CHAPTER 2
LITERATURE REVIEWS

2.1 *Salmonella* spp.

Salmonella, a genus within the *Enterobacteriaceae* family, was first identified by American veterinary pathologist Daniel Elmer Salmon in 1885 during his investigations into the etiology of swine cholera (Bintsis, 2017). The genus is classified into two species: *Salmonella enterica* and *Salmonella bongori*, the latter being primarily associated with cold-blooded animals (Grey, Tee, Phillips, Micalizzi and Armstrong, 2024). *Salmonella enterica* is further categorized into six subspecies: *S. enterica subsp. enterica, salamae, arizonae, diarizonae, houtenae*, and *indica*, as shown in **Figure 2.1**. Notably, nearly all *Salmonella* pathogens affecting humans and domestic animals are classified under *S. enterica* subsp. *enterica* (Fàbrega and Vila, 2013; Bintsis, 2017). The antigenic composition of *Salmonella* plays a crucial role in its classification and pathogenicity. The three primary antigens include: H (flagellar) antigen: determined by flagellar proteins and capable of phase variation, classified into phase 1 and phase 2, allowing *Salmonella* to alternate between these phases. O (somatic) antigen: located on the outer membrane and defined by the structure of lipopolysaccharides (LPS), essential for serogroup classification. Vi (capsular) antigen: a superficial antigen that overlays the O antigen and plays a role in virulence. The O antigen is primarily used for determining serogroups, while the H antigen complex is critical for defining serovars (serotypes) of *Salmonell*a strains (Giannella, 1996).

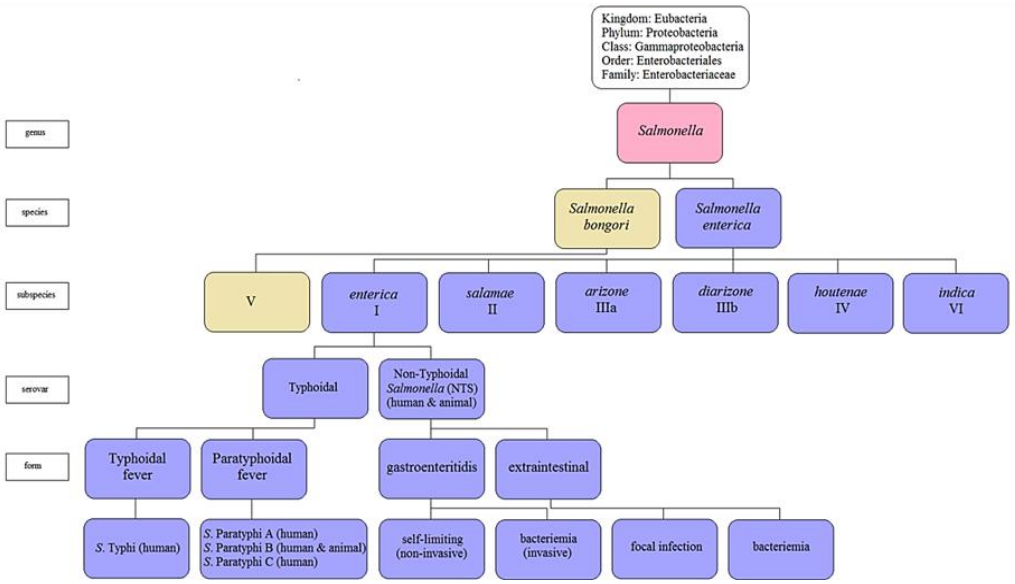

Figure 2.1 The Classification of *Salmonella* according to the most recent classification by the Centers for Disease Control and Prevention (CDC). The genus *Salmonella* is composed of two primary species: *Salmonella bongori* and *Salmonella enterica*.
Source: Wójcicki et al. (2021)

Salmonella is a rod-shaped, non-sporulating, facultatively anaerobic, gram-negative bacterium that exhibits motility through peritrichous flagella. The bacterial cells range in size from 0.7 to 1.5 μm in width and 2.2 to 5.0 μm in length, while colonies typically measure 2–4 mm in diameter (Arachchi and Wanigatunge, 2020; Grey et al., 2024). Metabolically, *Salmonella* ferments glucose and produces gas, utilizing citrate as its sole carbon source. However, it does not ferment salicin, sucrose, or lactose (Crump and Wain, 2016). The cell wall structure of *Salmonella* consists of lipids, lipopolysaccharides (LPS), proteins, and lipoproteins, with endotoxins *present* in the LPS and lipid components (Shaji, Selvaraj and Shanmugasundaram, 2023).

Salmonella grows on blood agar and MacConkey agar and can be selectively isolated using Xylose Lysine Deoxycholate (XLD) agar, Salmonella Shigella Agar (SSA), and Bismuth Sulfite Agar (BSA). On these selective media, *Salmonella* ferments glucose and mannose but does not ferment lactose or sucrose. The bacterium thrives at an optimal temperature of 35°C to 40°C but can survive within a broader range of 2°C to 54°C. While extremely low temperatures inhibit its growth, freezing does not necessarily eliminate the organism. *Salmonella* also exhibits acid resistance, withstanding pH levels as low as 1.0 to 4.6, and demonstrates high resistance to desiccation. Additionally, the bacterium is resistant to heat and alcohol, further

contributing to its environmental persistence and pathogenic potential (Cosby, Cox, Harrison, Wilson, Buhr and Cray, 2015; Oludairo et al., 2022).

Typhoid fever, also known as enteric fever, is a systemic infection caused by *Salmonella enterica* serotype *Typhi* (*S. Typhi*). According to the World Health Organization (WHO, 2023), typhoid fever affects over 9 million people worldwide and results in approximately 110,000 deaths annually. The disease remains a major cause of community-acquired bloodstream infections in south and southeast Asia and can lead to severe, potentially life-threatening complications (Ching, Bansal and Bhandari, 2017). Clinically, patients with typhoid fever typically present with prolonged fever, headache, abdominal discomfort, and general lethargy. Unlike other *Salmonella* serotypes, *S. Typhi* is human-specific and restricted to this host (House et al., 2001).

Nontyphoidal *Salmonella* (NTS) infections are primarily caused by *S.* Typhimurium, *S. Newport*, *S. Heidelberg*, and *S. Javiana* (Bush and Pertejo, 2024). Unlike *S. Typhi*, NTS infections are zoonotic and transmitted through contaminated food and water, particularly poultry, eggs, meat, and vegetables. Transmission can also occur via direct person-to-person contact (Dudhane, Bankar, Shelke and Badge, 2023). NTS commonly causes gastroenteritis, which is typically self-limiting and does not require antimicrobial treatment. However, in some cases, NTS leads to invasive infections, including bacteremia, osteomyelitis, and meningitis, which necessitate antimicrobial therapy (Chen, Wang, Su and Chiu, 2013). The genetic and genomic evolution of *Salmonella* has contributed to increased virulence and multidrug resistance (MDR), making NTS infections a significant public health concern. Antimicrobial resistance (AMR) in NTS varies across serotypes and antibiotics, with *S. Enteritidis* generally exhibiting greater susceptibility to antimicrobial agents compared to other serotypes. In contrast, *S.* Typhimurium shows a high resistance rate and is widely distributed globally (Chen et al., 2013). The rising prevalence of antibiotic-resistant *Salmonella* strains highlights the need for alternative treatment strategies and enhanced surveillance programs to mitigate the impact of AMR.

2.2 Virulence factors of *Salmonella* spp.

The pathogenicity of *Salmonella* is highly complex, involving a network of virulence genes that enable the bacterium to invade host cells, evade immune

responses, and establish systemic infections. Studies on *Salmonella* pathogenicity mechanisms have identified specific genes associated with virulence, which contribute to the bacterium's ability to cause disease (Kombade and Kaur, 2021). The severity of infection varies depending on both the host's physiological state and the virulence of the bacterial strain, which is determined by genetic elements known as virulence factors (Asten and Dijk, 2005).

A key component of *Salmonella* virulence is the presence of *Salmonella* Pathogenicity Islands (SPIs) clusters of virulence-related genes located on the *Salmonella* chromosome. These genetic regions play a crucial role in bacterial invasion, intracellular survival, and systemic dissemination. To date, 17 SPIs (SPI-1 to SPI-17) have been identified. These islands are typically inserted into tRNA genes, exhibit G+C content ratios between 37% and 47%, and display distinct codon usage compared to the core genome. Although the precise origin of SPIs remains unclear, it has been hypothesized that they were acquired through horizontal gene transfer, potentially from bacteriophages or plasmids (Amavisit, 2005; Ramatla et al., 2024; Marcus et al., 2000; Kombade and Kaur, 2021). Several SPIs have been extensively studied due to their critical roles in *Salmonella* virulence. For example:

- SPI-1 encodes genes responsible for host cell invasion and macrophage destruction, facilitating bacterial entry into epithelial cells.
- SPI-2 contains genes that enable *Salmonella* to survive and replicate within macrophages, contributing to systemic infection.
- SPI-3 plays a role in *Salmonella* survival within macrophages and in low-magnesium environments, which are critical for intracellular persistence.
- SPI-4 contains genes suspected to enhance *Salmonella*'s ability to persist within host tissues.
- SPI-5 houses genes involved in the pathogenesis of gastrointestinal infections, promoting inflammation and diarrhea.

The presence and function of these SPIs highlight the intricate molecular mechanisms that enable *Salmonella* to colonize hosts, evade immune responses, and establish infection. Understanding these virulence determinants is essential for

developing targeted strategies to combat *Salmonella* infections and mitigate their public health impact.

The Type III Secretion System (T3SS) is a highly specialized protein complex that plays a critical role in *Salmonella* pathogenesis. This structure consists of multiple subunits, comprising approximately 20 distinct bacterial proteins that collectively form the T3SS apparatus. These structural proteins provide the framework for the secretion system. In addition to these components, *Salmonella* utilizes translocator proteins to facilitate the transfer of bacterial effector proteins into the host cell cytoplasm (**Figure 2.2)**. These effector proteins manipulate host cell processes to promote bacterial survival and infection (Coburn, Sekirov and Finlay, 2007). The T3SS functions as a syringe-like mechanism, enabling *Salmonella* to translocate effector proteins directly into the intestinal epithelial cells of the host. Once inside the host cytosol, these effector proteins modulate signaling pathways to create a more favorable environment for bacterial invasion and persistence. This process is essential for *Salmonella* pathogenicity, as it allows the bacterium to evade immune defenses, establish infection, and maintain intracellular survival (Bao, Wang, Zhao and Liu, 2020; Kaur and Jain, 2012). The structural organization and function of the T3SS underscore its importance as a virulence factor in *Salmonella* infections. Understanding the molecular mechanisms of this system provides valuable insights into pathogen host interactions and may contribute to the development of targeted therapeutic strategies. Bao et al. (2020) have reported that *Salmonella enterica* encodes two T3SS gene clusters, designated as T3SS-1 and T3SS-2, located on SPI-1 and SPI-2, respectively. The T3SS-1 gene cluster plays a critical role during the early phase of infection, facilitating the invasion of intestinal epithelial cells and M cells, as well as initiating proinflammatory responses. In contrast, T3SS-2 is involved in later stages of infection, promoting intracellular survival and replication within host phagocytic cells.

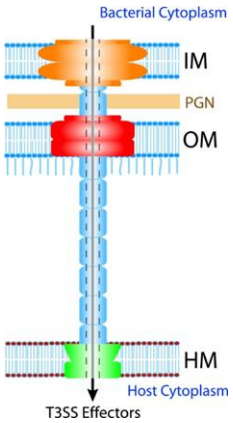

Figure 2.2 The Type III Secretion System (T3SS) consists of ring structures spanning both the inner membrane (IM) and outer membrane (OM) of the bacterium. A hollow cylindrical structure, known as the basal body, connects these membrane-spanning rings to the translocon complex (needle tip), which functions to form a pore in the membrane of the infected host cell (Bao et al., 2020).

Source: Alsubhi, (2021).

Fimbriae, also known as pili, are filamentous surface structures that play a crucial role in *Salmonella* adhesion and colonization. These structures range from 2–8 nm in width and 0.5–10 μm in length and are primarily composed of repetitive, helically arranged proteins known as fibrils. The genes responsible for fimbrial biosynthesis, structure, and assembly are typically organized within a 7–9 kb operon, comprising 8–11 genes (Asten and Dijk, 2005). In *Salmonella*, 13 predicted fimbrial loci have been identified, several of which are expressed in vivo and contribute to biofilm formation, host cell attachment, and intestinal colonization. However, these fimbriae do not appear to be directly involved in intracellular survival (Ibarra and Mortimer, 2009). Humphries, Townsend, Kingsley, Nicholson, Tsolis and Bäumler, (2001) demonstrated that fimbrial-mediated adhesion contributes to the infection process of *Salmonella* Typhimurium in intestinal epithelial cells. Their findings indicate that fimbriae are essential for bacterial invasion of tissue culture cells and may facilitate the targeting and selection of specific host cells during the early stages of infection.

Virulence plasmids are mobile genetic elements that enhance bacterial pathogenicity by encoding virulence factors such as toxins, adhesion molecules, and immune evasion mechanisms. These plasmids are commonly found in pathogenic bacterial strains, including *Escherichia coli*, *Salmonella*, and *Yersinia* (Clark, Pazdernik

and McGehee, 2018). Among *Salmonella* serovars, *S.* Typhimurium, *S. Enteritidis*, and *S. Choleraesuis* are recognized as significant human pathogens due to their possession of virulence plasmids. These plasmids harbor a distinct genetic locus known as the *Salmonella* plasmid virulence (SPV) locus, which comprises the *spvRABCD* gene cluster. The SPV genes play a critical role in promoting bacterial replication within host cells during extra-intestinal infections and contribute to systemic dissemination. In addition, virulence plasmids encode fimbrial genes (*pefBACDI*) and serum resistance factors (*traT*), further enhancing the pathogenic potential of *Salmonella* (Kaur and Jain, 2012). The role of the SPV locus in *Salmonella* virulence has been demonstrated by Silva, Puente and Calva, (2017), who investigated its function in zebrafish larvae infected with *S. Typhimurium*. Their findings revealed that the *spv* operon significantly increases bacterial virulence and survival by suppressing the host's innate immune response. Specifically, *S.* Typhimurium strains carrying an intact SPV locus were shown to inhibit neutrophil and macrophage activity, impairing the host's ability to clear the infection. Moreover, the *spv* genes interfered with early autophagosome formation, effectively neutralizing the host's immune defense mechanisms.

Fimbriae and virulence plasmids collectively play crucial roles in *Salmonella* pathogenicity by facilitating host cell adhesion, immune evasion, and intracellular survival. The SPV locus and its associated genes significantly enhance the bacterium's ability to persist and proliferate within host tissues, making them key factors in systemic *Salmonella* infections. A deeper understanding of these virulence determinants is essential for the development of targeted antimicrobial strategies to combat *Salmonella*-related diseases.

2.3 *Salmonella spp.* outbreaks

Salmonella infection, or salmonellosis, typically has an incubation period ranging from 12 to 72 hours, though in some cases it may extend to 60–120 hours. Outbreaks of salmonellosis are frequently associated with the consumption of contaminated food or water. Numerous animals including poultry, pigs, and cattle serve as reservoirs for *Salmonella* (Wei, Huang, Liao, Liu and Chiou, (2014), posing significant threats to both public health and economies worldwide (Popa and Papa, 2021).

Foodborne outbreaks commonly occur in enclosed communities where centrally prepared meals are served to large groups, such as student dormitories, elderly care homes, prisons, hospitals, and nursing facilities (Kunwar, Singh, Mangla and Hiremath, 2013). The Centers for Disease Control and Prevention (CDC) has identified nontyphoidal *Salmonella* as a major contributor to foodborne illnesses in the United States. Between 2012 and 2019, 27 *Salmonella* outbreaks were linked to beef, resulting in 254 hospitalizations and two fatalities. Ground beef was implicated in 73% of these cases, with non-intact raw ground beef responsible for 44% and intact raw beef for 22% (Canning et al., 2023). Previous studies have also associated *Salmonella* outbreaks with various food items, including eggs, cheese, dry cereals, packaged ice cream, fresh sprouts, fruit juices, cantaloupes, and other fresh produce (Acheson and Hohmann, 2001). Contaminated eggs, even those appearing normal, may carry *Salmonella* due to vertical transmission from infected hens via the ovaries (Vargas et al., 2020).

Most pathogenic strains belong to *Salmonella enterica*, which exhibits host specificity: *S. Enteritidis* is prevalent in poultry, *S.* Typhimurium in horses, *S. Anatum* in cattle, and *S. Weltevreden* in seafood (Karodia, Shaik and Qekwana, 2024). Transmission primarily occurs through ingestion of food or water contaminated with feces. In natural settings, *Salmonella* infects a wide range of animals, including birds, mammals, and humans. Environmental contamination often originates from manure of infected birds, rodents, and other wildlife (Lamichhane et al., 2024). According to Oludairo et al., (2023), approximately 60% of human pathogens are zoonotic, with wildlife playing a critical role in transmission. Of the 1,415 known human infectious agents, 61% are zoonotic, and over 70% of emerging zoonotic diseases originate from wild animal reservoirs. Wildlife can act as asymptomatic carriers of enteric pathogens, increasing environmental contamination risks, especially in water sources. These pathogens may also be transmitted during the handling, processing, or consumption of undercooked wild game. To mitigate the risk of *Salmonella* transmission in animal farming, Relaño et al. (2023) proposed various health control strategies, including acidification of animal feed using probiotics, prebiotics, or phytobiotics. Additional interventions include the use of bacteriophages, vaccination,
and implementation of stringent biosecurity measures particularly in poultry and swine farming.

Food contamination by pathogenic bacteria remains a significant global public health concern. *Salmonella* is consistently identified among the most prevalent agents of foodborne illness (Milho, Silva, Melo, Santos, Azeredo and Sillankorva, 2018). Canning et al. (2023) reported 53 *Salmonella* outbreaks in the United States from 1990 to 2014, resulting in 2,630 illnesses, 387 hospitalizations, and five deaths. Among affected individuals, 85% had contact with chicks, while 38% reported contact with ducklings. High-risk behaviors included keeping poultry indoors (46%) and kissing birds (13%). Popa and Papa, (2021) noted a *Salmonella* outbreak in Chile in 2019, linked to contaminated sushi, which resulted in 80 reported cases. Similarly, in the United States, over 1,000 cases were recorded in August 2019 due to indoor poultry exposure, with an additional 473 cases reported in June 2020. Oh and Park, (2017) highlighted an increasing number of outbreaks linked to diverse food items, including alfalfa sprouts, cucumbers, papayas, mangoes, and even dried goods such as peanut butter, chia powder, and pistachios. From 2008 to 2017, the incidence of *Salmonella* outbreaks linked to fresh produce increased approximately 4.3-fold. These findings emphasize the need for novel strategies to prevent and control *Salmonella* contamination across the food supply chain.

2.4 *Salmonella* Typhimurium

Salmonella Typhimurium is a major etiological agent of foodborne illnesses worldwide, frequently resulting in hospitalizations and fatalities. It is considered a generalist pathogen capable of causing gastroenteritis in both humans and a wide range of mammalian hosts (Won and Lee, 2017). Infection typically occurs via the ingestion of contaminated food or water, enabling the bacteria to traverse the gastrointestinal tract and invade the intestinal mucosa, thereby triggering disease symptoms (Fàbrega et al., 2013). Although it primarily colonizes the intestines of humans and warm-blooded animals, *S.* Typhimurium has also been isolated from reptiles and insects. Major sources of contamination include eggs, meat, dairy products, vegetables, and water (Popa and Papa, 2021). In the United States, *S.* Typhimurium is one of the leading causes of hospitalizations and deaths resulting from foodborne infections. Despite its significant public health burden, no effective vaccine currently exists for the prevention of *S.* Typhimurium induced gastroenteritis. Moreover, the pathogen's increasing resistance to multiple antibiotics limits treatment

options and underscores the urgency of developing novel therapeutic strategies (Makalatia et al., 2021; Anderson and Kendall, 2017; McClelland et al., 2001).

The *S.* Typhimurium LT2 strain, originally isolated in the 1940s, has served as a key model organism in the study of *Salmonella* biology. It was instrumental in the early discovery of phage-mediated transduction. Genomic analysis of the LT2 strain reveals a chromosome consisting of 4,857,432 base pairs (bp) and a virulence plasmid (pSLT) containing 93,939 bp, with both genomic elements exhibiting a G+C content of approximately 53%. Notably, the chromosome includes ribosomal RNA clusters accounting for 7% of its content (McClelland et al., 2001). The pathogenicity of *S.* Typhimurium is largely attributed to virulence factors encoded within *Salmonella* pathogenicity islands (SPIs), which are horizontally acquired gene clusters integrated into specific loci of the bacterial chromosome. To date, 23 SPIs have been identified, with SPI-1 and SPI-2 being the most extensively studied. These two SPIs encode components of the Type III Secretion System (T3SS), a needle-like protein complex often described as a molecular syringe. Through this apparatus, *Salmonella* delivers effector proteins into host cells to manipulate cellular processes, promote intracellular survival, and enhance bacterial replication (Shaji et al., 2023).

2.5 Antibiotic resistance of *Salmonella* bacteria

Antibiotic resistance poses a significant threat to global public health. The molecular mechanisms underlying the emergence of antibiotic resistance are complex, leading to alterations in both the structural and functional characteristics of bacterial cells. In their 2019 AR Threats Report, the Centers for Disease Control and Prevention (CDC) classify antibiotic resistance in drug-resistant, non-typhoidal *Salmonella* and *Salmonella* serotype Typhi as serious threats (Chaudhari, Singh and Kodgire, 2023). The misuse of antibiotics in animal production and clinical practice contributes to this resistance. Experts predict that antimicrobial-resistant pathogens could cause up to 10 million deaths worldwide by 2050 (Vargas et al., 2020). In the 1980s, non-typhoidal *Salmonella* were generally susceptible to antibiotics. In contrast, during the 1990s, clinical investigations by the CDC revealed increasing antibiotic resistance in *Salmonella* (Acheson and Hohmann, 2001).

In 2023, Fatima et al. conducted a study on the prevalence and drug resistance of *Salmonella* in raw meat samples from Lahore, Pakistan. They found

Salmonella in 57 out of 111 samples, including 64.28% in beef, 60% in buffalo and goat meat, and 45.83% in poultry. The researchers identified multiple pathogens, with *Salmonella enterica* serovar Typhimurium being the most common (45.4%). Strain identification using VITEK and 16S rRNA gene sequencing revealed that these *Salmonella* strains were multidrug-resistant (MDR) and extensively drug-resistant (XDR). This underscores the significant presence of drug-resistant *Salmonella* in raw meat from Lahore. Consistent with the findings of Qin et al. (2022), who investigated the prevalence and antibiotic resistance of *Salmonella* Typhimurium in China a major cause of global food poisoning data from 2011 to 2021 were collected from humans, animals, food, and the environment, with isolates mainly from Guangdong, Guangxi, Jiangsu, and Shanghai. Using a random-effects model, they estimated the resistance rate of *S.* Typhimurium to be 75% or higher. Antibiotics showing resistance included tetracycline, ampicillin, sulfisoxazole, and streptomycin. The study highlights the increasing severity of drug resistance in *S.* Typhimurium and emphasizes the necessity for rational antibiotic use and the development of alternative treatments.

2.6 Bacteriophages

Bacteriophages, or phages, are the most abundant entities in the biosphere and represent a ubiquitous aspect of prokaryotic life. Phages are viruses that infect and replicate within bacterial hosts. They have garnered significant interest among scientists due to their role in fundamental molecular biology research, their involvement in horizontal gene transfer and bacterial evolution, and their potential as novel therapeutic agents (Clokie, Millard, Letarov and Heaphy, 2011). Phages were first described in 1915 by Frederick Twort, who observed a substance capable of converting *Micrococcus* colonies into a clear form an effect caused by the destruction of bacterial cells by viruses. In 1917, Félix d'Hérelle reported a bacterial virus, which he named "bacteriophage," that lysed cells of *Shigella* spp. (Cisek, Dąbrowska, Gregorczyk and Wyżewski, 2017). Bacteriophages are classified based on morphology (**Figure 2.3**) and genetics. The majority belong to tailed phages that contain double-stranded DNA (dsDNA) and fall under the order *Caudovirales*. This order includes three families: 1. *Myoviridae*, recognized for their contractile tails; 2. *Podoviridae*,

distinguished by short tails; 3. *Siphoviridae*, characterized by long, non-contractile tails (Giri, 2021).

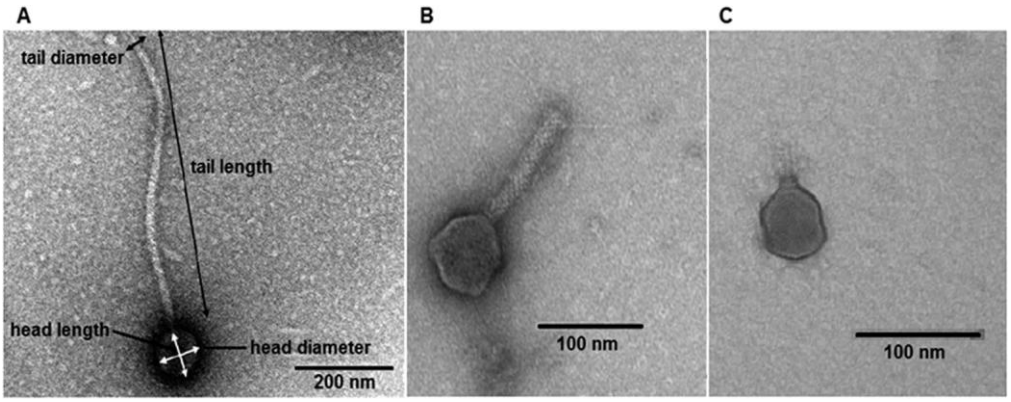

Figure 2.3 classification bacteriophages based on morphology;
A: *Siphoviride*, B: *Myoviridae* and C: *Podoviridae*.
Source: Kurek et al. (2016)

Bacteriophages exhibit two distinct life cycles: lytic (virulent) and lysogenic (temperate) (Britannica, 2023). The lytic cycle is characterized by viral specificity. Initially, lytic phages adsorb to host bacteria by binding their attachment sites to receptor sites on the bacterial surface. Certain bacteriophages employ enzymes to penetrate the bacterial cell wall, enabling them to inject their genome into the cytoplasm. The phage then replicates its genome and hijacks the host's metabolic machinery to synthesize viral enzymes and structural components. These components assemble during maturation, and eventually, a phage-encoded lysozyme degrades the bacterial cell wall, resulting in osmotic lysis and the release of new virions. In contrast, in the lysogenic cycle, bacteriophages integrate their DNA into the host bacterium's genome, becoming a noninfectious prophage. After genome injection, instead of immediately disrupting the host's cellular processes, the phage DNA incorporates into the bacterial chromosome. At this stage, a repressor protein inhibits the expression of genes required for replication. As a result, the prophage replicates passively along with the host genome, ensuring its transmission to all daughter cells (Gary Kaiser, 2024). A diagram illustrating both life cycles is presented in **Figure 2.4**.

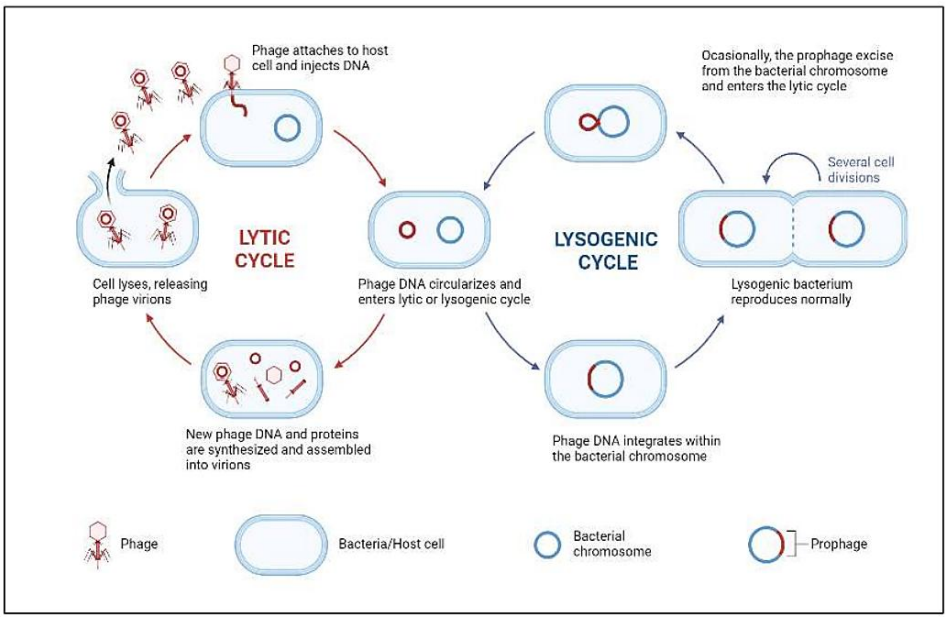

Figure 2.4 Diagram of the bacteriophage life cycle. Lytic cycle: The bacteriophage infects a bacterial cell, resulting in the synthesis of multiple new viral particles. The cycle concludes with the lysis of the host cell, releasing the newly formed virions. Lysogenic cycle: The bacteriophage genome integrates into the host bacterium's chromosome, forming a prophage. In this latent state, the viral DNA replicates passively alongside the host genome without causing immediate damage to the host. **Source:** Alsubhi, (2021).

2.7 Bacteriophages: a solution for bacterial treatment

Bacteriophages were first identified as antibacterial agents in 1915. However, following the discovery of penicillin in 1928 and its mass production in the 1940s, interest in bacteriophage therapy declined. Soon after the beginning of the antibiotic era, an increase in drug-resistant bacteria was observed. Unlike antibiotics, phages specifically target and infect particular bacterial species, making them a promising alternative for the treatment of bacterial infections (Alsubhi, 2021). In addition to their therapeutic potential, phages serve various functions. One such application is phage typing, a method that uses bacteriophages to identify bacterial species based on their specific interactions with bacterial receptors. This technique allows identification at both the genus and species levels (Merwe, Helden, Warren, Sampson and Pittius, 2014). Another application is phage biocontrol, which involves the use of phages to manage bacteria or fungi responsible for plant diseases. This strategy reduces the reliance on chemical treatments in the food industry and helps lower

microbial contamination in fruits, vegetables, and minimally processed foods without compromising sensory qualities such as taste and aroma (Adhya, Merril and Biswas, 2014). Phage therapy, which utilizes lytic phages to eliminate pathogenic bacteria, has proven effective in treating infectious diseases in both humans and animals (Lin et al., 2017).

Phage therapy refers to the use of bacteriophages, particularly lytic phages and their lytic proteins, to eliminate multidrug-resistant (MDR) bacteria. It can be applied either as a standalone treatment or in combination with antibiotics (Hibstu, Belew, Akelew and Mengist, 2022). According to Alsubhi (2021), phages may be employed alone or alongside antibiotic therapy. The first documented application of this treatment occurred in the Soviet Union, where phages were successfully used to treat patients with various bacterial infections. However, early publications mostly in Russian were not widely disseminated in Western scientific communities. Lin et al. (2017) highlight several advantages of phage therapy over conventional antibiotics, including host specificity, self-amplification, biofilm degradation, and low toxicity to humans.

At the Eliava Phage Therapy Center in Tbilisi, phages have been used to treat infections caused by *Enterococcus faecalis* (various serovars), *E. coli*, *Proteus vulgaris*, *P. mirabilis*, *Pseudomonas aeruginosa*, multiple *Salmonella* serovars, *Shigella flexneri*, and *Shigella sonnei*, with positive outcomes (Cisek et al., 2017). Several reviews, such as Liang et al. (2023), support the application of phage therapy in China, where significant therapeutic efficacy has been demonstrated in both veterinary and human clinical settings. Recent research by Young, Hall, Merabishvilli, Pirnay, Clark, and Jones, (2023) investigates phage therapy in diabetic patients at high risk of limb amputation. Patients consented to receive phage therapy alongside antibiotics, with phage solutions used to irrigate wounds instead of saline. The study reported positive outcomes in 9 out of 10 patients, showing improved infection control and wound healing without side effects. Nevertheless, limitations remain, as phages are sensitive to environmental stressors. According to Ranveer et al. (2024), phages can be inactivated at high temperatures and low pH, posing challenges for storage and application.

2.8 Salmonella bacteriophages

Salmonella is a foodborne pathogen responsible for significant disease outbreaks in both humans and animals. The pathogen has developed resistance to many commercially available antibiotics, presenting a substantial public health challenge. In contrast, salmonella-specific bacteriophages offer a promising alternative due to their high specificity, strong lytic activity, and safety for consumption (Baskaran and Karthik, 2022). More than 90% of salmonella phages isolated from the environment are tailed, lytic phages belonging to the order *Caudovirales* (Ackermann, 2007; Baskaran and Karthik, 2022). These phages are morphologically classified into three families: Myoviridae: These phages have icosahedral heads measuring approximately 60.7 nm and contractile tails. The tails measure 157 × 17 nm when uncontracted and include a sheath measuring 150 × 17 nm with 4 nm cross-striations when contracted. These phages lack collars and base plates and typically produce small plaques (<1 mm) without halos. Siphoviridae: These possess icosahedral heads (~62.5 nm) and long, rigid, non-contractile tails (120 × 7 nm) with 4 nm striations. Their tails feature a 20 nm wide baseplate with spikes and often form plaques with halos. Podoviridae: These also have icosahedral heads (~62.5 nm) and short tails (~13 nm) with a 20 nm wide base plate bearing three prongs. The plaques range from pinpoint size to 1 mm and are often accompanied by halos (Lappe, Doran, O'connor, O'hare and Cormican, 2009).

Non-typhoidal *Salmonella* is a leading cause of human diarrhea, with poultry being the primary reservoir. Therefore, eliminating *Salmonella* from poultry farms is vital for improving public health and food safety. Effective phage-based biocontrol depends on the isolation of lytic phages with high titers and broad host ranges (Gunathilake et al., 2024). To expand the phage library and ensure phage safety, continuous isolation of new phages is essential (Unverdi, Erol, Kaskatepe and Babacan, 2024). Mhone et al, (2022) examined the stability of ten *Salmonella enteritidis* phages isolated from Kenyan poultry farms under different pH levels, temperatures, and simulated gastric and intestinal fluid (SGF and SIF) conditions. Their results indicated a significant loss of phage activity at pH 2–3, while stability was

maintained at pH 5–9 and temperatures between 25–42°C for up to 12 hours. In SGF, infectivity declined after 20 minutes, whereas phages remained stable in SIF for up to two hours. Consistent with these findings, Kwaśnicka et al, (2020) reported two newly isolated bacteriophages vB_Sen-TO17 and vB_Sen-E22 from chicken feces. These phages infected multiple *Salmonella enterica* serovars and exhibited robust lytic activity. They efficiently adsorbed to host cells (>95%) within 10 minutes at 42°C and demonstrated stability across a wide range of environmental conditions, including pH levels 3–12, temperatures from −80°C to 60°C, and exposure to ethanol, chloroform, and DMSO. These features highlight their potential for phage therapy targeting *S. enterica* in poultry.

Several studies have explored the application of salmonella phages for pathogen control in poultry. Pelyuntha, Ngasaman, Yingkajorn, Chukiatsiri, Benjakul and Vongkamjan, (2021) isolated phages from various environmental sources at a broiler farm in Songkhla Province, including cloacal swabs, rice husk bedding, and wastewater treatment ponds. Phage cocktails formulated from these isolates effectively targeted five *Salmonella* serovars *Kentucky, Saintpaul*, *Schwarzengrund, Corvallis*, and Typhimurium reducing bacterial counts by 2.2–2.8 log units within six hours. Importantly, no resistance to the phage cocktail was observed. Similarly, Kumar et al. (2020) investigated phage NINP13076, isolated from sewage water, for its ability to reduce *Salmonella* contamination. Treatment with this phage significantly decreased *Salmonella* levels on raw chicken skin (from 6.7 to 5.4 log CFU/g in 3 hours) and in carrot salad (by 1 log CFU/g in 4 hours). These results support its potential use as a biocontrol agent in food safety applications. However, several challenges limit the commercial development of phage therapy. For instance, producing phages free from integrase genes, antibiotic resistance genes, or phage-encoded toxins remains difficult (Principi et al., 2019).

2.9 Extrusion technique

Microencapsulation techniques are commonly divided into three main categories. The first includes physical approaches like spray drying, extrusion, freeze-drying (lyophilization), supercritical fluid precipitation, and solvent evaporation. The second group comprises physico-chemical methods, such as coacervation, liposome formation, and ionic gelation. Lastly, chemical techniques involve processes like

interfacial polymerization and the formation of molecular inclusion complexes (Łętocha, Miastkowska and Sikora, 2022).

Extrusion techniques (external gelation), which include air jet, nozzle extrusion, centrifugation, electrostatic, and vibrational extrusion, have been widely employed in the formation of hydrogels. Among these methods, the extrusion or dripping technique is the most commonly utilized approach for producing alginate-based encapsulated hydrogels (**Figure 2.5**). In this technique, a suspension of hydrogel precursor is extruded through a nozzle, generating droplets that subsequently fall into a gelling solution typically containing divalent cations such as calcium chloride ($CaCl_2$) either by gravitational force or with external assistance. The size of the resulting microspheres is influenced by several parameters, including the concentration of the solution, the nozzle diameter, and the surface tension of the droplets (Paiboon, Surassmo, Ruktanonchai, Kappl and Soottitantawat, 2023; Lee, Ma, Khoo, Abdullah, Kahar and Hamid, 2021; Lee, Ravindra and Chan, 2013).

Bhushan, Parshad, Qazi and Gupta, (2008) demonstrate that immobilizing Arthrobacter sp. (ABL) cells, which produce lipase, within calcium alginate microspheres using the extrusion method enhances the enzyme's stability across a range of temperatures, pH levels, and storage durations when compared to the free enzyme. Similarly, Machado, Silva, Vicente, Soares, Pinheiro and Cerqueira, (2022) report that an extract of Spirulina sp. LEB-18 encapsulated using calcium alginate beads via the same technique shows higher bioaccessibility of phenolic compounds than in their free form. These studies indicate that the extrusion method is effective in protecting and maintaining the functional properties of bioactive substances.

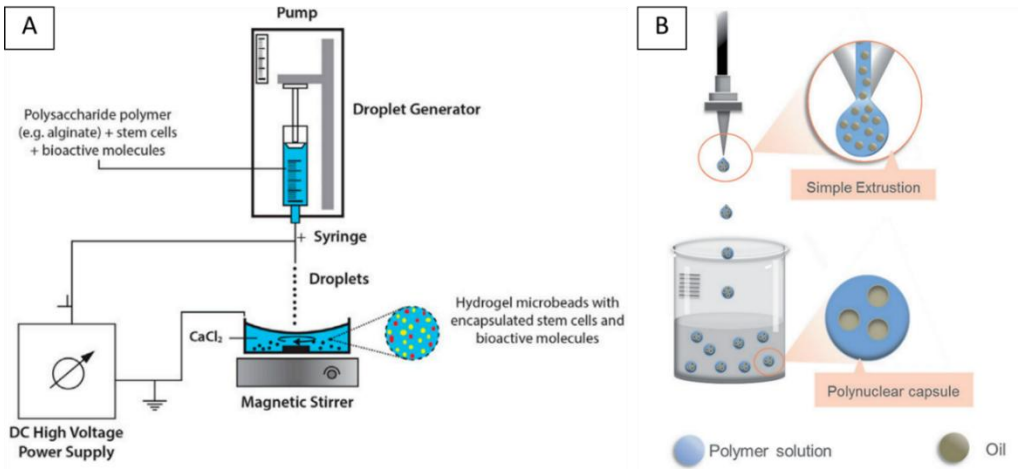

Figure 2.5 illustrates the encapsulation of an active compound within a hydrogel matrix, demonstrating two extrusion methods: (A) the electrostatic or electrospray droplet extrusion approach, and (B) the nozzle-based extrusion technique.
Source: Martins, Poncelet, Rodrigues and Renard, (2017) and Lee et al. (2021)

2.10 Production of Ca-alginate beads by the extrusion technique.

Alginate is a linear, anionic, water-soluble, and hydrophilic polysaccharide composed of 1,4-linked β-D-mannuronic acid (M-block) and 1,4-linked α-L-guluronic acid (G-block). These monomeric units can appear in homopolymer regions (M-blocks or G-blocks) or as alternating M-G sequences (**Figure 2.6**). One of alginate's most notable properties is its ability to form hydrogels in the presence of divalent cations, most commonly calcium ions (Ca^{2+}). The G-blocks in alginate chains preferentially bind with Ca^{2+} ions to form a cross-linked structure known as the "egg-box" model, which contributes significantly to gel strength (**Figure 2.7**) (Gajić, Savić and Svirčev, 2023; Tomić, Radić, Vuković, Filipović, Runic and Vukomanović, 2023). In contrast, M-blocks exhibit weaker interactions with divalent cations (Zhou et al., 2022). Alginate has been widely used due to its favorable characteristics, including biocompatibility, biodegradability, and non-toxicity. It is generally recognized as safe for biomedical and pharmaceutical applications. Among natural polymers employed for the preparation of microparticles, alginates have gained considerable attention owing to their excellent gelling behavior, viscosity, water retention capacity and stabilizing properties, which support their use in various industrial processes. Alginate microparticles are particularly suitable for encapsulation purposes, as they are environmentally benign and compatible with living systems (Łętocha et al., 2022; Frent et al., 2022).

The formation of alginate beads occurs through gelation, which may involve either covalent or ionic crosslinking. However, ionic crosslinking is more widely utilized because it offers a simpler process under mild conditions, typically performed at room temperature or up to 100°C. Notably, only the G-block segments, composed of consecutive guluronic acid residues, participate in the crosslinking process owing to their favorable three-dimensional configuration. This mechanism is often described by the "egg-box" model, which illustrates the coordination between divalent cations and G-blocks of neighboring polymer chains (Zhang, Grossier, Candoni and Veesler,

2020). Divalent cations such as calcium, barium and magnesium are commonly used as ionic crosslinkers to form ionic bridges between guluronic acid (G) units in the alginate polymer chain (Lee et al., 2021).

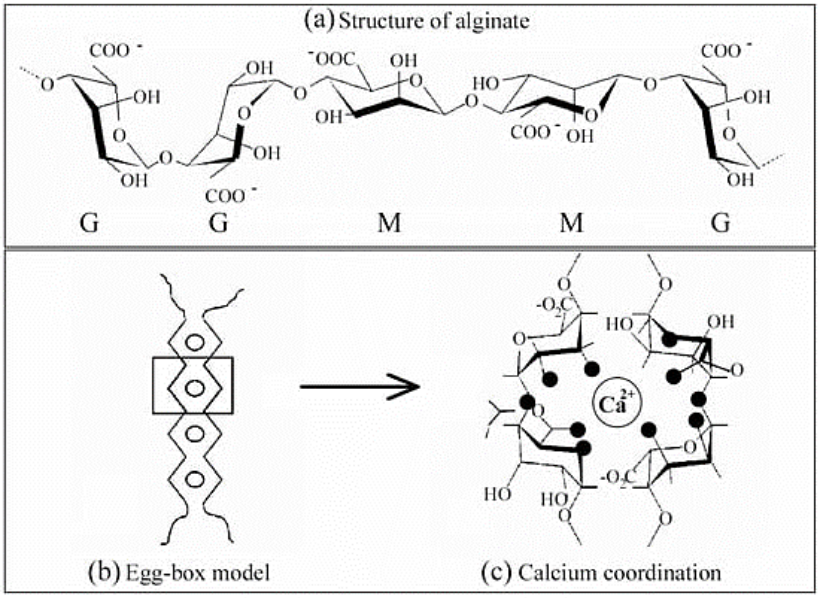

Figure 2.6 The structure of alginate and its gelation mechanism. (a) Schematic representation of M-blocks (β-D-mannuronic acid) and G-blocks (α-L-guluronic acid) in alginate chains; (b) The "egg-box" model illustrating cross-linking between G-blocks and Ca^{2+} ions; (c) Calcium coordination sites involved in the formation of alginate hydrogels.

Source: Hurtado, Aljabali, Mishra, Tambuwala and Aroca, (2022)

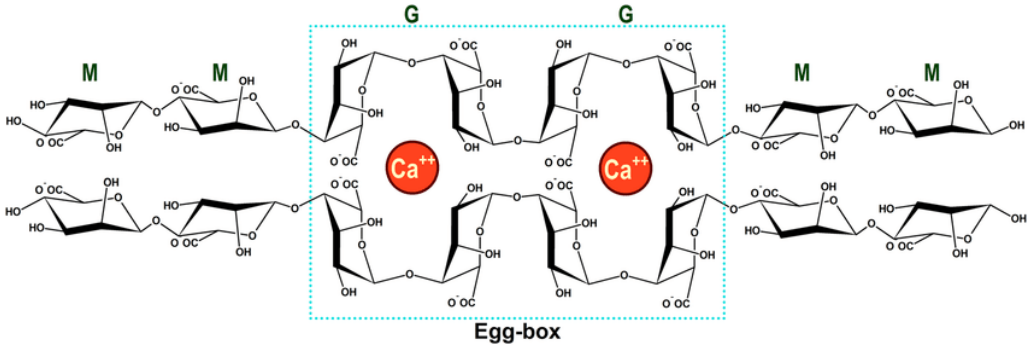

Figure 2.7 The gelation process of alginate is explained through the "egg-box" model, which illustrates the interaction between calcium ions (Ca^{2+}) and the alginate polymer chains. Calcium ions substitute sodium ions (Na^{+}) in the alginate matrix and subsequently form crosslinks by binding with guluronic acid blocks on adjacent alginate chains.

Source: Lee et al. (2019)

The mechanism underlying alginate gelation in the presence of divalent cations, such as calcium ions (Ca^{2+}), is well described by the "egg-box" model. In this model, guluronic acid (G) residues along alginate chains align in a way that allows them to chelate divalent ions within a structured cavity. These cavities are specifically formed by sequences rich in GG-blocks, which enable a highly specific and stable binding site for the cations. The cross-linking occurs as each Ca^{2+} ion simultaneously coordinates with carboxyl groups from adjacent G residues on two opposing polymer chains, resulting in a three-dimensional network that forms the basis of the hydrogel structure. It has been observed that at least 8 to 20 contiguous G residues are typically required to generate a mechanically stable and cohesive hydrogel network (Colin, Akpo, Perrin, Cornu and Cambedouzou, 2024).

2.11 Entrapment

Entrapment is a widely employed technique for the immobilization of materials and is considered one of the most popular methods for the preparation of immobilized systems. This approach involves incorporating target materials, such as biomass, into carrier matrices to form discrete particles or defined structures. The entrapment process serves to prevent material leakage and protect the entrapped substances from unfavorable environmental conditions. Based on the nature of the carrier system, entrapment methods can be classified into two main categories: gel entrapment and semipermeable membrane or capsule entrapment. This approach helps prevent leakage and protects the encapsulated material from adverse environmental effects. Common carriers and matrices used in entrapment include organic polymers, silica, membrane devices, and microcapsules (Schmidt and Mosbach, 1977; Liu, Yang, Zhang, Tang, He and Liang, 2022).

Entrapment is characterized by the immobilization of a material commonly termed the payload, active internal phase, or core within the structural framework of another substance, forming particles of nanoscale (nanoencapsulation), microscale (microencapsulation), or even millimeter dimensions. The enclosing material is referred to by various terms, including coating, membrane, shell, capsule, exterior phase, or matrix (Al, Saeinasab and Sefat, 2022). Entrapment has gained widespread

application across numerous fields. Rivera, Talavera, Jiménez, Cruz, Bernardino and Pacheco, (2021) described microbial entrapment, also known as bioencapsulation, as embedding microorganisms within a semi-permeable matrix that facilitates the transfer of nutrients and bioactive agents between the internal and external environments. While such structures are predominantly spherical, they can also appear in tubular or oval forms of varying sizes.

The main advantages identified in numerous studies include the protection of active ingredients from degradation (e.g., heat, acid, enzymes), controlled release, enhanced stability and shelf life, reduced side effects (e.g., some drugs do not irritate the stomach), and targeted delivery. Moreover, these materials have been shown to remain stable and can be utilized in various forms, including pure compounds, crude extracts, enzymes, whole cells, and microorganisms (Kailasapathy, 2009; Siddiqui, Singh, Bahmid, Mehany, Shyu and Assadpour, 2023; Pegg and Shahidi, 2007). Despite its benefits, entrapment has limitations. For instance, polymers may undergo denaturation, leading to leakage of the encapsulated substances. Furthermore, microencapsulation may hinder diffusion processes (**Figure 2.8**) (Schmidt and Mosbach, 1977; Homaei, 2015). Examples of situations where probiotic microorganisms confer health benefits when present in appropriate amounts (Nedović, Kalušević, Manojlović, Lević and Bugarski, 2010). However, their viability is often compromised by environmental stresses, including fluctuations in pH, mechanical force, transport conditions, and digestive enzymes. Entrapment provides a protective mechanism that mitigates these challenges without adversely affecting food texture or taste. It also enhances the survival of sensitive microorganisms, such as *Lactobacilli* and *Bifidobacteria*.

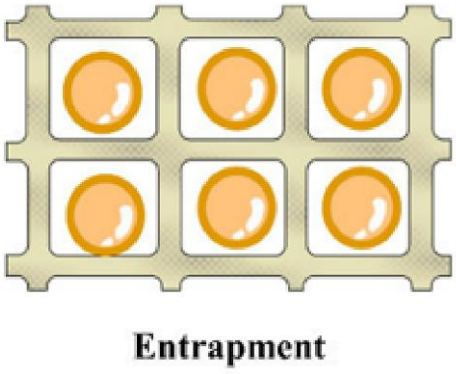

Figure 2.8 Illustration showing entrapment methods.

Source: Liu et al. (2022).

Gel entrapment is a form of irreversible immobilization in which materials are entrapped within polymer matrices or fibers. These matrices typically feature a lattice structure that allows the diffusion of substrates and products while preventing the escape of immobilized materials. Natural polymers such as alginate, chitosan, and agar are frequently employed in this context due to their capacity to form gels at relatively low temperatures (Chaudhary, Rana, Vaidya, Ghabru, Rana and Dipta, 2019). An effective entrapment method should ensure the retention of the encapsulated substance and maintain mechanical, chemical, and biological stability (Freeman, 1986). Recent studies have demonstrated the application of entrapment in food packaging. Alves, Cerqueira, Pastrana and Sillankorva, (2020) investigated the incorporation of bacteriophages and cinnamaldehyde (CNMA) into sodium alginate-based films to inhibit *Salmonella enteritidis* and *Escherichia coli*. The results revealed that CNMA did not impair phage activity, and the combined treatment effectively reduced bacterial populations. These findings underscore the potential of entrapment technologies in enhancing food safety.

The global rise in antibiotic resistance has revived interest in the therapeutic application of bacteriophages. However, phages are inherently unstable in solution and are vulnerable to degradation during processing and storage (Malik, Sokolov, Vinner, Mancuso, Cinquerrui and Vladisavljevic, 2017). Entrapment addresses these issues by protecting phages from the harsh gastric environment, including low pH and enzymatic activity. Entrapment is typically achieved using alginate-based microspheres or liposomes. Several studies have confirmed the viability of phage entrapment in food systems. For example, the integration of *E. coli* specific phages into whey protein isolate (WPI) films and chitosan matrices has been shown to preserve phage activity throughout storage (Alves et al., 2020). Encapsulated phages also exhibited enhanced antimicrobial efficacy when applied to vegetable surfaces, meat, and fish feed. Encapsulation of phage K in alginate has demonstrated significant antibacterial activity, particularly under acidic gastric conditions, where it outperformed non-encapsulated phages (Loh, Gondil, Manohar, Khan, Yang and Leptihn, 2021). Moreover, a phage cocktail encapsulated in alginate/$CaCO_3$ microcapsules was effectively used in broiler chickens infected with *Salmonella*. Pulit, Mituła, Śliwka, Łaba and Skaradzińska, (2015) further reported that phages

encapsulated in biopolymer matrices such as alginate and pectin, with optional oleic acid emulsification and high-methoxyl pectin coating exhibited high encapsulation efficiency (1.2×10^7 PFU/bead). These encapsulated phages retained infectivity after 30 minutes at pH 1.6, demonstrating superior resistance to pepsin digestion compared to free phages.

2.12 Entrapment with alginate beads

A major challenge in phage delivery systems is maintaining phage viability during processing, storage, and gastrointestinal transit. This limitation has prompted the development of various encapsulation strategies, including nanosomes, liposomes, and hydrogel-based systems. Among these, alginate (ALG) has emerged as a preferred biopolymer due to its biocompatibility, affordability, and low toxicity (Garcia et al., 2021). Alginate is a linear, anionic, water-soluble, and hydrophilic polysaccharide. Alginate is predominantly extracted from brown seaweeds belonging to the Phaeophyceae family, although certain bacterial genera, such as *Pseudomonas* and *Azotobacter*, also produce it. One of alginate's most notable properties is its ability to form hydrogels in the presence of divalent cations, most commonly calcium ions (Ca^{2+}) (Gajić et al., 2023; Tomić et al., 2023). Alginate is considered a highly effective material for entrapment applications. It exhibits resistance to acidic gastric conditions and allows for controlled release of live phages into the small intestine (Zhou et al., 2022). However, a key limitation of alginate gels is their relatively low mechanical strength, which can compromise their protective capability in harsh environments. To improve gel robustness, the typical method involves dropping alginate solution into a calcium chloride ($CaCl_2$) bath, which induces ionic cross-linking and significantly enhances the mechanical stability of the resulting beads (Ching et al., 2017). Nevertheless, this approach alone may not sufficiently shield phages from extreme gastric acidity. For this reason, the incorporation of additional polymers in combination with alginate has been recommended to enhance protective effects and overall encapsulation efficiency (Samtlebe, Ergin, Wagner, Neve, Küçükçetin and Franz, 2016).

2.12.1 Alginate beads modified by soy protein isolate and cassava starch

In 2022, Zhou et al. reported an early study on the encapsulation of bacteriophages using an ionic gelation technique, where calcium ions served as cross-linking agents. In this work, *Salmonella* spp. Felix O1 phages were successfully encapsulated within a matrix composed of chitosan, alginate, and calcium chloride. The encapsulated phages were shown to be safely delivered to the small intestine, demonstrating the potential of this method for targeted delivery applications (Garcia et al., 2021).

Similarly, Zhang et al. (2015) investigated the encapsulation of *Enterococcus faecalis* HZNU P2 using a composite of alginate and soy protein isolate (SPI). The study evaluated the survival of the encapsulated bacteria in simulated gastric fluid (SGF) and their release behavior in simulated intestinal fluid (SIF). At low pH values (2.5 and 2.0), the viability of encapsulated *E. faecalis* HZNU P2 remained stable after two hours of incubation, while the viability of free cells declined from 11 to 9.85 log CFU/mL. In SIF, the encapsulated bacteria were completely released within one hour, indicating effective protection and targeted release. Volić et al. (2018) explored the encapsulation of thyme essential oil using alginate–SPI beads formed by electrostatic extrusion followed by gelation with calcium ions. Alginate and SPI concentrations ranged from 1–2.5 wt.% and 0–1.5 wt.%, respectively. The beads were air-dried at room temperature until they reached a constant mass. The encapsulation efficiency, based on total polyphenol content, ranged from 72% to 80%. In SIF, 42% to 55% of the thyme essential oil was released over 2.5 hours, accompanied by bead swelling and partial matrix degradation. Consistent with these findings, Jin et al. (2023) reported that calcium alginate–SPI microgels exhibited higher encapsulation efficiency and greater storage stability of β-carotene compared to calcium alginate microgels alone. The addition of SPI resulted in enhanced resistance to SGF, evidenced by reduced gel size shrinkage in gastric conditions and minimized swelling in intestinal conditions. These results suggest that incorporating SPI into alginate-based systems improves the performance of controlled-release formulations and enhances protection in gastrointestinal environments.

Several studies have investigated the combination of cassava starch (CS) and alginate for use in encapsulation systems. Riyajan, (2017) highlighted the potential of this biopolymer mixture in various delivery applications. Vazquez et al. (2015)

explored the modification of alginate beads by incorporating different weight ratios of cassava starch (alginate: cassava starch; 1:0, 0.75:0.25, 0.5:0.5, and 0.25:0.75) for the encapsulation of chlorogenic acid (CGA). Among the tested formulations, the beads containing the highest cassava starch content (0.25:0.75) demonstrated the most effective control of CGA release under simulated gastrointestinal conditions. This outcome was attributed to increased viscosity resulting from the higher starch content, which reduced CGA diffusion and minimized leakage from the matrix. More recently, Fang et al. (2022) developed a novel oral drug delivery system for cancer therapy using carboxymethyl cassava starch–alginate beads enriched with magnesium ferrite ($MgFe_2O_4$) nanoparticles for the encapsulation of doxorubicin (Dox). In vitro experiments revealed that the beads remained stable in simulated gastric fluid (SGF) while enabling sustained drug release in simulated intestinal fluid (SIF). Additionally, cytotoxicity assays and confocal laser scanning microscopy showed that the delivery system did not adversely affect normal cells, but exhibited selective cytotoxic effects against colon cancer cells (HCT116). These findings support the application of cassava starch alginate-based systems in targeted and controlled drug delivery.

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