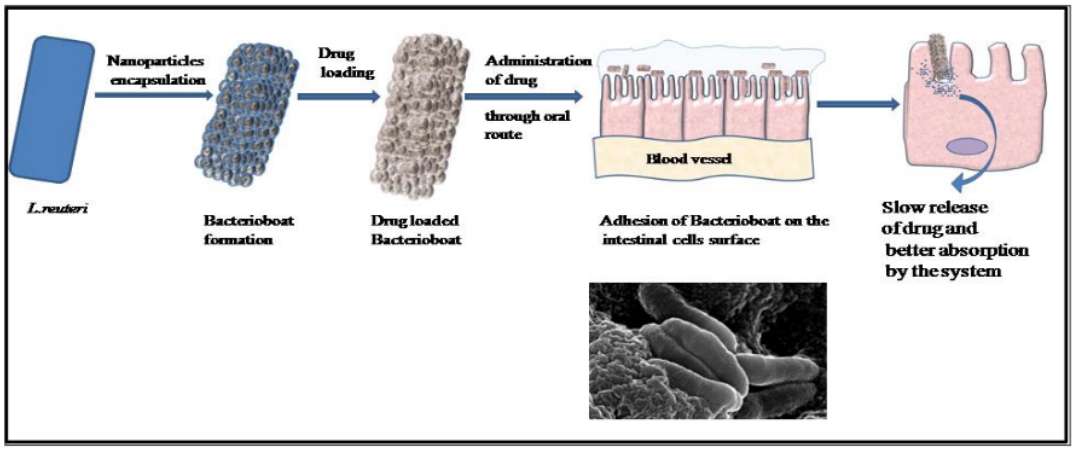

Figure 31: Representation of BB attachment on the intestinal lining. The schematic illustration shows the intestinal anchorage of BB and its sustained intestinal release of the drug and enhanced absorption of the drug for the orally administered BB system.

We developed an oral delivery method that will show sustained cargo release in the GI tract with a superior half-life period in the GI tract. Here, we have used metabolically active *L. reuteri* for this purpose. Metabolically active *L. reuteri* secretes biofilm in the gastric and intestinal conditions, allowing them to adhere to the microvilli's intestinal surface. A conclusive schematic representation demonstrating the advantages of the BB system in terms of intestinal anchorage and sustained release of the drug is presented. qPCR analysis with *L. reuteri*–specific primers revealed that the abundance of the bacteria was around 10-fold higher in the treated murine intestine sample compared to the untreated murine intestine. We have further used cell wall encapsulation using mesoporous carbohydrate nanoparticles, which can absorb various molecules (such as drugs and dye) released sustainably around the microvilli, resulting in efficient intestinal absorption of the drug. Enhanced and prolonged absorption resulted in an increase in the bioavailability of the drug in vivo. We have shown that the formulation can increase the bioavailability of 5-FU for up to 24 hours when applied through BB compared to the conventional oral dose where the traces of the drug were detectable in the serum for up to 8 hours. An increase in bioavailability also resulted in the requirement of administrable drug doses. Solid tumour size reduction and animal serval data suggested that even at half of the

recommended dose, the comparable (with recommended dose) tumour size reduction was observed when the drug was applied through BB, which resulted in better animal survival. The animal showed 10% enhanced survival when half of the recommended dose was applied through BB compared to the recommended dose, which may happen because of the reduction of the drug-related side effects. 5-FU is reported to cause a considerable increase in hepatic fat content, resulting in frequent hepatic steatosis in patients. Therefore, it would be interesting to know how applying drugs through BB resulted in liver damage and serum fat accumulation. Different parameters including SGOT, SGPT, albumin, globulin, alkaline phosphatase, liver weight, and BW analysis, showed a noticeable reduction of drug-related liver toxicity. Further, FUBB showed a noticeable reduction of drug-induced serum cholesterol and triglyceride level. There have been several medications with nanoformulations that have been authorized by the Food and Drug Administration (FDA) of the United States of America since the 1990s[173]. These drugs were found to be more effective and to have less adverse effects in comparison to the pharmaceuticals that are now available on the market. For instance, the Food and Drug Administration (FDA) and the European Medicines Agency (EMA) approved Doxil/Caelyx (Janssen) liposomal doxorubicin (PEGylated) in the year 1995. More recently, the FDA (2017) and the EMA (2018) approved Jazz Pharmaceuticals' liposomal versions of cytarabine[173]. These formulations exhibit several adverse effects, including the transport of nanoparticles inside the lymphatic system or circulation, as well as concerns about the stability and toxicity of the nanoparticles themselves as they are mostly administered via the intravenous method. As per the present system, chitosan nanoparticles remain in the gut (chitosan being a nontoxic, biodegradable, carbohydrate-based polymer used to form the nanoparticles over the surface of GRAS bacteria *L. reuteri*). *L. reuteri* , which has a probiotic potential and is a normal gut inhabitant, was used as a vehicle for EBB. Further, it is possible to generate BB with other microflora and may be explored for drug delivery and other purposes. BB can be stored after lyophilisation and loaded with various drugs without changing the drug's chemistry, making them an ideal candidate vehicle for oral drug delivery. In addition to that, BB does not considerably alter the body's immune response. Despite many advantages of intestinal drugs, absorption might have limited application for slightly acidic drugs primarily absorbed by the stomach, as low stomach pH is unfavourable for BB to bind. However, its surface coating has shown excellent sustained-release properties at the SGF, suggesting that BB made with suitable bacteria such as Prevotella spp., Streptococcus spp., and Veillonella spp. is among the other viable options for this purpose. Further, because the bacteria are metabolically active, their application through alternative routes, such as intravenous, intramuscular, and intradermal, is limited. At this point, further exploration and evolution are needed in preclinical and clinical trials to understand its true futuristic potential for clinical application

We applied for the patent on the outcomes of this project funded by DST/SERB. Project no. (ECR/2016/000486)

Title of the patent: Universal delivery system for drugs/ molecules of choice using live microbes and method thereof with Application **number 201911027084.** Date of filing of Application:05/07/2019 and Publication Date: 08/03/2021, and it is granted on 29/02/2024. The abstract of this application: The invention discloses a universal delivery system for drugs/molecules of choice using live microbes coated with porous nanoparticles, to which the same are attached. In one embodiment, the porous nanomaterial-coated microbes such as bacteria can be made and preserved like hollow capsules and then, as per requirement, can be incubated with any drug/molecule of choice. In a second embodiment, the nanoparticles can be loaded with the drug/molecule of choice separately and then coated onto the

surface of the cell wall of live microbes such as bacteria, e.g., *Lactobacillus reuteri, Bifidobacterium longum* or some other non-pathogenic intestinal bacteria. Moreover, the hollow porous nanoparticles (like chitosan or carbohydrate, lipids, proteins, or natural or synthetic inorganic, organic polymer) can be directly synthesised on the cell wall of the bacteria, and further cross-linking can be done for stabilisation. The coated microbes remain metabolically active and can form biofilm formation[58].

क्रम सं/SL No :011200825

INTELLECTUAL PROPERTY INDIA

सत्यमेव जयते

पेटेंट कार्यालय,भारत सरकार
The Patent Office, Government Of India

पेटेंट प्रमाण पत्र | Patent Certificate

(पेटेंट नियमावली का नियम 74) | (Rule 74 of The Patents Rules)

पेटेंट सं. / Patent No.	:	517173
आवेदन सं. / Application No.	:	201911027084
फाइल करने की तारीख / Date of Filing	:	05/07/2019
पेटेंटी / Patentee	:	THAPAR INSTITUTE OF ENGINEERING AND TECHNOLOGY
आविष्कारकों का नाम /Name of Inventor(s)	:	1.KAUR PARMANDEEP 2.CHOUDHURY DIPTIMAN

प्रमाणित किया जाता है कि पेटेंटी को, उपरोक्त आवेदन में यथाप्रकटित *UNIVERSAL DELIVERY SYSTEM FOR DRUGS/MOLECULES OF CHOICE USING LIVE MICROBES AND METHOD THEREOF* नामक आविष्कार के लिए, पेटेंट अधिनियम, 1970 के उपबंधों के अनुसार आज तारीख जुलाई 2019 के पांचवें दिन से बीस वर्ष की अवधि के लिए पेटेंट अनुदत्त किया गया है।

It is hereby certified that a patent has been granted to the patentee for an invention entitled *UNIVERSAL DELIVERY SYSTEM FOR DRUGS/MOLECULES OF CHOICE USING LIVE MICROBES AND METHOD THEREOF* as disclosed in the above mentioned application for the term of 20 years from the 5th day of July 2019 in accordance with the provisions of the Patents Act,1970.

अनुदान की तारीख : 29/02/2024
Date of Grant :

पेटेंट नियंत्रक
Controller of Patents

टिप्पणी - इस पेटेंट के नवीकरण के लिए फीस, यदि इसे बनाए रखा जाना है, जुलाई 2021 के पांचवें दिन को और उसके पश्चात प्रत्येक वर्ष में उसी दिन देय होगी।

Note. - The fees for renewal of this patent, if it is to be maintained, will fall / has fallen due on 5th day of July 2021 and on the same day in every year thereafter.

Main concept

- Use of GRAS bacteria non-pathogenic bacteria for biofilm production
- No genetic modification
- Designed to adhere to the intestine
- No seepage into blood
- No immune response
- Use of covalent bonding for encapsulation of bacteria on the cell wall
- Once made, no response to physiological stability, no change in particle stability
- No change in particle stability in response to change in physiological pH
- Drug can be loaded in situ or ex-situ during the formation of after the formation of particles
- An empty drug delivery system can be stored in a lyophilised condition, and the drug is loaded as per the requirement
- A variety of choice of drug can be loaded on the mesoporous surface of Universal Drug Delivery System
- The universal drug delivery system is highly metabolic active and shows encouragement in the small intestine that is highly dependent upon biofilm production during release, which takes place inside the intestine in a sustained manner
- Absorption of the drug, especially for the BCS 2,3,4 class of drugs, can be achieved through the system without any modification of the drug molecule
- Enhanced bioavailability of the drug using this delivery system
- Reduced drug-dependent toxicity of drug molecules using the system

6.2 . Technical Comparison with some of the advancements already achieved by different researchers in drug delivery systems with bacteria

S. No.	**Technical advancement / Property**	**Engineering Nanoparticle-Coated Bacteria as Oral DNA Vaccines for Cancer Immunotherapy- Nano letters (2015)**[66]	**Lactic acid bacteria: reviewing the potential of a promising delivery live vector for biomedical purposes (review 2015)**[161]	**Bacteria-based active drug delivery system-patent (2022)**[162]	**Engineered microbe-targeting molecules and uses thereof-Patent (2022)**[164]	**Universal drug delivery system (Present Invention)**[202] **Bacterioboat system**
1	Vector	Pathogenic Attenuated Bacteria-salmonella	Genetically modified bacteria, yeast, insect cells and mammalian cells to express protein	Genetically modified Bacteria	Immunologically modified Bacteria	Normal gut bacteria coated with nanoparticles

2	Nanoparticles / Delivering Moiety	Synthetic nanoparticles, constructed from cationic polymers and plasmid DNA by self-assembly	DNA insert in genetically modified organism	Biocompatible isomer micro bead containing the drug capsule and the bacterial capsule. No Nanoparticles	A microbe-targeting magnetic micro-bead No nanoparticles	Porous nanoparticles of polysaccharides, natural or synthetic, coated over the live bacterial cell wall
3	Drug Delivery/ Cargo	Oral delivery of DNA vaccines	therapeutic activity, from chemically synthesised molecules to recombinant proteins produced in bacteria, yeast, insect cells and mammalian cells	Isomeric micro bead containing drug capsule and bacteria capsule for targeted delivery	Microbe-binding molecules can be conjugated to a substrate, and the kits comprising can bind or capture a microbe or microbial matter and can thus be used in the diagnosis or treatment of an infection	Oral delivery of Drugs/ molecule of choice, e.g. Any conventional pharmaceutical drug or molecule of choice like ant cancerous drugs, anti-allergy, hypertension drugs, anti-helminthic drugs, especially of BCS (Biopharmaceutical classification system) class-II, III, IV can be loaded and delivered using this delivery system
4	Innovation	Engineered live attenuated bacteria for oral DNA vaccination in cancer immunotherapy via a shield like nanoparticle coating layer that enables bacteria to	This review paper provides an overview of using LAB-engineered (GENETICALLY MODIFIED) organisms for recombinant proteins in treating diseases.	Micro-bead that contains a drug capsule and bacteria capsule where bacteria may be Salmonella typhimurium	A microbe-targeting magnetic microbead	Nanoparticles are coated over the non-pathogenic bacterial cell wall

		efficiently evade phagosomes in the bloodstream and cause T-cell activation				
5	Mechanism	Immune response generating to make the vaccine effective	Review paper Genetically modified bacteria	Bacterial attenuation of pathogenic bacteria *Salmonella typhimurium* through genetic manipulation to avoid immune response in the human body.	microbe-binding entities consist of a carbohydrate recognition domain derived from lectin or a fragment thereof, connected to a segment of an Fc region.	Attached in the intestine, especially around the microvilli, and make biofilm stay in the intestine longer. It does sustained release over time.
6	Universality	No, differently engineered assembly for each vaccine	No	No	No	Yes, the same empty universal drug delivery system can be used for any kind of oral deliverable drug/ or molecule of choice.
7	A crucial factor for an effective mechanism	1. Only a low concentration of nanoparticle coating was found to facilitate cellular uptake of Salmonellae. 2. Synthetic polymer showing electrostatic interactions.	Genetic modification	Genetic modification	1. At least one microbe surface-binding domain 2. At least one linker between the microbe surface-binding domain and the substrate-binding domain	1. concentration range can be used. No narrow range but a wide range of concentrations used 2. Biofilm production by the live bacteria present in the universal drug delivery system to bind into the intestine and show sustained release of drug 3. porous

						nanoparticles for efficient uptake of drug or molecule and then sustained release in the body
8	Body clearance	Attenuated bacterial load to be removed by the body's immune system	Paper, do not comment on this	Suicidal genes/ antibiotic combined therapy to clear bacterial load	Removal with the magnet or some buffer solution is required	There is no need to clear from the body as the non-pathogenic gut microflora is used, though it is beneficial for gut health.
9	Genetic modification	Yes, to form attenuated strain	Yes	Yes	Immunological modification antigen antibody-like sensitive interactions	No need
11	Goes in circulation	Yes	Yes	Yes	Yes	No
12	Dosage requirement	N.A	N.A.	N.A.	N.A.	Lesser amount and number of dosages than the conventional form
13	Revivability	No	No	No	Yes, Microbe-targeting compounds may be reconstituted after treatment with a low-pH buffer	No
14	Utility	Vaccine delivery only	Designer drugs according to recombinant DNA	A target directivity against ischemic disease	For the sole purpose of diagnosing and/or treating an	Universal drug delivery system for all kinds of orally deliverable

				target directivity against cancer	illness that is caused by microorganisms	drugs to enhance their potential and to reduce the drug dosage-related toxicity
15	Immune Response	Needed immune response of the body for its functionality	It elicits an immune response	It elicits an immune response	Needed immune response of the body for its functionality	Neither needed nor causes an immune response, it makes drugs safer.

These are additional points.

1. Any biofilm-producing non -non-pathogenic gut microflora can be used for the development of a drug delivery system due to the presence of a mesoporous surface on the particles.
2. Any orally administered drug that can achieve metabolic activity in microbe is an absolute requirement for the system.

Critical points

1. Stability number
2. Size particles and pore size of particles
3. Sustained release from the mesoporous surface
4. Confocal microscopy showing the adherence of bacteria from Universal Drug Delivery System around the alveoli of mice intestine
5. lactic acid production

6.3 Technical Comparison with the conventional form and universal drug delivery system

S.No.	Technical point	Conventional form	Universal drug delivery system
1	Intestinal Absorption of less polar drugs	Shows very little absorption through the intestine	High absorption through the intestine 70-80%
2	Half-life in GI Tract	30 mins to 45 mins only	Half-life Up-to 48 hrs
3	Bioavailability of BCS-II drugs	Low as only 3 to 5 %of drugs get absorbed	As high as 70-80% of the drug/molecule of choice gets absorbed
4	Sustained Release	No	Yes, up to 48 hrs
5	Loading	Direct loading of the pharmaceutical formulation in a hollow capsule or coating to form a tablet or capsule.	Loading of pharmaceutical formulation in the universal drug delivery system, which comprises porous nanoparticles present over the bacteria's cell wall.

6	Requirement of dose	Standard dosage	Lesser than the standard dosage.
7	Frequency of dosage	Multiple dosages required	Prolonged stay in the intestine does not require multiple dosages.
8	Drug-related toxicity	Remains as such	Decreases due to the requirement of lesser dosage
9	GI Irritants	Remains as such	At least as the drug is encapsulated in the universal drug delivery system
10	Bio-Magnification	Causes bio-magnification in the environment and makes drug resistance in the environment	The drug/molecule of choice is absorbed through the intestine 70-80%, so do not cause bio-magnification and drug resistance in the environment.
11	Choice of Microbes	Always changes with the requirement and type of drug in case of recombinant or genetically modified organisms used to deliver drugs.	No change in the microbe is required. Change in microbe does not have a significant effect on the loaded drug /molecule of choice.
12	Immune response	It causes an immune response in the drugs, carrying pathogenic bacteria that go into the circulation for drug delivery.	Do not cause immune responses as the bacteria used for making universal drug delivery systems are non-pathogenic, and they are retained in the GI -Tract. No seepage in the circulation is there for a universal drug delivery system.
13	Nanoparticles related toxicity	The formulations of nanoparticles have several undesirable consequences, including the trafficking of these nanoparticles in lymphatic systems or circulation, as well as problems with the nanoparticles' stability and toxicity.	No toxicity issue as per the present system. Polysaccharide nanoparticles, like chitosan, are used, are non-toxic, and remain in the gut only.
14	Choice of route of delivery for BCS-IV drugs	For BCS-IV drugs, other than the oral route is used for drug administration.	Using a universal drug delivery system, the BCSIV drugs can also be given through the oral route.
15	Peptide-based delivery	The oral route is not favoured	Using a universal drug delivery system, peptide-based delivery can be done through an oral route.

16	Protection of drug from the stomach acid	No	Up-to large extent
17	Additional benefits	None	Probiotics improve the immunity of the person.
18	Overall health condition	Depends only on the drug administered	Better than when on conventional medication only.

6.4 Comparison with the available sustain release formulations

S. No.	Technical points	Available sustained-release formulation	Universal drug delivery system
1	Intestinal window	30 minutes to 1 hour	Up to 48 hr or more
2	Particle stability	30 minutes, then go out through faeces	Stays in the intestine for at least 48 hrs
3	Sustained release	Up to the time the particles are available in the intestine, i.e., up to I hr.	Up to the time, a universal drug delivery system is available for at least up to 48 hours.
4	Bioavailability	Bioavailability is greater than conventional forms, as Nano-formulations are more efficient.	More efficient than the present Nano formulations due to the biofilm formation of bacteria and its stability
5	Bio-magnification	Causes bio-magnification but is less than the conventional form	Least bio magnification due to prolonged and efficient release
6	Acid protection	Yes	Yes
7	Additional benefit	No	Probiotic bacteria pose better immunity to the system as the intestinal microflora prepares the universal drug delivery system.

6.5 Future Directions and Limitations

6.5.1 Translation of the research to the market through clinical trials

With the release of the patent, we have a prototype on which we want to translate the research outcome in the coming time. We will use the different microflora as well as different types of nanoparticles like liposomes, organic nanoparticles, inorganic nanoparticles or carbon dots to make the system more efficient and can have broader applications in the oral drug delivery system.

Throughout the video, I provide visual demonstrations of the system in action, showcasing its potential applications in various therapeutic areas. Finally, I discuss future directions for this innovation and its potential to revolutionise patient care by making treatments more effective and accessible.

For more detailed insights and visual demonstrations, you can watch the video here by clicking the link provided titled Bacterioboat- A Novel Drug Delivery System

https://youtu.be/u1oz300o-0w

6.5.2 Use of the other microflora to make BB for drug delivery

We also generated BB with other microflora, such as *Acinetobacter catcoacetlus, Escherichia coli, Bacillus subtilis, and Lactobacillus rhamnosus*, as shown in Fig.32. and these bacteria may also be explored for drug delivery and other purposes. However, further exploration and evolution are needed in preclinical and clinical trials to understand its true futuristic potential for clinical application.

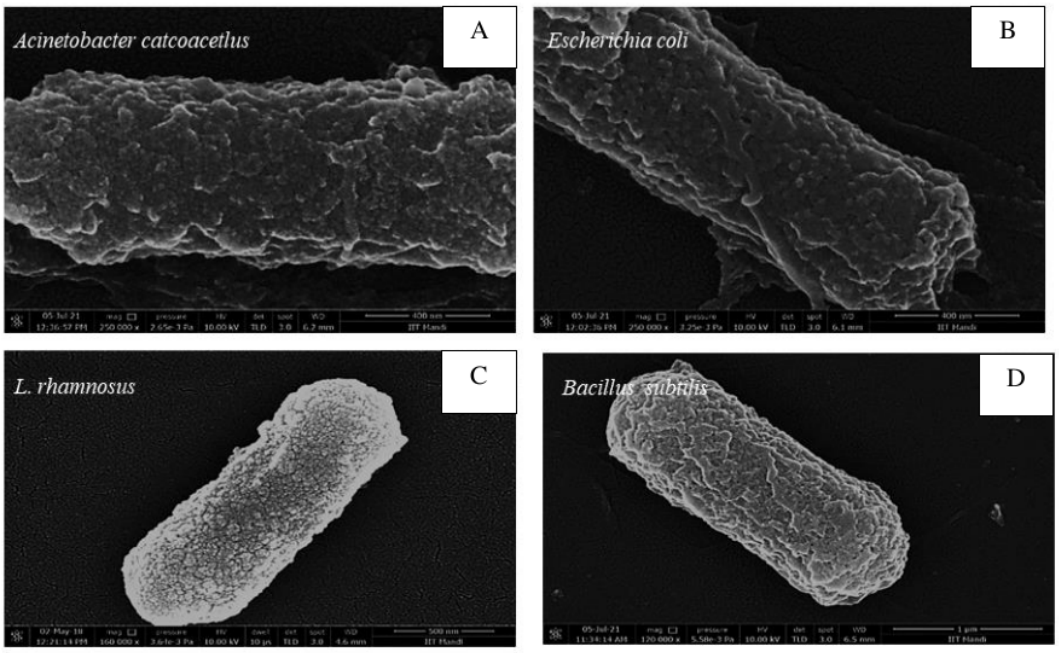

Figure 32: BB from other Bacteria (A) *Acinetobacter catcoacetlus* (B) *Escherichia coli* (C) *Lactobacillus rhamnosus* (D) *Bacilus subtilis*

6.5.3 BB for the Delivery of Anti-helminthic Drugs

We will employ the BB system for the Targeted Delivery of ABZ (Albendazole). Gastrointestinal infections caused by parasitic nematodes pose a considerable global health challenge, impacting human and animal populations. These widespread infections contribute to significant health complications, highlighting the need for effective prevention and treatment strategies. The burden of these parasitic diseases underscores their relevance in public health initiatives and veterinary care worldwide[110,198]. We are going to deploy the BB system to reduce the toxicity and, at the same time, increase its window in the intestine as it is low absorption drug, making the delivery of Albendazole more efficient. The work is in progress for the manuscript.

6.5.4 Limitations

This system has been devised for the oral drug delivery system. BB system cannot be used for the I.V. or other routes of drug delivery. Its working is limited to oral drug delivery as the system contains bacteria as an important part of its function, and biofilm formation is important for the system to function properly, which is possible inside the GI tract only.
Secondly, we are using the live microbe for drug delivery; hence, the system could be fatal for immunocompromised people. The normal microflora can cause disease in immunocompromised people. For example, exceptional cases like bubble baby syndrome condition in which the baby has an impaired immune system that only lives in a microbe-free environment inside the bubble, where even the microbe present in the environment is fatal for the system[199]. This system is not functional for SCID (Swere combined immunodeficiency syndrome) people[200].

6.6 Conclusion

The oral route of medication delivery is the most comfortable method of administration[1]. The brief half-life of the medications in the gastrointestinal system leads to their elimination from the body before their absorption, necessitating frequent administrations of the medication[201]. As a result, this leads to medication waste and undue damage to the body's organs. We developed a technique(UDDS) using living, metabolically active intestinal microorganisms to enhance the drug's retention period in the gastrointestinal tract[179]. In this drug delivery method, 5-FU-loaded 15-25 nm chitosan nanoparticles are coated on living *L. reuteri*. Both the drug release research and the FE-SEM investigation demonstrated that the drug-loaded chitosan nanoparticles were stable over the whole pH spectrum that is observed in the gastrointestinal tracts of people. This drug delivery system, when taken orally, will go and bind to the micro-villi areas of the small intestine. This is proven by confocal microscopy and Field emission electron microscopy (FE-SEM) investigation of the binding of this drug delivery vector to the mice intestine in vitro. A biofilm will be formed in the gut by metabolically active bacteria in the core, enabling the biofilm to securely connect the whole system to the villi area of the intestine. There, the chitosan nanoparticles will make it possible for the medicine to be released in the gut sustainably over an extended length of time[72]. This will result in an increase in the drug's half-life in the gastrointestinal system by a significant number of times. It is estimated that the drug loading efficiency of this drug delivery system is around 90 percent at its highest when the drug loading is performed after the lyophilisation of the system. On the other hand, the drug loading efficiency is approximately 75 to 80 percent at its highest when it is performed during the production of the system. The drug is absorbed by the mesoporous surface that is generated by the chitosan nanoparticles, and it demonstrates continuous release of the drug at a pH of 7.4. In this case, over 80 percent of the drug is released after 48 hours of incubation, which indicates that it has the potential to be an extremely effective drug delivery system of the future. Studies conducted in vivo on lab mice demonstrated that oral administration of 5-fluorouracil as FUBB leads to an increase in its efficacy, which in turn leads to an improvement in the shrinking of solid tumours, an increase in life expectancy, and a reduction in adverse effects. Because of its innovative design and development, this system is perfect for orally administrable medications with limited solubility or permeability, or both, and it even makes it possible to provide them at a lower dosage while still keeping them effective.

Our objective is to take it to the next level by translating the research that we have done on the BB universal drug delivery system into the market for the benefit of both human and environmental benefits.

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