Universidade do Minho
Escola de Engenharia

Yolanda Patrícia Manuel Leopoldo

Development of an alginate and shellac microencapsulation system for phages: targeting intestinal foodborne bacterial pathogens on ruminant livestock

julho de 2021

Development of an alginate and shellac microencapsulation system for phages: targeting intestinal foodborne bacterial pathogens on ruminant livestock
Yolanda Patrícia Manuel Leopoldo
UMinho | 2021

FCT
Fundação para a Ciência e a Tecnologia
MINISTÉRIO DA CIÊNCIA, TECNOLOGIA E ENSINO SUPERIOR

INL
INTERNATIONAL IBERIAN NANOTECHNOLOGY LABORATORY

Universidade do Minho
Escola de Engenharia

Yolanda Patrícia Manuel Leopoldo

Development of an alginate and shellac microencapsulation system for phages: targeting intestinal foodborne bacterial pathogens on ruminant livestock

Dissertação de Mestrado
Mestrado em Biotecnologia

Trabalho efetuado sob a orientação de
Doutor Hugo Oliveira
e
Doutor Pablo Fuciños

julho de 2021

DIREITOS DE AUTOR E CONDIÇÕES DE UTILIZAÇÃO DO TRABALHO POR TERCEIROS

Este é um trabalho académico que pode ser utilizado por terceiros desde que respeitadas as regras e boas práticas internacionalmente aceites, no que concerne aos direitos de autor e direitos conexos.

Assim, o presente trabalho pode ser utilizado nos termos previstos na licença abaixo indicada.

Caso o utilizador necessite de permissão para poder fazer um uso do trabalho em condições não previstas no licenciamento indicado, deverá contactar o autor, através do RepositóriUM da Universidade do Minho.

Licença concedida aos utilizadores deste trabalho

Atribuição

CC BY

https://creativecommons.org/licenses/by/4.0/

Agradecimentos

Chegado o fim desta etapa, gostaria de deixar aqui os meus sinceros agradecimentos a todos os que fizeram parte deste percurso:

Devo começar por agradecer à Professora Joana Azeredo por me ter recebido com tanta simpatia neste mestrado, que me proporcionou um grande crescimento pessoal e muitos aprendizados a nível académico.

Ao Hugo Oliveira e ao Pablo Fuciños pela oportunidade de trabalhar neste projeto e por tudo que aprendi com eles. À Carla Dias e à Anna Marín que me acompanharam inicialmente, na produção e ensaios com os fagos e na otimização do encapsulamento, respetivamente. Muito obrigada por tudo! Agradeço também ao projeto PhageSTEC, financiado pela FCT (POCI-01-0145-FEDER-029628), que

FCT
Fundação para a Ciência e a Tecnologia
MINISTÉRIO DA CIÊNCIA, TECNOLOGIA E ENSINO SUPERIOR

ajudou a suportar este trabalho.

À Marisol Dias, a minha companheira diária de laboratório, que foi essencial para o desenvolvimento deste trabalho, que não foi livre de frustrações mas acabou por ser muito gratificante. Não imaginava que ela e as meninas do INL (Patrícia, Mafalda, Inês e Micaela) acabariam por se tornar verdadeiras amigas ao longo do tempo e proporcionar-me-iam tantos momentos de alegria.

Deixo aqui também o meu reconhecimento aos meus amigos, que sempre acreditaram que eu seria capaz, apoiaram-me e incentivaram-me nos momentos de desânimo.

Por último, mas não menos importante, agradeço muito à minha família! Aos meus pais, Arminda e João Leopoldo, e às minhas irmãs Alícia, Edvânia e Márcia, que sempre me deram toda a estrutura e apoio necessários em todas as etapas da minha vida, e nesta não foi diferente. Aos meus tios, Moisés e Madalena Manuel, que me receberam aqui em Braga em sua casa com tanto carinho.

Sou muito grata a todos vocês! Um bem-haja a todos os mencionados e aos que não mencionei, mas que de alguma forma colaboraram para a conclusão e sucesso deste projeto, mesmo em tempos tão conturbados.

STATEMENT OF INTEGRITY

I hereby declare having conducted this academic work with integrity. I confirm that I have not used plagiarism or any form of undue use of information or falsification of results along the process leading to its elaboration.

I further declare that I have fully acknowledged the Code of Ethical Conduct of the University of Minho.

Desenvolvimento de um sistema de micro-encapsulamento de fagos, constituído por alginato e shellac, para eliminação de patogénios alimentares que colonizam o intestino de gado ruminante

Sumário

Este trabalho consistiu no desenvolvimento de um sistema de micro-encapsulamento para administração oral de bacteriófagos (fagos) a ruminantes, para protege-los durante a passagem pelo rúmen e abomaso, libertando-os posteriormente no intestino. Os ruminantes são um dos principais reservatórios de alguns dos patogénios alimentares bacterianos mais relevantes, como *Escherichia coli* patogénicas para humanos (ex. STEC, do inglês *Shiga toxin-producing E. coli*). As infeções por este tipo de bactérias estão frequentemente relacionadas a surtos de doenças alimentares, provocando complicações que podem ser fatais. A administração oral de fagos, que são agentes antibacterianos naturais e altamente seletivos, é uma das estratégias mais promissoras para evitar estas contaminações. O método de encapsulamento utilizado baseou-se na extrusão dos polímeros e permitiu formar micropartículas esféricas através da técnica de *prilling by vibration* seguida de gelificação iónica. Foram utilizados dois polímeros naturais, biodegradáveis e biocompatíveis, o alginato e a shellac (considerados aditivos alimentares seguros pela *European Food Safety* Authority), em condições de temperatura, pH e *stress* mecânico não prejudiciais para os fagos.

As micropartículas optimizadas são constituídas por uma mistura de 2 % (w/v) alginato and 3 % (w/v) shellac. Através de Microscopia Wide-Field foi possível determinar que o seu diâmetro médio era de 488 ± 16 μm e que estas possuíam uma morfologia esférica. Os testes *in vitro* realizados mostraram que a matriz polimérica manteve-se intacta à temperatura (38.5 °C) e aos valores de pH correspondentes ao rúmen (pH 5.8, 6.5 e 7) e ao abomaso (pH 3) e desintegrou-se no pH respetivo ao intestino (pH 7.5). Foi utilizada Microscopia Eletrónica de Varrimento, que permitiu verificar que as micropartículas apresentavam uma superfície porosa e um interior compacto. A análise por Microscopia Confocal mostrou que as nano-esferas fluorescentes de 200 nm de diâmetro, encapsuladas previamente para simular os fagos, mantiveram-se encapsuladas durante três dias de armazenamento e foram libertadas quando colocadas em tampão TRIS pH 7.5. Os dois fagos selecionados como prova de conceito foram, o T4 que é um fago modelo de *E. coli* e o CBA120 que é específico para STEC O157:H7, a estirpe mais comum de STEC. Após o encapsulamento dos fagos na matriz polimérica, testes de estabilidade foram realizados com fagos encapsulados e livres, provando que os fagos encapsulados resistiram mais ao pH 3, comparativamente aos fagos livres, que foram totalmente inativados no mesmo pH.

Estes resultados permitem concluir que as micropartículas de alginato e shellac desenvolvidas neste trabalho, apresentam grande potencial como sistema de encapsulamento que poderá ajudar os fagos a chegar ao intestino viáveis e em quantidades eficazes, eliminando assim as bactérias alvo aí presentes. Posteriormente, a técnica aqui desenvolvida poderá ser aplicada para diferentes fagos e bactérias patogénicas, no sentido de reduzir o elevado número de doenças alimentares que ocorrem mundialmente e são consideradas um problema de saúde pública pela Organização Mundial de Saúde, representando um elevado custo a nível socioeconómico.

Palavras-chave: Alginato; Encapsulamento de fagos; Ruminantes; Shellac; Zoonoses alimentares

Development of an alginate and shellac microencapsulation system for phages: targeting intestinal foodborne bacterial pathogens on ruminant livestock

Abstract

This work consisted in the development of a microencapsulation system for the oral administration of bacteriophages (phages) to ruminants, to protect them during their passage through the rumen and the abomasum, releasing them later in the intestine. Ruminants are a major reservoir for some of the most relevant bacterial foodborne pathogens, such as human pathogenic *Escherichia coli* (e.g., Shiga Toxin-producing *E. coli* - STEC). Infections with these types of bacteria are often related to foodborne outbreaks, causing complications that can be fatal. Oral administration of phages, which are natural and highly selective antibacterial agents, is one of the most promising strategies to prevent these contaminations. The encapsulation method used was based on the extrusion of the polymers and allowed to form spherical microparticles, through the prilling by vibration technique followed by ionic gelation. Two natural, biodegradable, and biocompatible polymers were used, alginate and shellac (considered safe food additives by the European Food Safety Authority), under conditions of temperature, pH, and mechanical stress not harmful for phages.

The microparticles optimized were composed of a mixture of 2 % (w/v) alginate and 3 % (w/v) shellac. By Wide-Field Microscopy, it was possible to determine that their average diameter was 488 ± 16 µm and that they had a spherical morphology. *In vitro* stability tests showed that the polymeric matrix remained intact at the temperature (38.5 °C) and pH values corresponding to the rumen (pH 5.8, 6.5 and 7) and abomasum (pH 3) and disintegrated at the pH corresponding to the intestine (pH 7.5). Scanning Electron Microscopy was used to verify that the microparticles had a porous surface and a compact interior. Confocal microscopy analysis showed that the 200 nm diameter fluorescent nano-spheres, previously encapsulated to simulate phages remained encapsulated for three days of storage and were released when placed in TRIS buffer pH 7.5. The two phages selected as proof of concept were the *E. coli* phage T4 as a model and the phage CBA120, which is specific for STEC O157:H7, the most common strain of STEC. Phages were encapsulated within the polymeric matrix, then stability tests with encapsulated and free phages were performed, proving that encapsulated phages resisted more at pH 3, compared to free phages, which were totally inactivated at the same pH.

These results allow us to conclude that the alginate and shellac microparticles developed in this work, present great potential as an encapsulation system that could help phages to reach the intestine in viable and effective quantities, thus eliminating the target bacteria present there. Subsequently, the

method developed here may be applied to different phages and pathogenic bacteria to reduce the high number of foodborne diseases that occur worldwide and are considered a public health issue by the World Health Organization, representing a high socio-economic cost.

Keywords: Alginate; Foodborne zoonoses; Phage encapsulation; Ruminants; Shellac

Table of contents

List of Abbreviations and Acronyms

CE	Competitive Exclusion
CM	Confocal Microscopy
CN	Concentric Nozzle
ECDC	European Centre for Disease Prevention and Control
EFSA	European Food Safety Authority
EHEC	Enterohemorrhagic *Escherichia. coli*
EU	European Union
FAO	Food and Agriculture Organization of the United Nations
FDA	Food and Drug Administration
FOS	Fructooligosaccharides
FVN	Flow Vibration Nozzle
GIT	Gastrointestinal Tract
MPs	Microparticles
SEM	Scanning Electron Microscopy
SN	Single Nozzle
STEC	Shiga Toxin-producing *Escherichia. coli*
TOS	Trans-galacto-oligosaccharides
VFA	Volatile Fatty Acids
WHO	World Health Organization
WM	Wall Material

List of Figures

List of Tables

Chapter 1: State of the art

Chapter 1: State of the art

1.1 Foodborne Zoonoses: their impact on public health and the world economy

Zoonotic diseases or zoonoses are a group of infectious diseases that are naturally transmitted between animals and humans and can occur as isolated cases or regional, national, and global outbreaks. For the World Health Organization (WHO), the greatest risk of transmission of zoonoses occurs at the human-animal interface, through direct or indirect human exposition to animals, their products (e.g. meat, milk, eggs, etc.) or their environments (WHO 2020). Foodborne diseases are zoonoses caused by the consumption of food contaminated with pathogens, and there are over 200 diseases caused by contaminated food. According to the WHO, these diseases represent a wide spectrum of pathologies that result from the ingestion of food contaminated with microorganisms such as bacteria, viruses, protozoa, and helminths, or toxins and metabolites synthetized by microorganisms, or even chemical products such as heavy metals (Miller and Cawthorne 2017; WHO 2015). The clinical manifestations of the mentioned illnesses can be sporadic symptoms or chronic complications. The most common are the gastrointestinal symptoms, and the severe consequences include kidney and hepatic insufficiency, neurological and brain disturbs, reactive arthritis and cancer (Miller and Cawthorne 2017; WHO 2020). The contamination can occur at any stage of the food process chain, like production, delivery, consumption of the food product, or during unsafe food storage and processing. It can result from environmental contamination, like water, soil, or air pollution, leading to a high annual number of human infection cases, several of which resulting in death (Miller and Cawthorne 2017). These diseases are a growing public health issue due to their high morbidity and mortality rates, and the burden is disproportionately carried out by low- and middle-income countries. Children under five years old normally are the most affected. The number of notified cases is not representative of the real number of individuals affected because a significant number of cases are not identified nor reported (Hedberg 2011).

In the first global report on the burden of foodborne diseases, WHO concluded that in 2010, 31 identified hazards (among microorganisms, toxins, and chemicals) caused 600 million cases of foodborne diseases and 420 thousand deaths worldwide, being almost 40 % of the cases and 30 % of deaths of children under five years old (Preneuf and Morales 2018; WHO 2015). It is estimated that each year, nearly one in ten people worldwide get ill from contaminated food (Miller and Cawthorne 2017). At the European Union, as claimed by the European Food Safety Authority (EFSA), there are annually reported

over 350,000 occurrences, and the five main bacterial agents are *Campylobacter, Salmonella, Yersinia,* Shiga Toxin-producing *Escherichia coli* (STEC), and *Listeria* (EFSA 2020) (**Figure 1**). Considering 2018, at "The European Union One Health 2018 Zoonoses Report", it was verified a total number of 572 deaths, and the number of deaths for each zoonosis is presented in **Figure 1**. The most relevant were Listeriosis, causing almost half of the deaths (229 deaths), West Nile fever (137 deaths), and Salmonellosis (119 deaths). This document also contains data about Portugal, being the two main etiologic agents *Campylobacter* and *Salmonella*. It is also important to note that in 2018 (**Figure 1a**) there was a significant increase in the number of cases of STEC infections compared to the previous year (**Figure 1b**).

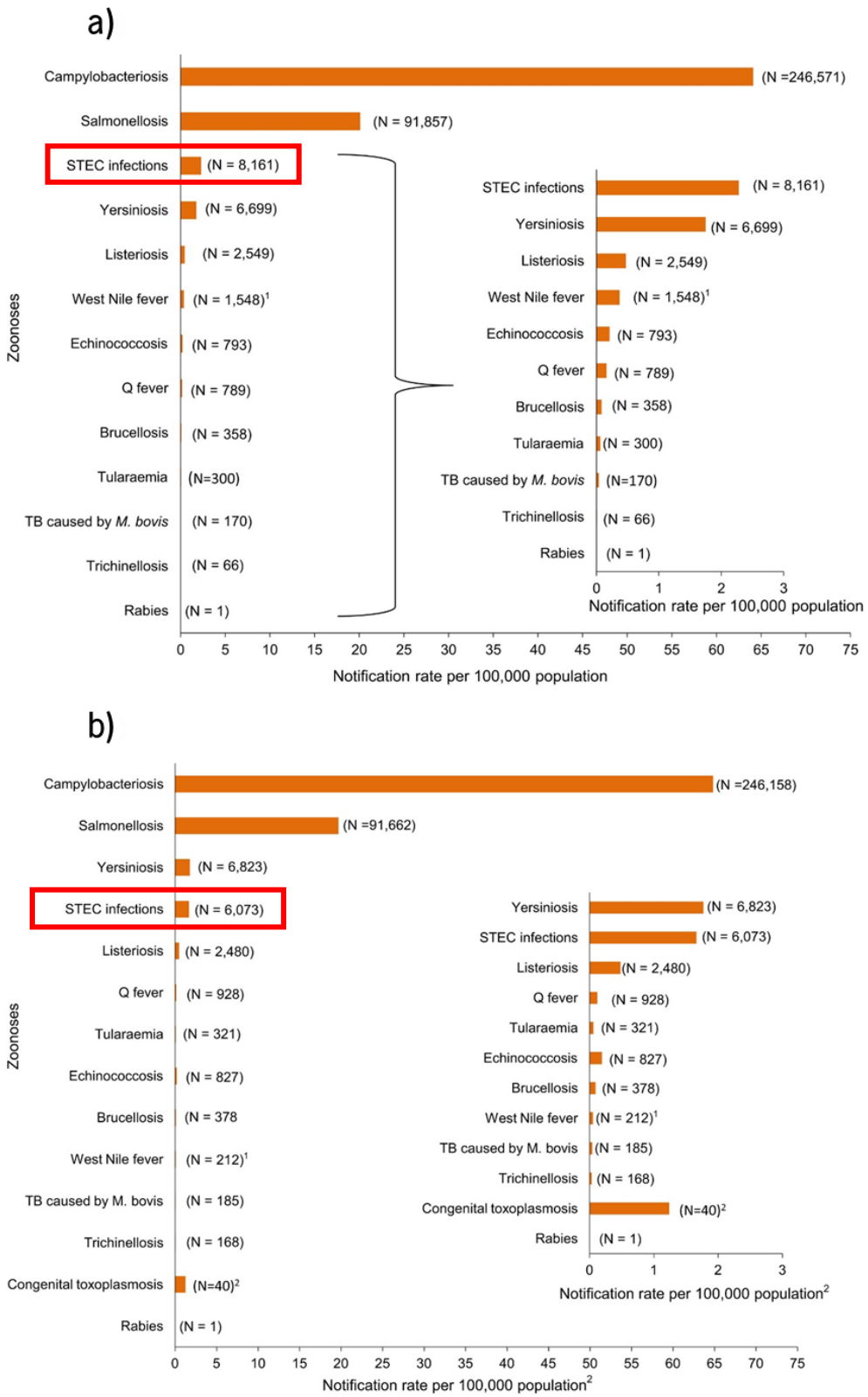


***Figure 1**: Reported numbers and notification rates of confirmed human zoonoses in the EU in 2018 (**a**) and 2017 (**b**).*

***Note:** Total numbers of confirmed cases are indicated in parenthesis at the end of each bar. **1 Exception**: West Nile Fever where the total number of cases were used. **2 Exception**: Congenital toxoplasmosis notification rate per 100,000 live births. Adapted from: "The*

European Union One Health 2018 Zoonoses Report" and "The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2017".

The global socioeconomic impact of this problem includes loss of productivity, pressure on healthcare systems, impacts on tourism and the economic trade, and is difficult to calculate accurately due to the lack of information reported to the authorities (WHO 2015). However, according to the World Bank, US$ 110 billion is lost each year in productivity and medical expenses resulting from contaminated food in low- and middle-income countries (Preneuf and Morales 2018). In the WHO global report, 54 % of the total burden has been attributed to agents of diarrheal diseases, of which the majority of the etiologic agents are bacteria, including STEC (WHO 2015). This information makes clear the importance of preventing these types of infections. For this purpose, several strategies (e.g. the "*Codex Alimentarius"*), activities, programs, and manuals ("WHO Five Keys to Safer Food") have been provided by WHO to help Member States building capacity to prevent, detect and manage food-related hazards.

1.2 The ruminants' role in the transmission of foodborne pathogens

The colonization of food-producing animals, such as chickens, pigs, and cattle, by pathogens, represents a risk for the environment, for the individuals exposed (like breeder producers and slaughterhouse workers), and to the final consumer. One of the most important ways of contamination is the ingestion of meat from ruminant animals (e.g. bovine, ovine, goats) contaminated with pathogenic bacteria. For example, in England and Wales, between 1996 and 2000, were registered 1.7 million cases of foodborne diseases, and beef was the vector of 7 % of the cases. Therefore, ruminants are among the principal food-producing animals and reservoirs of bacteria like pathogenic *E. coli*, which is one of the most significant foodborne pathogens (**Figure 1**) (Heredia and García 2018). These bacteria colonize the gastrointestinal tract (GIT) of the animal and may contaminate meat at the slaughterhouses through the contact with the feces or intestinal fluids, as shown in **Figure 2** (Heredia and García 2018; Kaper 1998).

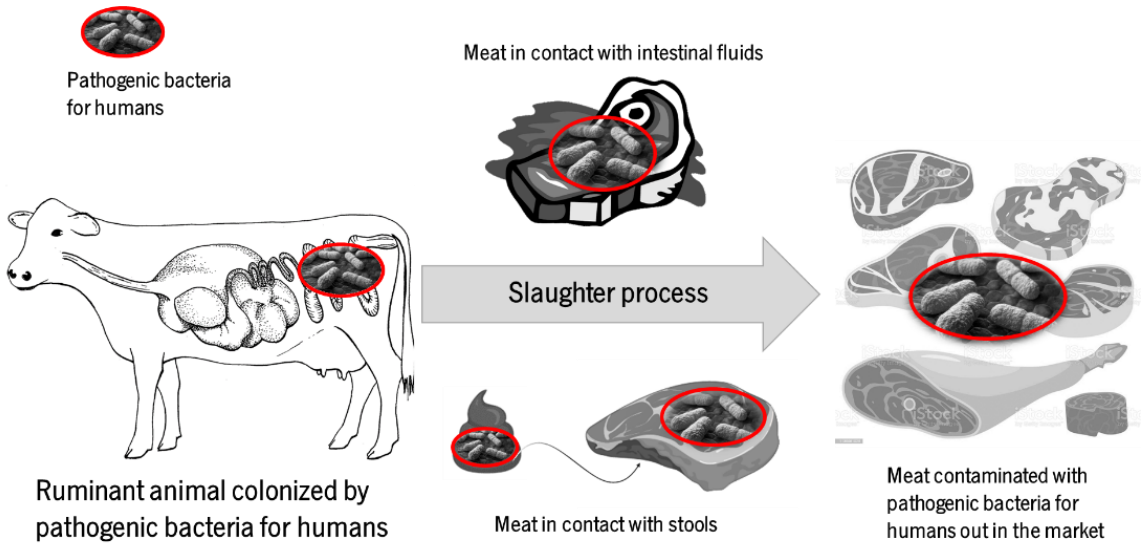


***Figure 2**: Route of contamination of beef.*

If the animal (e.g. cow) is contaminated with bacterial pathogens that colonize the intestine, it may not show symptoms of disease, so it goes on to the slaughter process. During this process, the meat may be exposed to contaminated feces or intestinal fluid, becoming contaminated. In the subsequent steps, if no decontamination measures are applied, the product goes to the shelves. In case the bacteria persist in the confection process, it will end up infecting the consumer, causing foodborne disease. Adapted from: "Animals as sources of food-borne pathogens: A review" (Heredia and García 2018).

E. coli species are gram-negative, facultative anaerobic bacteria, with optimal growth temperatures between 37 °C and 39 °C, very similar to the normal temperatures of the human organism. They belong to the principal commensal bacteria present in the human intestines; it helps the vitamin's synthesis and competes with and suppresses the growth of pathogenic bacteria. However, some *E. coli* strains have acquired virulence factors, causing diseases in the human GIT, urinary tract, or central nervous system (Kaper, Nataro, and Mobley 2004). One of the main reservoirs of *E. coli* strains are healthy ruminant animals. When these bacteria reach the human GIT, they cause infections with severe consequences that depending on the strains include hemorrhagic colitis, acute abdominal cramping, vomiting, and acute renal failure. STEC (e.g. serogroup O157 e serotype H7) is one of the most frequent strains of enterohemorrhagic *E. coli* (EHEC), but there are many other representatives of well-studied foodborne pathogens (Bibbal et al. 2015; Heredia and García 2018; Sillankorva, Oliveira, and Azeredo 2012).

1.3 Strategies against foodborne pathogens

As a consequence of the world population increase and the social and diet changes in the last years, the demand for products from animal origin as well as the import and export of such products have increased (Dhama et al. 2013). These factors contribute to an intense production and processing that can lead to the application of inefficient techniques, increasing the risk of food contamination (Heredia and García 2018). Currently, the food industry applies some strategies during food processing in order to prevent pathogens propagation. Safety measures can be applied as pre- or postslaughter interventions (**Figure 3**). Although farmers have been traditionally applying postslaughter interventions, the preslaughter ones are considered more efficient to prevent the propagation of pathogens throughout the food chain. Their great efficiency is due to the potential elimination of cross-contamination, pathogens shedding through feces, and contamination of other products and surfaces.

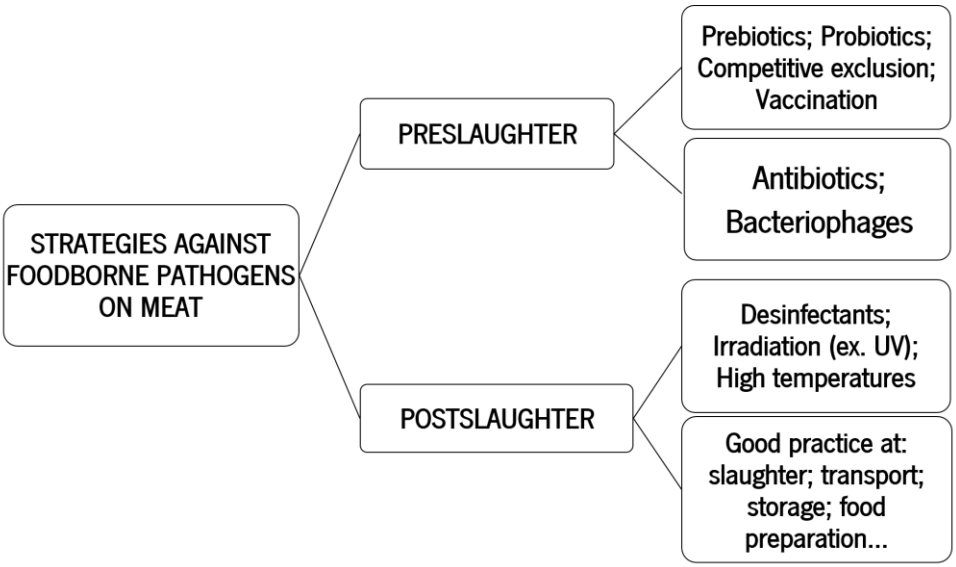


***Figure 3**: Schematic overview of the several strategies used in the food industry to eliminate foodborne pathogens on food.*

They can be employed before or after the slaughter process. In both stages, there are innumerous options and some of them can be used preslaughter or postslaughter (e.g. bacteriophages).

Some examples of preslaughter methods are the administration of prebiotics and probiotics and the competitive exclusion. These strategies have been used to remove pathogens like STEC, and there are commercial products available in the market. The three techniques are based on the concept of antagonistic microbial interactions, and they can be applied separately or synergically (symbiotics). As an example, the Biomin IMPO *(ME BIOMIN GmbH)* is a commercial product that is administered to broilers, pigs, and calves that combines fructooligosaccharides (FOS) and *Enterococcus faecium* (Callaway et al. 2003; Markowiak and Ślizewska 2018; Radzikowski 2017; Uyeno, Shigemori, and Shimosato 2015).

Alternatively, vaccines, antibiotics, and bacteriophages are also used, with the latter two being used as bactericidal agents to combat foodborne pathogens.

1.3.1 The use of prebiotics and probiotics

Prebiotics are nutrients like sugars or organic compounds that are not metabolized by the host organism but are digestible by a specific fraction of microorganisms from the intestinal flora (e.g. *Bifidobacteria*, *Butyrivibrio,* or *Lactobacillus*) and can be included in the animal's diet (Callaway et al. 2003, 2008). In order to be classified as a prebiotic, a compound needs to fulfill the following set of criteria: **i)** it needs to resist gastrointestinal digestion, including gastric acidity and hydrolysis by mammalian enzymes, so it can reach the large intestine; **ii)** once the compound reaches the intestine it must be selectively fermented by intestinal microflora; **iii)** it has to selectively stimulate the growth and activity of the intestinal microorganisms that are beneficial to the animal health and well-being (Roberfroid 2018). Additionally, in ruminants, it is essential that the compound resists the degradation by the fermentative microorganisms present in the rumen, so it is essential to develop strategies to ensure that it reaches the intestine (Callaway et al. 2003). Examples of nutrients classified as prebiotics are FOS, trans-galacto-oligosaccharides (TOS), or inulin (Callaway et al. 2003, 2008; Roberfroid 2018). PROFEED *(Beghin Meiji)* is an example of a FOS-based product available in the market that is administered to pigs and calves (Markowiak and Ślizewska 2018). Studies have proven that prebiotics can help eliminate and inhibit pathogenic bacteria in the intestine by giving some competitive advantages to certain microorganisms (De Vaux, Morrison, and Hutkins 2002). These advantages can be related to the direct competition for nutrients, the production of blocking factors, or even the production of antimicrobial compounds like bacteriocins or colicins (Callaway et al. 2003).

Following the previous principle, some microorganisms classified as probiotics can also be included in animal feeds. These microorganisms are not pathogenic to the animals but can inhibit, by competition or antagonism, the intestinal growth of bacteria harmful to humans. Probiotics can be **1)** live cultures of yeast or bacteria, **2)** heat-treated (or otherwise inactivated) cultures of yeast or bacteria, or **3)** fermentation end products from culturing yeast or bacteria (Callaway et al. 2013). The most used probiotics for administration to animals are lactic acid bacteria and *Bifidobacteria* (Callaway et al. 2003, 2008; Markowiak and Ślizewska 2018; Radzikowski 2017; Walker and Duffy 1998), and other microorganisms like *Bacillus subtilis* or *Saccharomyces cerevisiae* (Callaway et al. 2003; Markowiak and

Ślizewska 2018). Probiosacc C-1 is one of the many probiotics products available on the market; it is constituted by *S. cerevisiae* and is administered to calves (Markowiak and Ślizewska 2018).

Competitive exclusion (CE) is a specific type of probiotic strategy that consists in the inoculation of non-pathogenic microorganisms on the intestinal tract of the animal. A CE culture may be composed of one or more strains or species of bacteria, but it should be derived from the animal of interest (e.g., a bovine CE culture from bovine or a swine CE from swine) (Callaway et al. 2013). The introduced microorganisms have better adaptation mechanisms to the GIT. Therefore, by the attachment to the epithelium and the synthesis of metabolites (e.g. volatile fatty acids) or antimicrobial compounds, CE species prevent and reduce the colonization of pathogens (Callaway et al. 2003, 2008). Although there are positive results described in the literature, these methods have not been totally efficient because there are several factors during the animal growth process, like the administration of antibiotics, which interfere with these methods and reduce their efficacy (Callaway et al. 2003, 2008).

1.3.2 Antibiotics

Antibiotics can be considered as a direct way to combat pathogenic microorganisms. It was confirmed that the antibiotic neomycin sulfate on livestock, reduces the quantity of *E. coli* O157:H7 (Elder et al. 2002), although its use to eliminate foodborne pathogens on ruminants has some controversies. Over the past years, these compounds have been extensively administered to livestock in order to enhance the efficiency and the growth rate of the animals. Since bacteria inherently have several natural adaptation mechanisms to resist antibiotics, the wide application of these compounds has increased the occurrence of horizontal transference of resistance genes. This phenomenon contributes to the increase of antibiotic-resistant bacteria (Callaway et al. 2003; Witte 2000). On the other hand, as the gastrointestinal microorganisms belong to different groups, it is often impossible to identify precisely the etiologic agent. In these cases, wide-spectrum antibiotics are used, and this can compromise the commensal intestinal microflora, which consequently enables the invasion by opportunist pathogens. So in the upcoming years, the use of antibiotics in the livestock industry is expected to decrease and be subject to stricter regulations (Callaway et al. 2003).

1.3.3 Vaccines

The vaccination (immunization) seems to be a promising technique and it can be utilized alongside other techniques synergistically. Nowadays there are already vaccines available on the market aimed to immunize livestock against several foodborne pathogens like *Salmonella* and STEC (Gyles 1998; House et al. 2001). However, the stockbreeders do not use these vaccines because they are not economically favorable. As an example, there are two vaccines on the market to combat EHEC. One of them is a formulation of Type III Secretion System (T3SS), a protein secreted by EHEC during the infection, called Econiche® (Bioniche Life Sciences Inc., Belleville, Ontario, Canada). The other one is a formulation of Siderophore Receptor and Porin (SRP), a membrane protein of EHEC, called Epitopix® (Epitopix LLC, Willmar Poultry Company (WPC), Minnesota, EUA) (Chin-fatt, Topp, and Menassa 2018). Nevertheless, this strategy needs improvements, because some studies verified that bacteria such as *E. coli* O157:H7 and *Salmonella* prevailed on animals exposed to their antigens (Gyles 1998).

1.3.4 Bacteriophages

Another technique with great potential is the use of phages. These viruses recognize and infect specifically bacterial cells through the recognition of bacterial membrane receptors. They were first identified in 1915 by William Twort, and in 1917 by Felix d'Herelle (Clokie et al. 2011). Phages are the most abundant and most diverse biological entities; it is estimated that there are 4.80×10^{31} phages on Earth (Cobián Güemes et al. 2016). They are constituted by proteins that form the capsid and nucleic acids, DNA or RNA, that are inside the capsid (Sillankorva et al. 2012). Phages have been isolated from several ecosystems, including from the GIT of food-producing animals (Adams et al. 1966; Klieve and Bauchop 1988; Orpin and Munn 1974) and a wide variety of raw products (e.g. beef and chicken) (Atterbury et al. 2003; Hsu, Shieh, and Sobsey 2002).

There are several phage morphologies and the majority include a tail, but there have also been described phages without a tail (Ignacio-Espinoza and Fuhrman 2018). The tailed ones are classified as *Caudovirales*, being the *Myoviridae, Siphoviridae,* or *Podoviridae* among the most well-known (**Figure 4**). These biological units are not considered living beings, since they need to use the enzymatic machinery of a host cell to replicate its genetic material and multiply. Depending on their life cycle, phages can be temperate or virulent. The first incorporates its genetic information into the host cell genome and keeps the cell intact (lysogenic cycle). The second produces new viral particles upon infection and then induces the host cell lysis in order to release the newly formed viral particles (lytic cycle) (Sillankorva et al. 2012).

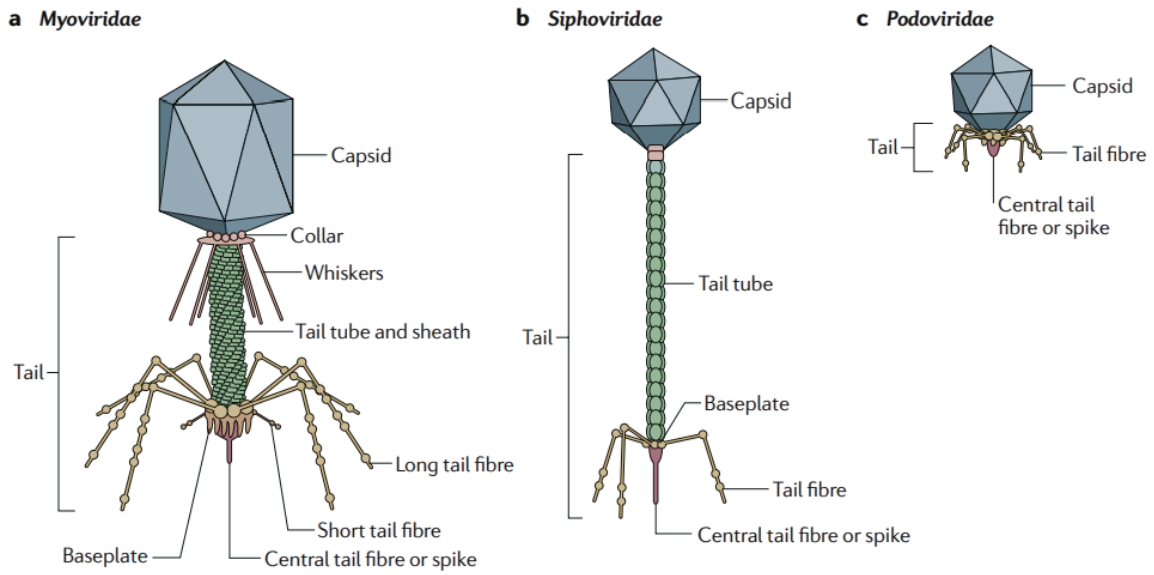


***Figure 4**: Representative structures of tailed phages.*

All the tailed phages have a capsule that surrounds and protects the genome and connects to the tail. ***a|*** *The phages of the Myoviridae family (are the only tailed phages with a contractile-tailed sheath).* ***b|*** *The phages belonging to the Siphoviridae family.* ***c|*** *The members of the Podoviridae family. From: "Targeting mechanisms of tailed bacteriophages" (Nobrega et al. 2018).*

For the application as a strategy against pathogenic bacteria, lytic phages are the most suitable as they will lead to the effective elimination of the target cells. Since phages are specific for bacteria, they do not constitute a risk for animal cells or the respective commensal microflora. In addition, as they are formed only by proteins and nucleic acids, they are not toxic. For these reasons, since phages were discovered in 1915, they have been used in human and veterinary medicine, and agriculture (Callaway et al. 2003; Sillankorva et al. 2012). Phages have many advantages as biological control agents: **i)** their process of isolation, culture, and manipulation are relatively easy and have a low associated cost; **ii)** they multiply after infection, persisting as long as there exist target bacteria in the environment; **iii)** they can continuously adapt to the bacterial defense mechanisms, among other benefits (Sillankorva et al. 2012).

Pathogenic bacteria have been controlled in animals with lytic phages during primary production (phage therapy). This strategy is applied immediately before the slaughter or during the growth of the animal to reduce the probability of cross-contamination (Sillankorva et al. 2012). Several studies performed in different animals and using phages for distinct bacteria were published; some specific for *E. coli* O157:H7 elimination are described in **Table 1**. One example is the study conducted by Rivas et al. in 2010, where they tested the use of the phages e11/2 and e4/1c *in vivo* (livestock) and *ex vivo* (a rumen model) and verified the reduction of *E. coli* O157:H7. In Europe and the USA, some applications

of phages in the food industry and animal production have been recognized for pathogens, including *Salmonella*, *Campylobacter*, *E. coli,* and *Listeria* (Islam, Wang, and Sabour 2018). In the USA, the Food and Drug Administration (FDA) has approved several products; examples are EcoShieldTM a food additive, and PLSV-1TM for veterinary use (Intralytix Inc., Baltimore, MD USA) (Farooq et al. 2019). However, in the EU, no phage-based product has been approved yet (Pinto, Almeida, and Azeredo 2020). Despite promising results, the majority of attempts of phage application *in vivo*, administered to ruminant animals, did not demonstrate satisfactory effects respecting the elimination of pathogens on the animal feces (Sillankorva et al. 2012). This can be due to several factors that will be enumerated in section 1.4.

***Table 1**: Examples of the application of free bacteriophages as preslaughter strategy to eliminate the food pathogen E. coli O157:H7 on ruminants.*

Year	Animal/Product	Phage (s)	Strategy	Highlights	Reference
2003	Ruminant (lamb)	DC22	Oral administration	No reduction in fecal elimination after 30 days	(Bach et al. 2003)
2006	Ruminant (sheep)	CEV1	Oral administration	In 2 days, there was a reduction of 2 CFU's log	(Raya et al. 2006)
2006	Ruminant (cattle)	Phage cocktail (KH1, SH1)	Oral/rectal administration	No reduction in CFU's when applied orally. Combined oral/rectal treatment reduced CFU's, but did not eradicate	(Sheng et al. 2006)
2009	Ruminant (bullock)	Phage cocktail (rV5, wV7, wV8, wV11)	Oral/rectal administration	Although the bacteriophage counts detected in the oral and oral+rectal treatments were higher than in the control group and the group with rectal administration, there was no significant difference between treatments (P > 0.05) in the number of *E. coli* O157-positive samples	(Rozema et al. 2009)
2010	Ruminant (cattle)	Phage cocktail (e11/2, e4/1c)	Oral administration	Rapid decrease of CFU's within 24 to 48 hours, but no decrease in fecal elimination levels	(Rivas et al. 2010)
2011	Ruminant (sheep)	Phage cocktail (CEV1, CEV2)	Oral administration	Cocktail eradicated (> 99.9%) the pathogen, being more effective than CEV1 alone	(Raya et al. 2011)

1.3.5 Postslaughter strategies

As postslaughter strategies, antimicrobial compounds or decontamination processes are often applied directly to the meat. Some examples are phages, disinfectants, UV radiation, exposure to high temperatures, among others (Chin-fatt et al. 2018). It is also important to ensure the use of appropriate practices during the slaughter, as well as the sterility of the surfaces that products come in contact with, adequate conditions of transport, storage, and preparation of food. The reduction of food colonization (biocontrol) during industrial processing can be performed through the application of phages directly on the contact surfaces or on the food itself (**Table 1**), where they remain viable for a prolonged period, depending on the physico-chemical conditions involved (Sillankorva et al. 2012).

1.4 Challenges and possibilities of oral administration of phages to ruminants

Despite the investment in the application and optimization of the techniques listed above, there are still a high number of outbreaks of foodborne diseases around the world every year. This demonstrates that eliminating foodborne pathogens is not an easy task, leading to the urgent need to develop new and more effective techniques. Since phages have several advantages, many efforts have been made to increase the efficiency of their possible applications, such as their encapsulation (Dini et al. 2012; Stanford et al. 2010).

Focusing on the oral administration of phages to ruminants, with the aim of killing intestinal bacteria, such as STEC, one perceives a reduced efficiency of this method. This can happen due to the difficulties that phages face to reach the target site since the route to the intestine is hindered by the digestive process, mainly in the rumen and abomasum (**Figure 5**). This can happen for different reasons: **i)** the aggregation to food particles and other debris; **ii)** through the inactivation of phages by fermentative microorganisms present in the rumen or products of its metabolism and **iii)** the low pH conditions, presence of enzymes, and other compounds characteristic of the abomasum. These factors contribute to the fact that only a small number of viable phages reach the intestine, preventing the effective elimination of pathogens at the intestine site (Goodridge and Bisha 2011; Sheng et al. 2006; Sillankorva et al. 2012; Smith, Huggins, and Shaw 2009).

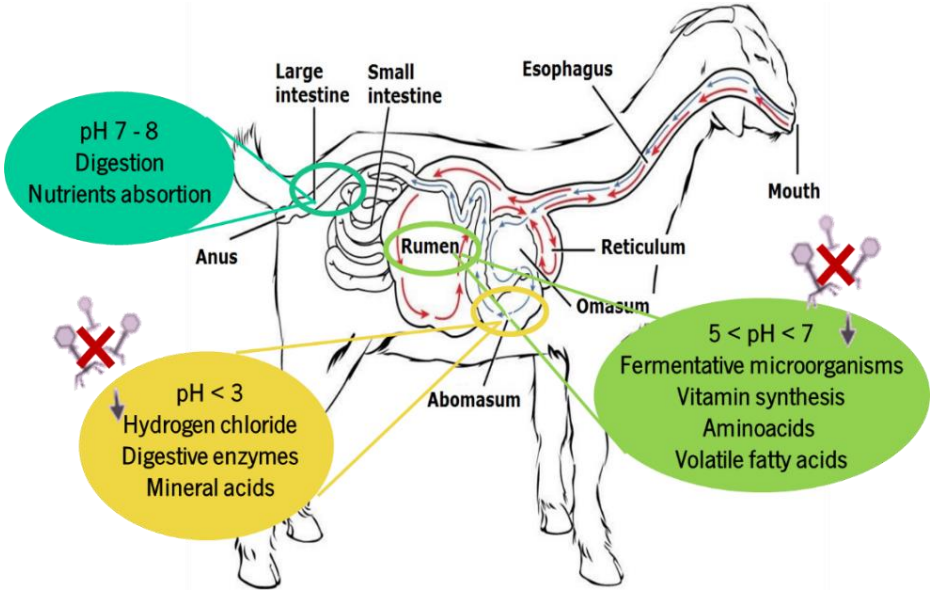


***Figure 5**: Illustrative image of the digestive system of a ruminant and synthesis of the characteristics of the rumen, abomasum, and intestine.*

The rumen and abomasum present characteristics that make difficult the arrival of phages to the intestine, hence the reduced efficiency of oral administration of phages to eliminate intestinal pathogens. The arrows represent the route of food intake (red - when there is regurgitation, blue – after regurgitation). Adapted from: https://toaaps.weebly.com/ruminant-digestive-system.html.

1.4.1 Advantages of encapsulating the phages

To overcome these obstacles, many studies evaluated the impact of the encapsulation of phages on the efficiency of the oral administration process of these agents. Encapsulation can protect bioactive compounds from adverse conditions and has already been used in the food area to encapsulate several nutrients, such as pre- and probiotics, among other substances (Fuciños et al. 2014; Gibbs et al. 1999; Whelehan and Marison 2011). It has been verified that this technique can stabilize phages during the passage through the digestive system of ruminants and increase the effectiveness of oral administration (Dini et al. 2012; Hussain et al. 2017; Stanford et al. 2010; Yongsheng et al. 2008).

An encapsulation process can result in several types of products, such as gels, films, liposomes, and particles. Encapsulation can be defined as a process that allows the complete entrapment of pre-selected materials (solids, liquids, and gases) within a porous or impermeable matrix or membrane, originating final products of sizes in the order of nanometers (below 0.1 µm), micrometers (between 0.1-1000 µm) or millimeters (above 1000 µm) (Whelehan and Marison 2011). Structurally, the particles produced can be classified as beads (matrix type) (**Figure 6b**) or capsules, also called core-shell particles that can be reservoir type (**Figure 6a**), or coated matrix type (**Figure 6c**) (Gibbs et al. 1999). Beads

are solid particles whose geometry can be clearly defined by their diameter, having the encapsulated material distributed throughout the structure of the matrix, i.e., it has no defined core or shell. The capsules, on the other hand, are spherical particles constituted by a defined core, containing the encapsulated material or consisting of the encapsulated material itself, surrounded by a shell.

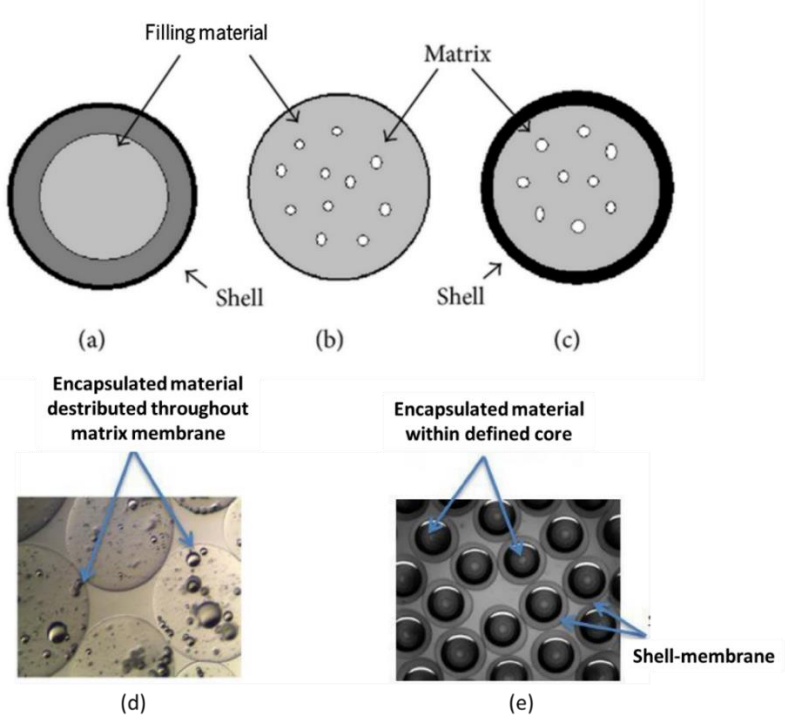


***Figure 6**: Schematic representation of encapsulation systems: (**a**) reservoir type, (**b**) matrix type, and (**c**) coated matrix type.*

*Illustrations of the structural differences between beads (**d**) and capsules with core and casing (**e**). From: https://www.researchgate.net/figure/Schematic-representation-of-encapsulation-systems-a-reservoir-type-b-matrix-type_fig4_256501985 and "Buchi Encapsulator B-390/B-395 Pro -Laboratory Guide".*

The six main benefits of encapsulating a compound are summarized in **Figure 7** and are: **i)** protection (stabilization) of the encapsulated material from environmental conditions (heat, humidity, oxygen, etc.) and interactions with other compounds useful in food, biotechnology, pharmaceutical and cosmetic industries; **ii)** controlled release of the encapsulated material that has an infinity of applications in industries such as food, pharmaceutical, agriculture, textile and cosmetics; **iii)** delivery to target sites, which allows the encapsulated material to be delivered directly to the place where it is needed, which is very useful for applications in the pharmaceutical, medical and biotechnological sectors; **iv)** allowing the encapsulated material to act as an extraction aid for product removal, this has applications in agriculture, environment and bioremediation; **v)** improving the flow properties the of encapsulated material, with applications in the food, pharmaceutical and agriculture industries; **vi)** improving the organoleptic

properties, masking its taste or unpleasant smells, improve its appearance and texture, extensively explored in the food and pharmaceutical industries. Encapsulating a material can also help to convert a liquid into a solid particle which improves handling, use, and storage and prevent direct contact between the encapsulated material and the manipulator, thus allowing safer handling of toxic materials (Whelehan and Marison 2011).

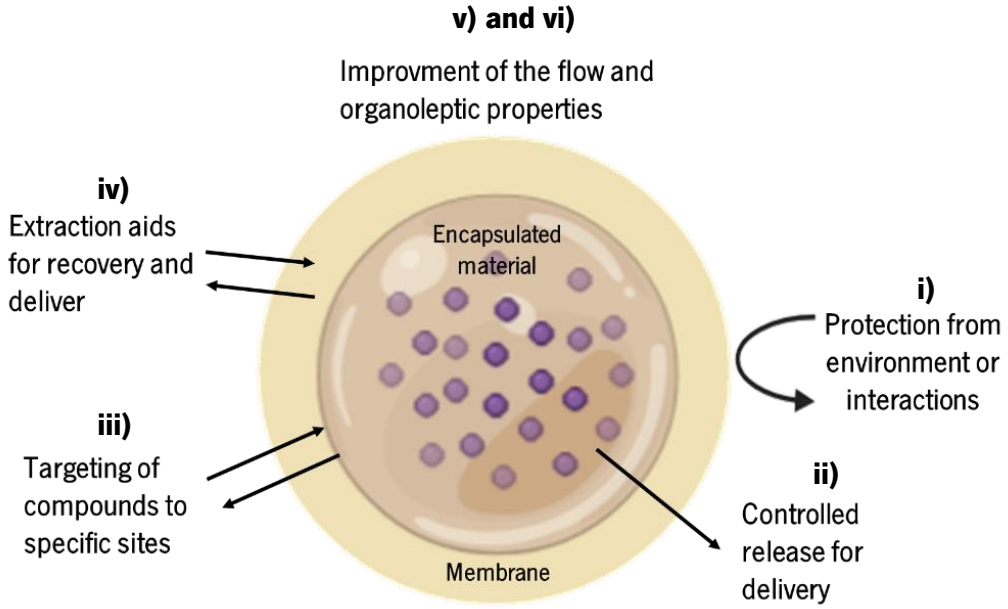


***Figure 7**: Schematic representation summarizing the six main advantages of encapsulation.*

Adapted from: "Buchi Encapsulator B-390/B-395 Pro -Laboratory Guide".

1.5 Principal methods and techniques applied for nano and microencapsulation

In order to preserve the integrity, stability, and biological activity of certain compounds, several encapsulation methods and techniques are applied. Examples are emulsification, extrusion, spray drying, spinning disk atomization, sonication, electrospinning, polymerization, and others, as well as combinations of them. It is important to note that the success of the encapsulation system developed depends mainly on the wall material (WM) used as encapsulating agent, the optimized process conditions, and the encapsulation technique employed, so all these parameters can affect the process and the encapsulation efficiency (EE) (Gbassi and Vandamme 2012). Encapsulation methods can be classified into three groups: **1)** Chemical methods including interfacial polymerization and *in situ* polymerization;

2) Physical methods as spray drying, freeze-drying/lyophilization, and solvent evaporation and **3)** Physico-chemical methods such as emulsification and extrusion-gelation (Ozkan et al. 2019).

1.5.1 Chemical Methods - Polymerization techniques

Several publications refer to methods based on polymerization techniques, which are widely used for manufacturing polymeric products of special design and controlled morphology, using natural or synthesized polymers. Therefore, they can be applied to nano and microencapsulation. Polymerization can occur through physico-chemical, physico-mechanical, or chemical processes (Mirzataheria, Mahtabanib, and Lotfalieic 2014), so there are many different polymerization techniques. Interfacial polymerization (Ozkan et al. 2019) and *in situ* polymerization (Nguon et al. 2018) are both chemical processes.

Interfacial polymerization occurs through the interface of hydrophilic and lipophilic monomers in an oil-water emulsion, reacting and forming a polymeric membrane on the surface of the droplet or particle. Some advantages of this process are the absence of a catalyst and the low temperatures required (Ozkan et al. 2019). In addition, other advantages include the possibility to control the average size of the capsule and the thickness of the membrane, achieving a high loading of active compound, versatile and stable mechanical and chemical properties of the membrane, low cost, easy scalability, simplicity, and reliability of the process (Perignon et al. 2015). Some drawbacks associated with this method are the difficulty in producing large oil-water interfaces; for this reason, proteins and enzymes are inactivated, leading to significant losses on proteins' biological activity during polymerization reactions. This technique also lacks control of the polymerization characteristics such as yield and quality of the polymeric membrane. Moreover, this method also requires washing steps to remove monomers, by-products, organic solvents, etc., leading to losses of water-soluble active compounds and other substances (Yeo, Baek, and Park 2001).

In the *in situ* polymerization process, a solution of the monomeric or oligomeric WM is added to the core phase, the latter being dispersed to the desired size. Controlled deposition and precipitation of the polymer take place at the interface, using precipitants or a change in pH, temperature, or quality of the solvent. Several encapsulation processes rely on this polymerization technique and have been discussed in several reviews (Nguon et al. 2018). Arshady and George (Arshady and George 1993) distinguished three cases of *in situ* polymerization based on the solubility of the monomer and the

polymer: suspension polymerization, precipitation polycondensation, and dispersion polycondensation. The suspension polymerization occurs when the monomer is insoluble in the dispersion medium; it forms suspended monomer droplets that polymerize and create polymer microparticles (MPs). The polymerization reactor and stirring rate are thus important parameters in maintaining a uniform size distribution. Precipitation polycondensation, takes place when the monomer but not the polymer is soluble in the dispersion medium. As the reaction proceeds, flocculation and aggregation of a low molar mass polymer produce particles with characteristic broad size distribution and irregular shape. Lastly, dispersion polycondensation, when the dispersion medium is a good solvent for the monomer, but a poor solvent for the polymer. Under such conditions, swelling of the polymer rather takes place and microcapsule growth occurs by the sustained addition of monomer and oligomer to the particle. MPs with a narrow size distribution are formed under these conditions (Arshady and George 1993; Nguon et al. 2018).

1.5.2 Physical Methods

1.5.2.1 Spray drying

Spray drying is a method that consists of atomization of a liquid product, which is rapidly dried by a hot gas (usually air) to obtain a very fine powder (10-50 μm) or large size particles (2-3 mm) depending on the starting feed material and operating conditions (Gharsallaoui et al. 2007; de Vos et al. 2010). The liquid can be a solution, an emulsion, or a suspension. This process follows three main steps: **1)** atomization that can be made by different types of atomizers (pneumatic atomizer, pressure nozzle, spinning disk, fluid nozzle, and sonic nozzle); **2)** air contact and formation of the droplets; **3)** evaporation of the water and dry product/humid air separation (Gharsallaoui et al. 2007). The temperature of the hot air inlet is between 150 °C and 220 °C, and the evaporation occurs very quickly, so the exposure of the MPs to these high temperatures is short, usually just a few seconds. This reduces the possibility of damage of thermo-sensitive compounds, reducing significant losses with special emphasis when chitosan is used. In the end, the temperature decreases to moderate values around 50-80 °C (Azeredo 2005; Desai and Park 2005; Estevinho et al. 2013; Gharsallaoui et al. 2007; de Vos et al. 2010).

It is possible to encapsulate active compounds through the spray drying process. Compared to some other methods for microencapsulation, this is a relatively rapid, low-cost, and reproducible technology, allowing an easy scale-up. For these reasons, this method is preferred in industrial processes

(Pu, Bankston, and Sathivel 2011; Rattes and Oliveira 2007; Schafroth et al. 2012; de Vos et al. 2010). It is used, for example, in the food industry since the 1950s to protect flavors from degradation and oxidation or to dry solid suspensions, but is also applied to encapsulate probiotics and bioactive molecules (Gouin 2004; Pu et al. 2011). This method allows a large variation of the WM, the use of common equipment, and the production of good quality particles. The particles are normally of the matrix type, where the encapsulated material is distributed in the encapsulating agent, which can be gums, gelatin, maltodextrin, alginates, chitosan, etc., and can be compact or hollow. The limitation of this method is the fact that the WM should be highly soluble in water (Azeredo 2005; Desai and Park 2005; Estevinho et al. 2013; Gharsallaoui et al. 2007).

1.5.2.2 Spinning disk atomization

Spinning disk or cup atomizers have been applied successfully in the chemical (De Beer et al. 2014; Jacobsen and Hinrichsen 2012), metallurgical (Duan et al. 2015; Zhang et al. 2013), and food (Akhtar, Murray, and Dowu 2014) industries. In this process, the liquid is fed to the center of a rotating disk or cup, where the centrifugal force causes the liquid to spread; due to this instability, three breakup modes of the liquid film can occur around the rim: direct-drop mode, ligament mode, and sheet mode. The transition from one mode to another occurs by changing the operating parameters and geometrical configurations (Li, Sisoev, and Shikhmurzaev 2018). Also concerning this technique, the most attractive advantage is the ability to be fully applicable to low viscous liquids, high viscous liquids, suspensions, and emulsions (Ahmed and Youssef 2012; Sun et al. 2015). This allows the encapsulation of bioactive compounds with a variety of WMs once it can be applied to many chemical reactions like organic syntheses (Dehkordi and Vafaeimanesh 2009; Mohammadi, Harvey, and Boodhoo 2014) or catalyzed reactions (Chiang et al. 2012).

1.5.2.3 Freeze-drying or Lyophilization

A drying technique such as freeze-drying is the most adequate for dehydration and microencapsulation of heat-sensitive materials and active compounds (Desai and Park 2005), aiming to the long-term preservation of heat-sensitive food and other biological materials based on the phenomena of sublimation (Ceballos, Giraldo, and Orrego 2012). It is a multistage process consisting of four main operations such as freezing, sublimation, desorption, and finally, storage (Mascarenhas, Akay, and Pikal

1997). Similar to other methods, the efficiency of protection or controlled release depends mainly on the composition and structure of the WM (Young, Sarda, and Rosenberg 1993) which can be chosen among polymers such as arabic gum, maltodextrin, emulsifying starches, whey protein, etc. The success of a freeze-drying process lies in the preservation of most of the initial characteristics of the raw material, like its shape, dimensions, appearance, taste, biological activity, etc. (Ceballos et al. 2012). Freeze-drying presents several disadvantages and it is important to refer that this technique requires a high energy input and a long processing time (at least 48 hours). Also, there is the formation of a barrier with a high-porous structure between the active agent and its surroundings, consequently, offering little protection of the compound when a prolonged release is required (Zuidam and Shimoni 2010).

1.5.2.4 Solvent evaporation

Microencapsulation using the solvent evaporation technique is employed mainly in the pharmaceutical industry to obtain controlled release formulations such as liposomes. There are many methods for microencapsulation based on this technique, and the selected method depends on the hydrophilicity and hydrophobicity of the encapsulated compound (Li, Rouaud, and Poncelet 2008; Miladi et al. 2013). Emulsification is an example, and it is possible to perform a single emulsion (W/O) or a double emulsion (W/O/W) (Alex and Bodmeier 1990; Pisani et al. 2008). In the case of a double emulsion, the homogenization is performed in two steps; in the first step, water-soluble drugs are incorporated in the inner aqueous phase (W1), and polymer or lipophilic drugs are added into the oil phase (O), then both phases are homogenized by proper agitation to form the primary emulsion (W1/O). After that, the primary emulsion is emulsified with the outer aqueous phase (W2), containing an appropriate stabilizer to form a double emulsion (W1/O/W2). The formation of double emulsion (particulate dispersion) is followed by evaporation of the organic solvent (O) from the dispersed phase leading to the point of insolubility and, consequently, hardening of the polymer and encapsulation of the active compound. The solvent can be evaporated under reduced pressure via a rotary evaporator or by simple stirring at room temperature, depending on the boiling point of the organic solvent. The external aqueous phase act as a dispersion medium, and the agitation can be provided either by mechanical stirring or sonication depending upon the nature of the compound to be encapsulated and the intended particle size (Iqbal et al. 2015).

1.5.2.5 Electrospinning

Electrospinning is a simple and versatile technique characterized by the application of an electrical field to continuously draw a polymer solution or melted polymer droplet that is previously extruded at the needle tip by a syringe pump into a fine fiber followed by its deposition in a grounded collector (Huang et al. 2006; Wen et al. 2017). Fibers can have diameters from several micrometers to a few nanometers, usually in hundreds of nanometers (Huang et al. 2006). Due to its outstanding features, such as an extremely high surface area to volume ratio, polymer nanofibers obtained through electrospinning have been proposed for a variety of applications, especially in drug release systems (Huang et al. 2003; Li and Xia 2004; Zhang et al. 2005). Taking into account the aforementioned, this technique has a significant interest in the microencapsulation of drugs and other sensitive bioactive compounds (Wen et al. 2017). Moreover, several advantages have been attributed to electrospun polymeric nanofibers when compared to other dosage forms. For example, **1)** therapeutic compounds can be conveniently incorporated into the carrier polymers using electrospinning; **2)** the drug release profile can be finely tailored by modulating the morphology, porosity, and composition of the nanofibers membrane (Kim et al. 2004). Besides, **3)** the very small diameter of the nanofibers can provide a short diffusion passage length (Nair, Bhattacharyya, and Laurencin 2004); and **4)** the high surface area is useful for mass transfer and efficient drug release (Verreck et al. 2003).

1.5.2.6 Sonication

Ultrasonic technology, such as sonication, is a versatile method and has been proven effective for encapsulating materials in particles (beads/capsules) dispersed in water, with specific physical and functional properties. Sonication can be used to produce highly stable emulsions, functional polymeric particles with environmental sensitivity, and MPs for encapsulating drugs for target delivery (Leong, Martin, and Ashokkumar 2017). This type of formulation has several applications in nutritional foods (Chemat, Zill-E-Huma, and Khan 2011), imaging (Goldberg, Liu, and Forsberg 1994), energy production (Pollet 2010), and therapeutic or diagnostic medicine (Pandey et al. 2015; Suslick and Price 1999). The mechanism behind this technique is known as acoustic cavitation, which consists of the formation and collapse of bubbles influenced by ultrasound that promote the internalization of materials through a process known as encapsulation (Leighton and Apfel 1994; Leong et al. 2017). The number and intensity of these cavitation events can be controlled by the application of a broad range of frequencies, and it

allows the control of some properties like particle size, structure, and surface roughness (Leong et al. 2017).

Sonication can be used to develop specific functionality in different materials; for example, it can be used to disperse different organic/oil phases into various aqueous phases in a controlled manner to create emulsified products that are very shelf-stable and attractive (Kentish et al. 2008). Sonication can also be used to aid the formation of polymeric systems that respond to specific environmental conditions, such as pH and temperature. These systems are useful for controlling drug release in biological environments. The combination of emulsification and polymerization can be promoted by ultrasound to form protein cross-linkages, resulting in the formation of protein microspheres. These entities can be biocompatible and biodegradable to enable their use as drug-delivery vehicles (Bhanvase et al. 2011; Leong et al. 2017).

1.5.3 Physico-chemical Methods

1.5.3.1 Emulsification

An emulsion consists of a mixture of two immiscible liquids, like water and oil, to create small droplets dispersed in a continuous phase (Solans, Morales, and Homs 2016; Stroeve and Varanasi 1984), where molecules and small particles can be encapsulated within these droplets. Emulsions are of great interest as applications for several areas like separation technology (Gaitzsch, Gäbler, and Kraume 2011; Stroeve and Varanasi 1982), or encapsulation of sensitive molecules for food (Benichou and Garti 2001; Garti 1997), cosmetics, and pharmaceutics. It is also applied for the preparation of functional food products, with fat reduction (Benoy, Elson, and Schneider 1972; Florence, Jenkins, and Loveless 1976; Marti-Mestres and Nielloud 2002). It is possible to obtain different types of emulsions, oil-in-water (O/W) mainly used to encapsulate insoluble or poorly water-soluble active agents, water-in-oil (W/O) suitable for encapsulation of hydrophilic agents, or water-in-oil-in-water (W/O/W) the so-called double-emulsions.

As mentioned above, there is the possibility of creating multiple emulsions, where a water-in-oil emulsion is dispersed in a continuous water phase. This very complex system normally is achieved by two sequential steps; at the first one, the inner W/O emulsion is produced using conventional high shear emulsification devices like high-pressure homogenizers and rotor-stator systems to achieve small sizes (microcapsules) and narrow size distributions (Van Der Graaf, Schroën, and Boom 2005; Schuch et al. 2013). The second step is the most complex because, in this stage, the dispersed phase is an emulsion

itself and during the process, the distribution of the size of the droplets and the amount of water encapsulated in the oil can be affected; this can influence the properties and functionality of the double emulsion. Another challenge in the production of multiple emulsions is the influence of the complex structure of the dispersed phase on the droplets breakup (Schuch et al. 2013; Stroeve and Varanasi 1984).

1.5.3.2 Melt extrusion or injection

This technique is widely used in the food industry and many other areas. The difference between melt extrusion or injection and the common extrusion is that the first two consist in the incorporation of volatile compounds, like flavors, fragrances, or other sensitive molecules into a thermoplastic matrix, forcing this mass through an orifice (extrusion) or into a mold (injection) to shape the encapsulation system. The efficiency and quality of the overall process are determined considering the result of the combination of system morphology, i.e. the way the encapsulated material is dispersed in the matrix and the physical state of this matrix (Castro et al. 2016). Benczedi and Bouquerand (2003) demonstrated that lemon, lime, and tangerine flavors presented higher stability and great shelf life (4 years at 20 °C) with the melt extrusion method in comparison to the spray-drying technique.

1.5.3.3 Prilling by vibration

Extrusion is a process that does not require high temperatures or the use of organic solvents (de Vos et al. 2010) and has minimal emission of odor-contaminated exhaust air. Besides, extrusion allows better control of the state of the matrix and a reduced energy and water consumption (Castro et al. 2016). This process is widely used for probiotic and phage encapsulation. Consists of mixing a compound in hydrocolloid solutions of natural or synthetic polymers, carbohydrates, lipids, etc., the resulting mixture pass through a nozzle, and the droplets formed are collected in a bath solution that, in some cases, should be a gelling bath (Krasaekoopt, Bhandari, and Deeth 2003; Risch 1995). This process results in matrix-type beads where the entrapped material is present in the whole particle structure, and consequently, are exposed to environmental conditions that could negatively affect their viability. To overcome this problem, it is possible to perform a co-extrusion under the same conditions but using a concentric nozzle system. In this system, capsules are produced by vibration technologies to break the laminar liquid jet into equal-sized droplets that are collected at the end of the process forming core-shell

capsules (Silva et al. 2018). Referring to the drawbacks of this method, it is important to mention that it has a limited loading capacity (never exceeding more than 15 % - 20 % and can lead to the coalescence of the droplets containing the active compound (Emin and Schuchmann 2013a, 2013b).

The encapsulation of several compounds, using various materials as carriers, can be performed through the prilling by vibration technique (Whelehan and Marison 2011) using encapsulator equipment. This technique is based on the principle of disrupting the laminar flow stream of a liquid by applying a controlled vibrational frequency as illustrated in **Figure 8**. Through the disruption of the liquid jet, small particles are formed (droplets), which as mentioned above can originate beads or capsules, depending on the pulsation chamber used, it can be the single nozzle (SN), concentric nozzle (CN), or flow vibration nozzle (FVN) (**Annex 1** - BUCHI Encapsulator model B-395 Pro). Due to the availability of nozzles with different diameters, it is possible to pre-select a wide range of particle sizes (80 µm - 4 mm). The technology also allows the reproducibility of homogeneous particles, which have a small size distribution (<5 % standard deviation of the mean size for alginate solutions) through a one-step process. For these reasons, it is one of the most widely used techniques to produce beads and capsules at a laboratory scale, whether it is for the encapsulation of enzymes, drugs, chemicals, flavors and fragrances, vitamins, oils, cells, or microbes, in a wide range of different carriers. It has been used for more than two decades to develop new innovative products (Del Gaudio et al. 2005, 2009, 2014; Martinez et al. 2004; Mirtič et al. 2018; Partridge et al. 2005; Rodríguez-Dorado et al. 2018).

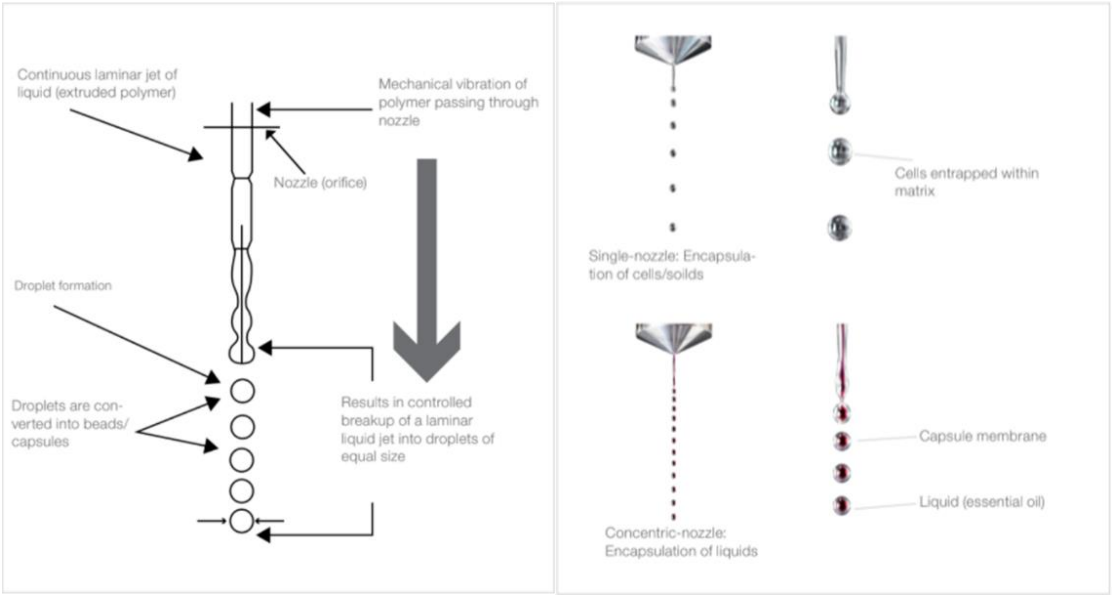


***Figure 8**: Schematic illustration of the operating principle of the prilling by vibration technique.*

This technique uses vibrating frequencies for controlled disruption of the laminar flow of a liquid to form droplets of equal size. Adapted from: "Microencapsulation using vibrating technology", Journal of Microencapsulation.

To avoid coalescing of the droplets during jet disruption or when they are collected into the gelling solution, an electrical charge is induced on the surface of the droplets using an electrostatic voltage system. This system applies an electrical potential between the nozzle and an electrode, placed directly under the nozzle (Brandenberger et al. 1999; Serp et al. 2000; Whelehan and Marison 2011). As the droplets pass through the electrode, they are charged and deflected from the vertical position, resulting in their distribution over a larger area in the solution. The size of these particles and their production rate depends mainly on the internal diameter of the nozzle, the flow rate of extruded liquid, and the viscosity of the liquid, which varies according to the type and concentration of the carrier, and the vibration frequency applied. All these parameters can be controlled, allowing the operator to determine the size and characteristics of the particles produced (Serp et al. 2000; Whelehan and Marison 2011). A detailed description of how each parameter influences the production of particles is intuitively presented in **Annex 2** - Optimization parameters.

1.5.4 Examples of phage encapsulation studies

Until now, the encapsulation of phages in polymers such as methacrylate (synthetic polymer) (Stanford et al. 2010), gelatin, chitosan, different types of alginates (biopolymers), and others have been performed. These encapsulation systems were produced using phages specific for different pathogens, for oral administration to pigs, birds, or ruminants (Colom et al. 2017; Dini et al. 2012; Outokesh et al. 2006; Vinner et al. 2019; Yongsheng et al. 2008) (**Table 2**). In most cases, it was concluded that optimization and additional studies would be necessary to guarantee better results since the strategies were not able to definitively eliminate the pathogens nor to decrease shedding through the feces (Sillankorva et al. 2012). When developing this type of technique, it is essential to consider the type of pathogens and the environment from which they are intended to be eliminated to select the phage, as well as the type of WM and the encapsulation technique.

***Table 2**: Examples of phage encapsulation studies for biocontrol.*

Year	Animal/Product	Phage(s)/ Pathogen	Encapsulation strategy	Highlights	Reference
2008	*In vitro*	Felix O1/ *Salmonella*	Extrusion with prilling by vibration, followed by ionic gelation ($CaCl_2$ bath)	Chitosan-alginate MPs The phage's viability after exposure to acidic conditions was preserved	(Yongsheng et al. 2008)
2010	Ruminant (bullock)	*Phage cocktail (wV8, rV5, wV7, wV11)/ E. coli* O157:H7	Spray drying (with a rotary atomizer)	Gelatin capsules; oral administration No reduction in the fecal elimination of NREC O157:H7, but the duration of the fecal shedding was reduced by 14 days	(Stanford et al. 2010)
2016	Chicks	Felix O1/ *Salmonella*	Extrusion, followed by ionic gelation ($CaCl_2$ bath)	Ca^{2+} alginate-whey protein beads Most of the phage went through GIT and was detected in the feces in 4 h in low levels, being excreted until 24 h	(Ma et al. 2016)
2017	Chickens	Phage cocktail (UAB_Phi20, UAB_Phi78, UAB_Phi87)/ *S. Typhimurium*	Air atomization	Alginate/$CaCO_3$ beads; oral administration Encapsulated phages had greater and more lasting efficacy than a cocktail of the non-encapsulated phages	(Colom et al. 2017)
2018	-	Phage T3/ *E. coli* and phage K/ *S. aureus*	Liposomes	Encapsulation yield of the T3 phages was affected by the aggregation of the phages Phage K interacted with the large number of outer bound phages	(Cinquerrui et al. 2018)
2018	*In vitro*	f3αSE/ *S. enteritidis*	Extrusion with a syringe, followed by ionic gelation ($CaCl_2$ bath)	Alginate beads Encapsulated phages resisted much longer (100 h) to high temperatures compared to non-encapsulated phages	(Soto et al. 2018)
2019	*In vitro*	Felix O1/ *Salmonella*	Spray drying (with a pneumatic atomizer)	Eudragit S100® and trehalose tablets The potential of pH-sensitive beads, for oral delivery of bacteriophages targeting gastrointestinal applications, was demonstrated	(Vinner et al. 2019)

2019	Birds (meat)	ϕIBB-PF7A/ *fluorescens*	*P.* Phages incorporated into sodium-alginate films	Sodium-alginate films; applied on the surface of the meat The incorporated phages decreased the growth of bacteria in the first 2 days and up to 5 days of exposure	(Alves et al. 2019)

1.6 Biopolymers as encapsulation materials

The use of biological materials as carriers has been explored in the last years in the development of new encapsulation systems for the protection and transport of sensible biological compounds. Lipids (e.g. fatty acids and waxes), proteins (e.g. casein, gelatin, and keratin), polysaccharides (e.g. alginates, cellulose, and chitosan), and resins (e.g. shellac) are some examples (H. Wu et al. 2019). Recently, the use of polymer-based capsules from natural sources has aroused a lot of interest due to their renewability, biodegradability, biocompatibility, and nontoxicity (Messaoud et al. 2016). It is very important to take into account the final application, the nature, and the physico-chemical properties of the carrier before selecting an adequate material (Khanvilkar, Ranveer, and Sahoo 2016). The use of polysaccharides from natural sources is extensive and of great importance in the encapsulation field (Madene et al. 2006; Martau, Mihai, and Vodnar 2019). With these materials, it is possible to achieve successful encapsulation, controlled delivery, and release of the encapsulated material to the external environment (Hu et al. 2008). In addition, these materials have properties, such as low viscosity at high solids contents, good solubility, and drug interaction behaviors that are desirable in an encapsulating agent (Madene et al. 2006; Rinaudo 2008). Besides, their diversity, low cost, and widespread use in food products make them the preferred choice for encapsulation. Hydrogels are prepared using polysaccharides, which are water-soluble polymers with the ability to form gels under well-defined conditions (Rinaudo 2008).

1.6.1 Alginate

Alginate is a naturally derived copolymer and one of the most used polysaccharides to prepare encapsulation systems based on capsules or beads. This is due to its interesting properties such as wide availability, biocompatibility, gelation under mild conditions, low cost, resistance to contamination, and food-grade status (it is approved by the EFSA as a food additive) (Chávarri et al. 2010; Ding and Shah

2008; Kailasapathy 2002; Pawar and Edgar 2012; Pitigraisorn et al. 2017; de Vos et al. 2010). It is extracted from brown algae such as *Macrocystispyrifera*, *Ascophyllum nodosum*, *Laminaria hyperborean*, or isolated from bacterial species such as *Azotobacter* and *Pseudomonas*. Chemically it is considered an anionic, unbranched binary polysaccharide, composed of linear chains of 1,4-linked β-D-mannuronic (M) and α-L-guluronic acid (G) (Shilpa, Agrawal, and Ray 2003). These uronic acid residues are distributed in a pattern of homopolymeric blocks (G or M-blocks) and heteropolymeric blocks (MG-blocks) (**Figure 9**) (Pawar and Edgar 2012).

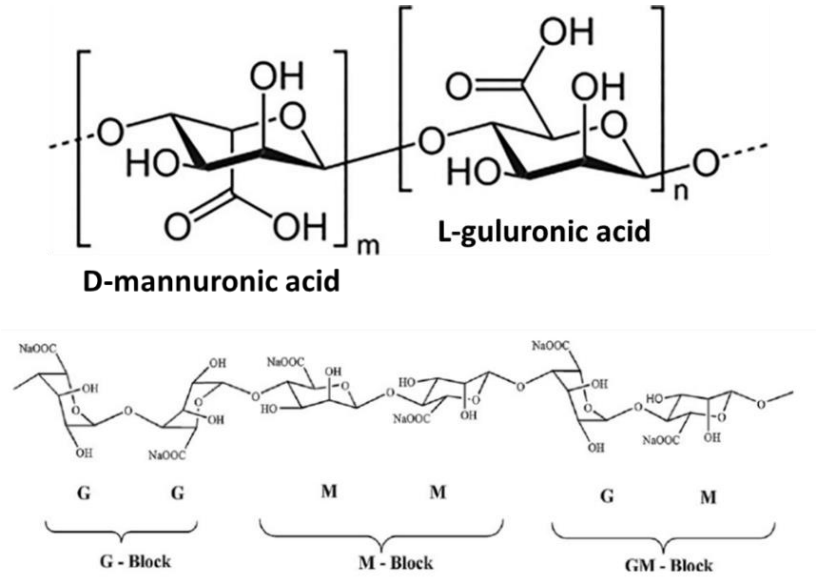


***Figure 9**: Structural monomers of alginate polysaccharide chain.*

D-mannuronic (M) and L-guluronic (G) acids can be arranged along the chain in homopolymeric blocks (G or M-Blocks) or heteropolymeric blocks (GM-block).

The most attractive functionality of the alginic acid is its capacity of forming a hydrogel, which is induced simply by cross-linking with divalent cations such Ca^{2+}, Sr^{2+} and Ba^{2+}, except Mg^{2+} (Coradin, Nassif, and Livage 2003; Zhao et al. 2010). At neutral pH, the carboxylic acid functional groups are deprotonated so the polymer has a global negative charge, usually compensated by sodium (Na^{+}) ions, but with the addition of divalent cations, the cross-linking is induced, leading to gel formation (Coradin et al. 2003). The described process is usually reversible through the addition of chelating agents such as sodium citrate that are able to withdraw most of the divalent ions, originating the initial sodium alginate polymer (Lim and Sun 1980). Usually, alginate gel beads are prepared by ionic gelation, which can occur

externally, by dropping a sodium alginate solution into an aqueous solution of Ca^{2+} made from calcium chloride ($CaCl_2$), or internally by reversing the process, i.e. dropping the Ca^{2+} solution into a sodium alginate solution (Lupo et al. 2015). The obtained cross-linked networks – calcium alginate hydrogel - has been proven to exhibit a gel phase transition at pH values around 2.5 - 3 (similar to ruminants' abomasum fluid pH), which means that they collapse (shrinks) at pH values below those mentioned, but rapidly swollen in higher pH values. This feature makes them an appealing WM for phages encapsulation, applied to GIT delivery systems (Moghtader, Eğri, and Piskin 2017).

Alginate capsules possess selective retention and release properties that make them such a good carrier for biotechnology applications (Morales et al. 2017). Bearing in mind that the gel beads functionality depends heavily on their permeability and physico-chemical stability, it is important to note that their relatively high porosity is not suitable for release in some industrial applications, e.g. release and drug delivery applications for low molecular weight drugs or phages (Mancini, Moresi, and Rancini 1999; Messaoud et al. 2016). For this reason, several strategies have been developed in order to overcome this problem and improve encapsulation efficiency. Several studies have been made exploring the combination of alginate with nanoparticles (Degen et al. 2015) or further natural polymers such as chitosan and shellac, etc., to control the physico-chemical properties of the final matrix (Messaoud et al. 2016; Peniche et al. 2004; Tan, Chan, and Heng 2009).

1.6.2 Shellac

Shellac is the refined form of Lac, which is a resinous secretion of lac insects of the genus *Kerria*, mostly cultivated in host trees in South-East Asia. In India, the major producing country, it is mostly the *Kerria lacca* with the two insect strains Kushmi and Bysakhi and in Thailand *Kerria chinensis* (Cole, Hogan, and Aulton 1995; Morales et al. 2017). Actually, shellac is a general term used to denominate many different products depending on insect strain and refining method (Buch et al. 2009). This renewable, non-toxic, biodegradable polymer consists of a complex mixture of aliphatic and alicyclic acids, with the main components being aleuritic acid as well as jalaric and shellolic acids (**Figure 10**) (Al-Gousous, Penning, and Langguth 2015; Patel, Remijn, et al. 2013; Specht et al. 1999). It is not soluble in water, but it is possible to obtain aqueous solutions, dissolving shellac in an adequate alkali, remembering that the nature of the alkali strongly influences the final solution properties (Al-Gousous et al. 2015; Penning 1996; Specht et al. 1999). Its constitution in esters and carboxyl groups is what makes

shellac insoluble at neutral and acidic aqueous solutions but soluble in aqueous alkalis and alcohols (Kumpugdee-Vollrath, Tabatabaeifar, and Helmis 2014).

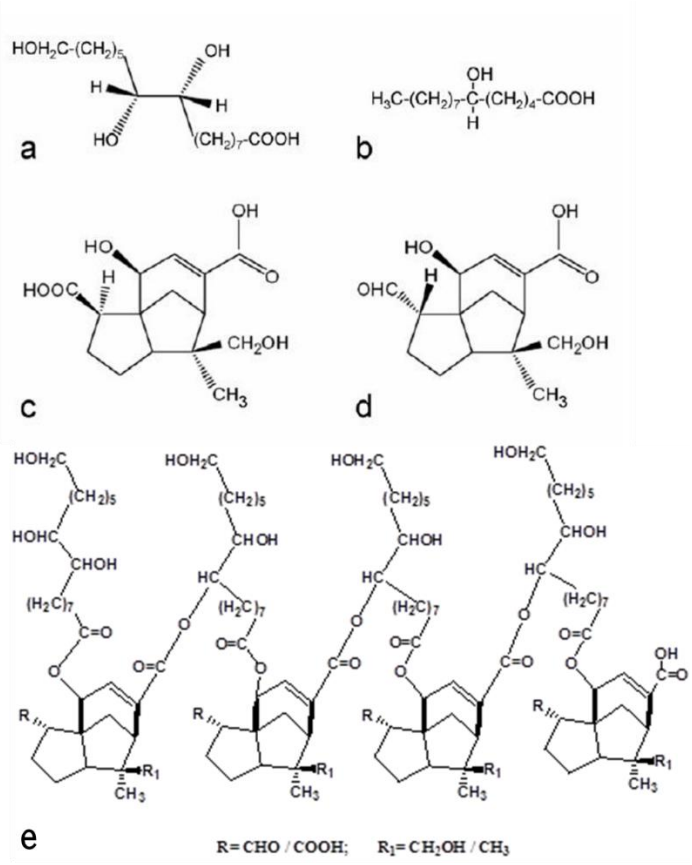


***Figure 10**: Chemical composition of Shellac.*

*Main components: **a**) aleuritic acid, **b**) butolic acid, **c**) shellolic acid, **d**) jalaric acid. And **e**) polymeric chain arrangement.*

On account of its desirable characteristics, this natural polymer is considered a food additive by the JECFA (Joint FAO/WHO Expert Committee on Food Additives), an international expert scientific committee that is administered jointly by the FAO and the WHO. It has been widely employed for years in several industry sectors, as adhesives, coating materials (Al-Gousous et al. 2015), insulating materials, sealants, oleogelator (Patel, Schatteman, et al. 2013), and thermoplastic material (Lausecker et al. 2016). Recently, it has gained a lot of interest for the development of a variety of encapsulation systems, acting as WM for capsules produced by several techniques, alone or combined with other polymers (Hamad, Stoyanov, and Paunov 2012; Henning et al. 2012; Leick et al. 2011; Xue and Zhang 2008, 2009). Shellac is one of the polymers that can be combined with alginate on composite beads (Morales et al. 2017) or coated capsules (Messaoud et al. 2016). Additionally, it has demonstrated a protective

effect in gastric fluid, and release in the intestinal environment (Phan The et al. 2008; Qussi and Suess 2005), which is an advantage when used in MPs for protection through the GIT (Schell and Beermann 2014; Stummer et al. 2010).

1.6.3 Chitosan

Similar to alginate, chitosan is a natural hydrophilic polymer capable of forming hydrogels through crosslinking. Chitosan is obtained from the partial deacetylation of chitin, which constitutes the insect's skeleton and shell of crustacea such as crab, shrimp, and crawfish. It is a cationic linear polysaccharide with a variable content of β(1-4) linked D-glucosamine and N-acetyl-D-glucosamine monomers (**Figure 11**) (Hoemann et al. 2007). This polymer is very advantageous for applications as a biomaterial because it is amenable to enzymatic and chemical modifications, acts as an adhesive because it is positively charged at physiological pH, is biocompatible, biodegradable, and can be readily processed into different shapes (Chandy and Sharma' 1990; Costa-Pinto, Reis, and Neves 2011). In fact, chitin, and consequently chitosan, is the second most abundant natural biopolymer after cellulose and is mainly derived from shellfish processing waste (Ren et al. 2017; C. Wu et al. 2019); it was also proven to have certain antimicrobial properties attributed to amino groups in the molecules (Haghighi et al. 2020; Shahidi, Arachchi, and Jeon 1999).

Several studies explored the use of chitosan to produce encapsulation systems for drug delivery applications, alone (Lee et al. 2011; da Rosa et al. 2013; Tozaki et al. 2002) and combined with several other biopolymers like alginate and shellac (Chávarri et al. 2010; Yuan et al. 2021). Since chitosan and alginate have opposite charges, these two biopolymers tend to form polyelectrolyte complex membranes throughout electrostatic interactions (**Figure 11b**); hence it has been widely applied at the microencapsulation field for the intestinal delivery of a variety of drugs (Li et al. 2007). This biopolymer is soluble in aqueous acidic solutions (e.g. formic acid and acetic acid) (pH <6.5) but is insoluble in water and organic solvents (Shahidi et al. 1999; Wang et al. 2006).

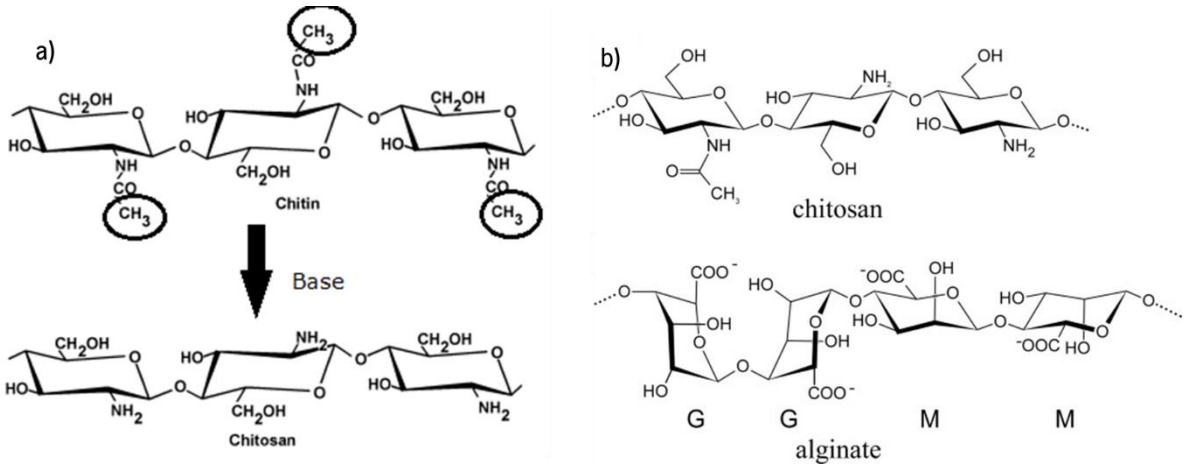


***Figure 11**: Process of chitin deacetylation originating chitosan molecules **a**) and chemical interaction of alginate and chitosan molecules **b**).*

Chapter 2: Project aims

Chapter 2: Project aims

The present work proposes the development of an innovative system of microencapsulation for phages to prevent food pathogens that colonize the intestine of ruminant animals. It is intended to test different biopolymers whose physico-chemical characteristics are modulated by pH, such as alginates, shellac, chitosan, and some synthetic polymers, approved for administration to animals, that can protect phages from digesting conditions of the ruminants. These biopolymers must be biodegradable, non-toxic, economical, and must not interfere with phages' viability. The proposed encapsulation technique is called prilling by vibration and it will be carried out using BUCHI Encapsulator equipment, model B-395 Pro (BÜCHI Labortechnik AG, Flawil, Switzerland).

Two different approaches are proposed:

1. A system consisting of two protective layers (coated matrix type), each adapted to one of the compartments of the GIT. The outer layer should provide resistance to the rumen, so it should be resistant to the physiological characteristics there and be soluble only in the abomasum. The internal layer, in which the phages will be entrapped, should present resistance to the low gastric pH of the abomasum and must be soluble in the intestine in order to release the phages so that they can infect and kill the pathogenic bacteria colonizing this environment.
2. A system consisting of a single protective layer (matrix type) optimized to protect the phages at rumen and abomasum and release them at the intestine.

In summary, throughout this work, we intend to develop and optimize a technique that allows targeted phage delivery, increasing the effectiveness of oral phage administration, targeting foodborne pathogens with high prevalence in the ruminant intestine. In the future, this method may be applied in the area of food biotechnology with the ultimate goal of reducing the alarming number of food-borne diseases that occur annually worldwide.

The work developed was integrated into the PhageSTEC project, a collaboration between the Centre of Biological Engineering (CEB) of the University of Minho and the Food Processing Group (FPG) of the International Iberian Nanotechnology Laboratory (INL).

Chapter 3: Materials and Methods

Chapter 3: Materials and Methods

3.1 Phage production by double agar technique and phage titration

The procedures that involved bacteria (*E. coli* BL21 and NCTC 12900) and phage (*E. coli* phage T4 and CB120) were conducted under aseptic conditions, inside a laminar flow chamber. All materials were sterilized in the autoclave at 121 °C for 15 minutes. Tryptic Soy Broth (TSB, Oxoid™) was used to grow bacteria. Tryptic Soy Agar (TSA) with 1.5 % (w/v) agar or Molten Top-Agar (MTA_TSB) with 0.6 % (w/v) agar were used to grow bacteria in plates and to produce the phages.

Both phages (T4 and CBA120) were produced by the double agar technique (**Figure 12**). First, TSA plates with bacterial lawns (*E. coli* BL21 for T4 and NCTC 12900 for CBA120) were prepared by adding 100 µL of overnight suspension into TSA plates. The plates were then spotted with 100 µL of phage stock suspensions and mixed with MTA_TSB. After incubation overnight at 37 °C, 3 mL of SM buffer (1 M Tris-HCl buffer at pH 7.5, 0.58 % (w/v) NaCl, and 0.2 % $MgSO_4.7H_2O$) were added to the plates for 8 hours to recover the phage into the liquid. The SM buffer was then removed together with the top agar layer into falcon tubes.

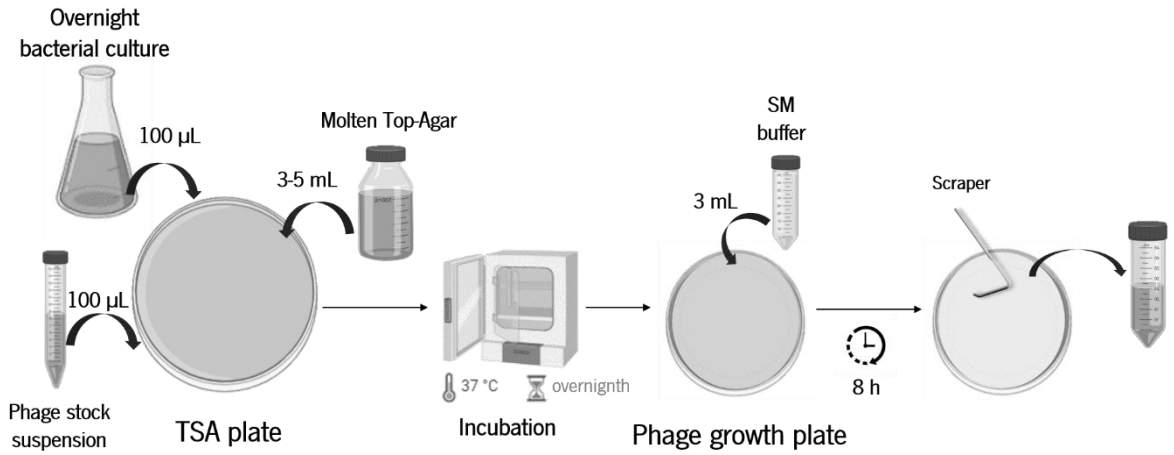


***Figure 12**: Phage production.*

It was accomplished by inoculating phage from a phage stock suspension to TSA plates with bacterial lawns and incubating overnight at 37 °C. SM buffer was poured onto phage growth plates, after 8 hours the SM buffer and the top-agar layer were collected, centrifuged, and filtered to obtain a phage suspension.

These tubes were centrifuged for 10 minutes at 4 °C and 9000 g (refrigerated Sigma 3-16K centrifuge) and the supernatants (phage suspension) were recovered. Before storage at 4 °C, the phage suspension was filtered using a 0.22 µm syringe filter. Phage titer was determined by the small drop plaque assay. Performing a serial dilution of the phage suspensions on SM buffer and each dilution (10 µL) was added into bacterial lawns plates for counting after incubation overnight at 37 °C. After incubation, the Plaque Forming Units per milliliters (PFU/mL) were determined using **Equation 1**. For the dilution where the number of phage plates was between 10 and 100, the PFU/mL were determined.

***Equation 1**: Phage titer equation.*

Relating the number of phage plaques, the dilution factor and the volume of the phage sample, it allows quantifying the plaque-forming units per milliliter (PFU/mL) present in a phage suspension.

$$\textbf{Phage titer}\left(\frac{\textbf{PFU}}{\textbf{mL}}\right) = \frac{\textbf{Nº of phage plaques} \times \textbf{Dilution factor}}{\textbf{Volume of phage sample (mL)}}$$

3.2 Stability of free T4 and CBA120 phages at different pH and temperature

Stability tests were performed by incubating the free phages, for 24 hours, at different pH values (1, 3, 6, 7, 9, 11, and 13) using a universal buffer (0.15 M potassium chloride, 0.01 M potassium dihydrogen phosphate, 0.01 M sodium citrate, 0.01 M boric acid) with pH adjusted, at 37 °C. In all experiments, the phage was diluted and plated on host lawns for enumeration.

3.3 Encapsulation methods

Before the encapsulation of the phage particles, different encapsulation parameters and wall materials were tested. The technique applied for the MPs formation was the prilling by vibration, using the BUCHI Encapsulator B – 395 Pro equipment (**Figure 13**), followed by ionic gelation. The encapsulation agents used were alginate and shellac gum.

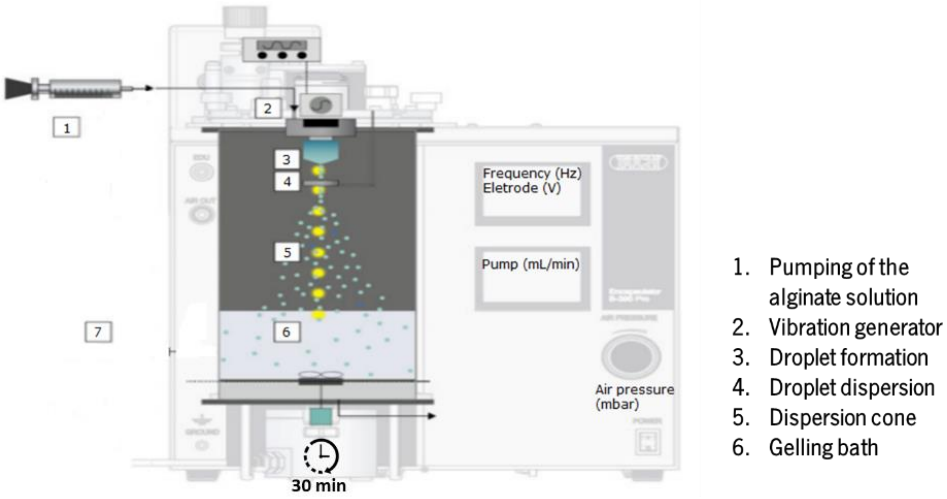


***Figure 13**: Schematic representation of the equipment used for encapsulation.*

3.3.1 Matrix type system

3.3.1.1 Production of alginate microparticles with Single Nozzle

The system parameters: polymer concentration, nozzle size, vibration frequency, amplitude, electrode voltage, and pump flow rate, were combined according to the equipment manual specifications (**Annex 2** - Optimization parameters) in seven different conditions that are synthesized in **Table 4**. The alginate MPs were produced using two solutions of sodium alginate, at 1 % (w/v) and 2 % (w/v) final concentrations, dissolving alginic acid sodium salt (Sigma-Aldrich alginic acid sodium salt powder) in distilled water under overnight agitation at 500 rpm using a magnetic stirrer. For the ionic gelation, an aqueous solution of Ca^{2+} was prepared by dissolving $CaCl_2$ (Supelco Merck Calcium chloride anhydrous, granular ~2 – 6 mm) in distilled water at a final concentration of 0.1 M. After the alginate solutions were completely homogenous and without air bubbles, the SN pulsation chamber was employed to produce MPs of alginate by cationic internal gelation. Alginate solutions were loaded into 10 mL syringes, extruded through the 120, 150, and 200 µm nozzles and collected into 100 mL of the gelling solution of $CaCl_2$ in continuous agitation of 200 rpm. Amplitude was set on 3, vibration frequency varied between 1000 Hz and 2500 Hz, three different voltages were used (400, 900, and 1000 V), and pump flow rates of 2 and 4 mL/min were employed. After extrusion, the MPs were allowed to stay in the gelling bath for 30 minutes for complete polymerization of alginate polysaccharide chains. In the end, the MPs were recovered using a 100 µm cell strainer and stored in distilled water at 4 °C.

3.3.1.2 Production of alginate and shellac microparticles with Single Nozzle

Similar to the production of the alginate MPs, alginate and shellac MPs were produced using the encapsulator SN pulsation chamber and the system parameters were combined in five different conditions as shown in **Table 5**. For this procedure, two sodium alginate stock solutions (2 % and 4 %) were prepared as previously, after complete homogenization they were mixed with the stock aqueous solution of ammonium shellac salt without preservatives (SSB AQUAGOLD ammonium shellac salt, 25 % (w/v) solids contents, pH 7.3). Four different polymer mixtures were obtained: 1 % alginate + 12.5 % shellac; 1.5 % alginate + 0.5 % shellac; 3 % alginate + 1.5 % shellac and 2 % alginate + 3 % shellac. Three gelling solutions were prepared, the first one dissolving $CaCl_2$ at a final concentration of 0.1 M, in distilled water and ethanol in a ratio of 1:1 (v/v) as described by Morales et al. 2017. The others were prepared by dissolving $CaCl_2$ at a final concentration of 1.5 % (w/v), in distilled water with 0.1 % (v/v) Tween 80, as indicated in the Encapsulator B395 Laboratory Guide, but one of them had the pH adjusted to 7 using a 4 M HCl solution. Different nozzle sizes (120, 200, and 300 µm) were used, combined with different vibration frequencies (1200, 1000, and 700 Hz), amplitudes (3 and 4), electrode voltages (900, 1700, and 2000 V) and pump flow rates (2.3, 5.7 and 6 mL/min). The mixtures of the two polymers were loaded into a syringe and the extrusion followed by an ionic gelation process was performed as described above.

3.3.1.3 Determination of the gelling bath optimum pH

The pH value of all the working solutions (2 % alginate, 3 % alginate + 1.5 % shellac mixture, 3.2 % $CaCl_2$, and 10 % shellac) was measured using a pH meter. Then three 3.2 % $CaCl_2$ solutions were prepared as described by Silva et al. (Silva et al. 2016), and the pH values were adjusted to 7, 5, and 3 using 4 M HCl. The SN pulsation chamber was used to produce matrix type MPs, using a 3 % alginate + 1.5 % shellac mixture, 120 µm nozzle, 2.3 mL/min flow rate, 1000 Hz and 2000 V. At the first experiment, the MPs were produced and collected in the gelling bath with pH 7, 5 or 3. The second experiment differed on the electrode voltage value, now 1500, and consisted in collecting the MPs on the gelling solution at pH 7, and after 30 minutes transferring to gelling solution pH 3 for 15 minutes. The MPs morphology and size were analyzed before and after incubation at pH 3.

3.3.1.4 Production of alginate and shellac microparticles using Flow Vibration Nozzle

The production of alginate and shellac MPs was performed using the BUCHI Encapsulator B – 395 Pro with the FVN configuration. The FVN system consists of passing an airflow through the external nozzle, which enables the formation of smaller MPs compared to the SN, using the same nozzle diameters. For this method, the common parameters for all the tested conditions were: a formulation of 3 % alginate + 1.5 % shellac; gelling solution composed of 1.5 % (w/v) $CaCl_2$, 0.1 % Tween 80 and pH 7; internal and external nozzles of 200 µm and 400 µm, respectively; 1400 Hz; 1000 V and amplitude 8. The effect of the polymer flow rate (FR) and air pressure (AP) on the MPs size was analyzed following a Full Factorial design with two central points (Box, Hunter, and Hunter 2005). The range and codification of the variables are shown in **Table 3**.

***Table 3**: Experimental domain and codification of independent variables in the factorial design.*

Coded Values (both for FR and AP)	**Natural Values**	
	FR (mL/min)	**AP (bar)**
+1	2	0.4
0	1.8	0.35
–1	1.6	0.3

Codification: $V_c = (V_n - V_0) / \Delta V_n$; Decodification: $V_n = V_0 + (\Delta V_n \times V_c)$

V_c = coded value; V_n = natural value; V_0 = natural value in the centre of the domain;

ΔV_n = increment of V_n per unit of V_c.

The following empirical model, with the MPs size (in µm) as the dependent variable, was fitted to the experimental data using the software Protimiza Experimental Design (https://experimental-design.protimiza.com.br):

$$\text{MPs Size} = b_0 + b_1\,FR + b_2\,AP + b_{12}\,FR\,AP$$

where FR is the Polymer Flow Rate (mL/min) and AP is the Air Pressure (bar). The signification of the coefficients (b_0, b_1, and b_{12}) was evaluated by the Student's t-test ($\alpha = 0.05$). After the fitting, non-significant parameters were removed, and best-fit values recalculated. The consistency of the model was tested by the Fisher's F-test ($\alpha = 0.05$) using the following mean squares (MS) ratios (automatically calculated by Protimiza):

	The model is acceptable if
F_1 = Model MS / Residuals MS	**$F_1 \geq F_{tab}$**
F_2 = Lack of fitting MS / Experimental error MS	**$F_2 \leq F_{tab}$**

The mean diameters were analyzed by Image J.

3.3.2 Coated matrix type system

3.3.2.1 Production of alginate and shellac core-shell microcapsules with Concentric Nozzle

Alginate stock solutions (4 %, 2 %, and 1 %) were prepared as described above and shellac solutions (10 % and 4 %) were obtained diluting the stock shellac solution in distilled water, then combinations of these solutions were prepared at different final polymer concentrations. Two gelling solutions were used, one consisting of 0.1 M $CaCl_2$ dissolved in an ethanol/distilled water mixture (1:1 v/v), with 0.1 % of Tween 80. The other was composed of 3.2 % $CaCl_2$ dissolved in distilled water as described by Silva et al. 2016. The CN pulsation chamber was used to produce core-shell MPs with different internal (200, 150, and 120 µm) and external (500, 400, 300, 200 µm) nozzle sizes and wall material concentrations (**Table 7**). In this procedure, a syringe pump controlled the flow rate of the core polymer (2 % or 1 % alginate) solution, while the shell polymer mixtures (2 % alginate + 2 % shellac; 3 % alginate + 1.5 % shellac, and 2 % alginate + 1 % shellac) was extruded by air pressure adjusted to the desired flow rate. Amplitude was set on 3 for all the conditions, vibration frequencies of 100, 200, 600 and 800 Hz were used, combined with electrode voltage values varying between 1200 and 2200 V, and

core flow rates of 1.5, 2.3, and 4 mL/min and variable shell flow rates. The MPs were collected into the gelling bath and kept under magnetic stirring for 30 minutes.

3.4 Microparticles stability during *in vitro* gastrointestinal simulation

In order to verify if the MPs resist the ruminants' GIT temperature and pH, an *in vitro* simulation was performed at 38.5 °C and 100 rpm. To simulate the rumen compartment, 0.1 M MES buffers were prepared at three different pH, 5.8, 6.5, and 7. The abomasum and intestinal fluids were mimicked by 0.1 M citrate buffer pH 3 (0.00856 mol of trisodium citrate dihydrate, 0.0414 mol of citric acid), and 0.1 M TRIS buffer at pH 7.5, respectively. First, individually 0.5 g of MPs were incubated in 15 mL of MES buffers for 6 hours, in 0.1 M citrate buffer pH 3 for 4 hours, and in TRIS buffer at pH 7.5. Then, sequentially 0.5 g of MPs were incubated in 15 mL of MES buffer at pH 6.5 for 6 hours, then transferred to 15 mL of 0.1 M citrate buffer pH 3 for 4 hours, and finally transferred to 15 mL of TRIS buffer at pH 7.5. Samples were collected every hour at the first experiment and at the end of the incubation on each pH at the second one.

3.5 Fluorescent nano-spheres encapsulation

For the encapsulation of the fluorescent nano-spheres (Fluoresbrite® YG Microspheres 0.20 µm, Polysciences, Inc., Germany), the 2 % alginate + 3 % shellac mixture was prepared as previously. The nano-spheres, of 200 nm of diameter used to simulate phages were added at a final concentration of 5.68×10^{8} particles/mL and the production of MPs was performed as described above. All the procedures were carried out in an environment without light, so it would not affect the spheres' fluorescence. The MPs produced were submitted to the sequential incubations to simulate GIT conditions.

3.6 Microparticles characterization

3.6.1 Determination of the microparticles size and density

The morphology of the MPs was analyzed by Wide-Field Upright Microscope (optical microscope) and the mean size was determined using the microscope software tool for this purpose. For the determination of the MPs' density (mass *per* volume), a previously determined mass of MPs was put into a 10 mL volumetric flask, then a controlled volume of water was added until the final volume was completed. The density of the MPs was determined using **Equation 2**.

3.6.2 Scanning electron microscopy (SEM) and Confocal microscopy (CM)

For the SEM analysis, a small portion of the MPs was previously dried for approximately 24 hours in a desiccator on top of pieces of silica wafers, then were observed on SEM at 3 kV using an Everhart-Thornley detector (ETD). In the CM analyses the MPs containing the fluorescent nano-spheres were observed after storage in distilled water for 3 days and after approximately 1 and 5 minutes in 0.1 M TRIS buffer pH 7.5.

3.7 Phage encapsulation and *in vitro* gastrointestinal simulation

Inside a laminar flow chamber, the phage suspensions of T4 (10^{10} PFU/mL) and CBA120 (1×10^{11} PFU/mL), obtained as described in section 3.1, were mixed by gentle magnetic stirring with the formulation of WM (2 % alginate + 3 % shellac), at a final ratio of 1:10. After this, MPs were produced with the optimized conditions for the SN. After 30 minutes of gelation, a sample of the gelling bath was collected and some of the produced MPs were dissolved in 0.1 M phosphate buffer pH 7; both were stored for posterior analysis. Also, 1.5 g of MPs with encapsulated phages were incubated sequentially in 15 mL of each buffer. After rumen (0.1 M MES buffer pH 6.5) and abomasum (0.1 M citrate buffer pH 3) simulations, the MPs were filtered and each of the corresponding liquid phases was stored in the fridge for posterior analysis. Finally, the MPs were transferred to 0.1 M TRIS buffer pH 7.5 for intestine simulation and a sample was also stored for analysis. In the end, each sample was diluted in SM buffer and the PFU/mL were count as previously described.

Additionally, free CBA120 was incubated in 0.1 M MES buffer pH 6.5 at 38.5 °C during 5 hours, after 5 hours it was transferred into 0.1 M citrate buffer pH 3 and incubated during 3 hours. Finally, it was incubated in 0.1 M TRIS buffer pH 7.5. Another experiment was performed, mixing CBA120 in 0.1

M citrate buffer pH 3 and in 0.1 M TRIS buffer pH 7.5 individually. In all experiments, the phage was diluted and plated on host lawns for enumeration.

3.8 Statistical analysis

The results presented are the averages and standard deviation calculated from replicate measurements. Results were evaluated by one-way analysis of variance (ANOVA) with a significance level set at 0.05, using the software GraphPad Prism v8.2.1 (GraphPad Software, Inc., CA, USA).

Chapter 4: Results and Discussion

Chapter 4: Results and Discussion

4.1 Phage production

Phage production is an essential step to obtain a sufficient amount of viral particles for subsequent characterization, titration, or possible biotechnology applications. It is accomplished using a bacterial host compatible with the selected phage. In this study, *E. coli* BL21 and *E. coli* NCTC 12900, two non-pathogenic strains, were used as hosts for phages T4 and CBA120, respectively. These host-phage pairs are well studied and widely used in laboratory procedures, so there are several standardized and successful protocols for phage production in the literature (Plattner et al. 2019; Taj et al. 2014; Woodward et al. 2003).

A suspension of T4 at a final concentration of 8×10^{10} PFU/mL and a suspension of CBA120 at a concentration of 10^{11} PFU/mL were successfully obtained.

4.2 Stability of free T4 and CBA120 phages at different pH

The results of the stability tests of phage T4 and CBA120 are presented in **Figure 14**. Both phages maintained viability at neutral pH values. However, they were completely inactivated at extreme acidic (pH 1 and 3) or alkaline (pH 13) conditions. Since the abomasum of ruminant animals has a pH between 1 and 3 (Chaucheyras-Durand et al. 2010), these results demonstrate the importance of a protection mechanism for phages to ensure that they reach the intestine.

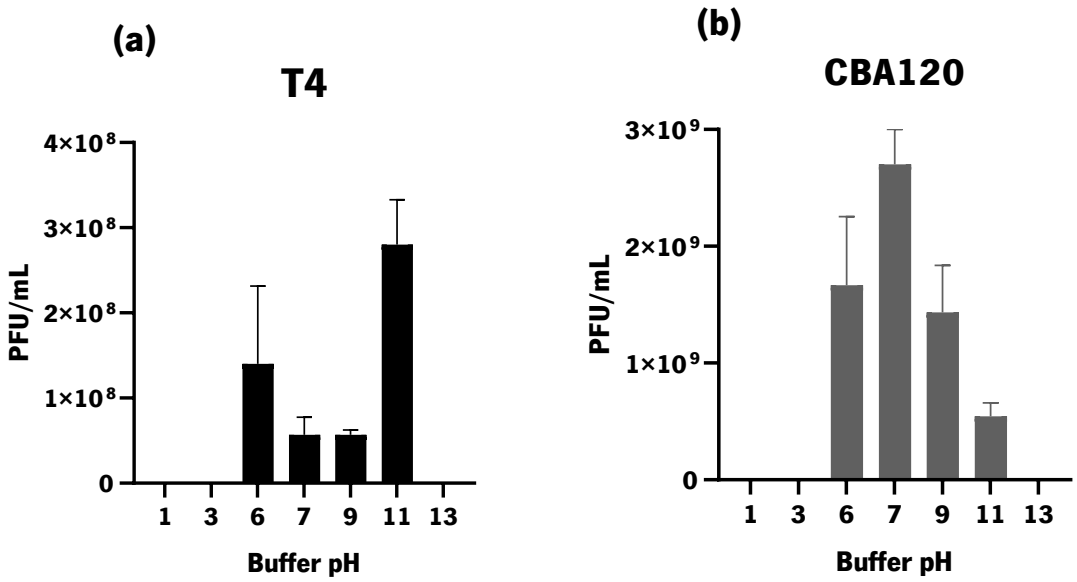


Figure 14: *Phage T4 and CBA120 stability in different pH.*

*Effect of different pH on T4 (**a**) and CBA120 (**b**) after 24 h, measured as phage relative survival, i.e., amount of phages enumerated. Averages and standard deviations of three repeated experiments are given.*

4.3 Optimization of the encapsulation method

4.3.1 Matrix type system

4.3.1.1 Alginate microparticles produced with Single Nozzle

At the beginning of the optimization process, the SN was employed using the parameters described at **Table 4**. Using this nozzle, the size of the beads produced is a function of the nozzle size, the vibration frequency and liquid flow rate used, as well as the liquid viscosity. A general rule of thumb is that the final bead size is approximately twice the size of the nozzle. As it is possible to observe in **Figure 15**, the MPs produced by 1 % alginate alone presented a very irregular morphology and more heterogeneous size variation, compared to the o 2 % alginate, showing that an increase in the alginate concentration allowed the formation of a more stable and compact hydrogel. In **Figure 16** there are presented the mean diameters and density (± Standard Deviations) of the MPs obtained with each condition (**a**-**f** in **Table 4**). For the majority of the conditions with 1 % alginate, mean diameters are very

similar (**a** 327 ± 79 µm; **c** 329 ± 55 µm and **d** 325 ± 68 µm), except for the condition **b** (199 ± 25 µm). This smaller mean diameter, compared to the other MPs produced with 120 µm nozzle (**a**), can be justified by the increase of the vibration frequency (to 2500 Hz), which is known to influence the bead size this way (Del Gaudio et al. 2005, 2014). When the concentration of alginate was increased (2 %), an improvement in the MPs shape was noted (**Figure 15 e.** and **f**). Mean diameters of 459 ± 41 µm (**e**) and 576 ± 89 µm (**f**) were achieved. Usually, diameters between 30 µm and 2000 µm are used for rumen by-pass, although the ideal range is described to be 200-400 µm, so the 1 % MPs are inside this range, but the 2 % MPs are not.

The density was measured using **Equation 2**, for conditions **c.** and **f.** due to the better results observed compared to similar conditions. For the 1 % MPs the density was 1.08 ± 0.06 g/cm^3 and for 2 % MPs it was 1.06 ± 0.01 g/cm^3. Although there was a significant difference in the mean diameter (**Figure 16**), there was no significant difference in the density between the two conditions. These values are according to the recommended range (1.0 – 1.7 g/cm^3) (Belverdy, Alamouti, and Azizi 2019) but very close to the minimum value. To overcome this problem and increase the MPs' density, it is possible to combine alginate with the addition of a high-density weighting agent (e.g. kaolin, chromium sesquioxide, or barium sulfate).

***Equation 2**: Microparticles density equation.*

According to the method described above, this equation quantifies the density (mass per volume) of the microparticles, relating the mass in grams (g) and the water volume in cubic centimeters (cm^3).

$$\textbf{Microparticles Density}\left(\frac{g}{cm3}\right) = \frac{\textbf{Mass of microparticles } (g)}{\textbf{10 } - \textbf{Water volume } (cm3)}$$

***Table 4:** Optimization parameters, using the single nozzle to produce matrix type microparticles of 1 % and 2 % alginate.*

MATRIX TYPE SYSTEMS (ALGINATE)							
Condition	**Gelling solution**	**Polymer concentrations (w/v)**	**Amplitude**	**SN size (µm)**	**Vibration frequency (Hz)**	**Electrode voltage (V)**	**Pump flow rate (mL/min)**
a	0.1 M $CaCl_2$	1 % Alginate	3	120	1000	400	2
b					2500		
c				150		1000	4
d					1800		

e	2 % Alginate	200	1200	
f				900

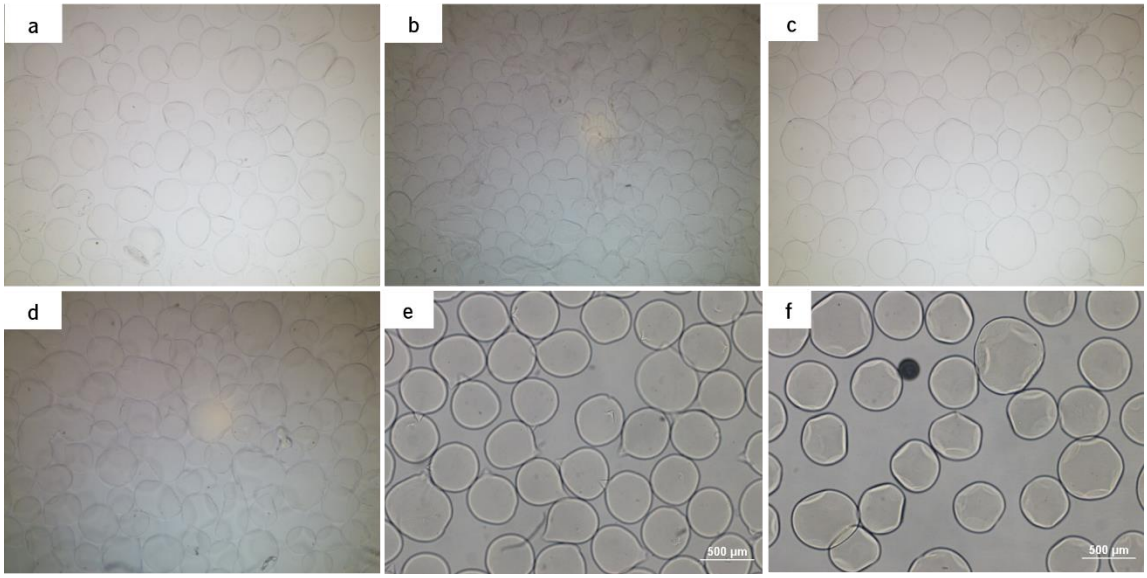


Figure 15: *Results of the different conditions listed in* ***Table 4*** *for the production of microparticles of alginate.*

Two different alginate concentrations (1 % and 2 % (w/v)) were used.

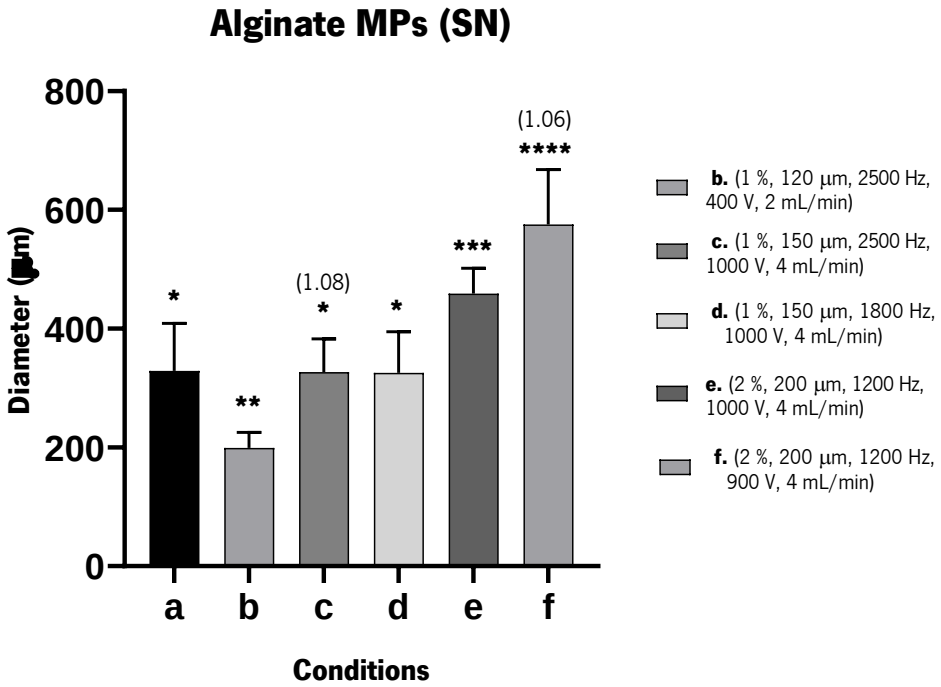


Figure 16*: Mean diameters and standard deviations of the different conditions (a.-f.). Densities of conditions* ***c*** *and* ***f*** *are indicated in parenthesis above the respective bar.*

4.3.1.2 Alginate and shellac microparticles produced with Single Nozzle

Since the alginate hydrogel has a high porosity, which can interfere with the protection of the phages in gastric environments and to obtain higher densities, a solution was to produce a polymer mixture of alginate and shellac and produce particles using the SN as well. Two different blends were prepared, mixing a 2 % alginate solution and the shellac (25 %) stock solution, at 1:1 and 3:1 ratio. The gelling solution consisted of $CaCl_2$ dissolved on a mixture of distilled water and ethanol; ethanol was used to promote shellac precipitation, as previously reported by Xue and Zhang 2008. The results of the conditions described in **Table 5** are presented below (**Figure 17**), and the mean diameters and mean densities measured are presented in **Figure 18**. Here the two variables were the concentrations of the polymers and the nozzle size; the equipment parameters used at conditions **a.**, **b.** and **c.** were the ones optimized at previous section used at condition **f.** with the exception of the flow rate that was increased to 6 mL/min due to the increase of the viscosity of the solutions (according to **Annex 2** - Optimization parameters). A relatively small diameter variation was only observed when a mixture of 1 % alginate and 12.5 % shellac and a 300 µm nozzle were used (condition **a.**). The mean diameter was 785 ± 38 µm, which is significantly higher than the maximum recommended value, and the mean density was 0.86 ± 0.03 g/cm^3, which is under the ideal density considered ideal for rumen by-pass. In the other two conditions, the WM was composed of 1.5 % alginate and 0.5 % shellac and two different nozzles were used (300 µm and 200 µm). Condition **c.**, due to the smaller nozzle used, lead to a smaller mean diameter of 644 ± 98 µm and higher densities, around 1.38 ± 0.02 g/cm^3, compared to condition **b.**, 1019 ± 152 µm and 1.09 ± 0.01 g/cm^3, both with a significant size variation. The parameters at conditions **d.** and **e.** were optimized in order to obtain smaller mean diameters and narrow size variations. The results prove that it was successfully achieved, MPs whit very homogeneous spherical shapes (**Figure 17**) and mean diameters of 474 ± 60 µm (**d.**) of 488 ± 16 µm (**e.**). It was achieved by decreasing the nozzle diameters and increasing the vibration frequency.

Table 5: *Optimization parameters, using the single nozzle to produce matrix type microparticles of alginate and shellac.*

MATRIX TYPE SYSTEMS (ALGINATE+SHELLAC)							
Condition	**Gelling solution**	**Polymer concentrations (w/w)**	**SN size (µm)**	**Vibration frequency (Hz)**	**Amplitude**	**Electrode voltage (V)**	**Pump flow rate (mL/min)**
a.	0.1 M $CaCl_2$ + 50 % (v/v) Ethanol	1 % Alginate + 12.5 % Shellac	300	1200	3	900	6

b.		1.5 % Alginate + 0.5 % Shellac	300				
c.		1.5 % Alginate + 0.5 % Shellac	200				
d.	$CaCl_2$ 1.5 % (w/v) + 0.1 % (v/v) Tween 80	3 % Alginate+1.5 % Shellac	120	1000	3	2500	2.3
e.	$CaCl_2$ 1.5 % (w/v) + 0.1 % (v/v) Tween 80, pH 7	2 % Alginate+3 % Shellac	200	700	4	1700	5.7

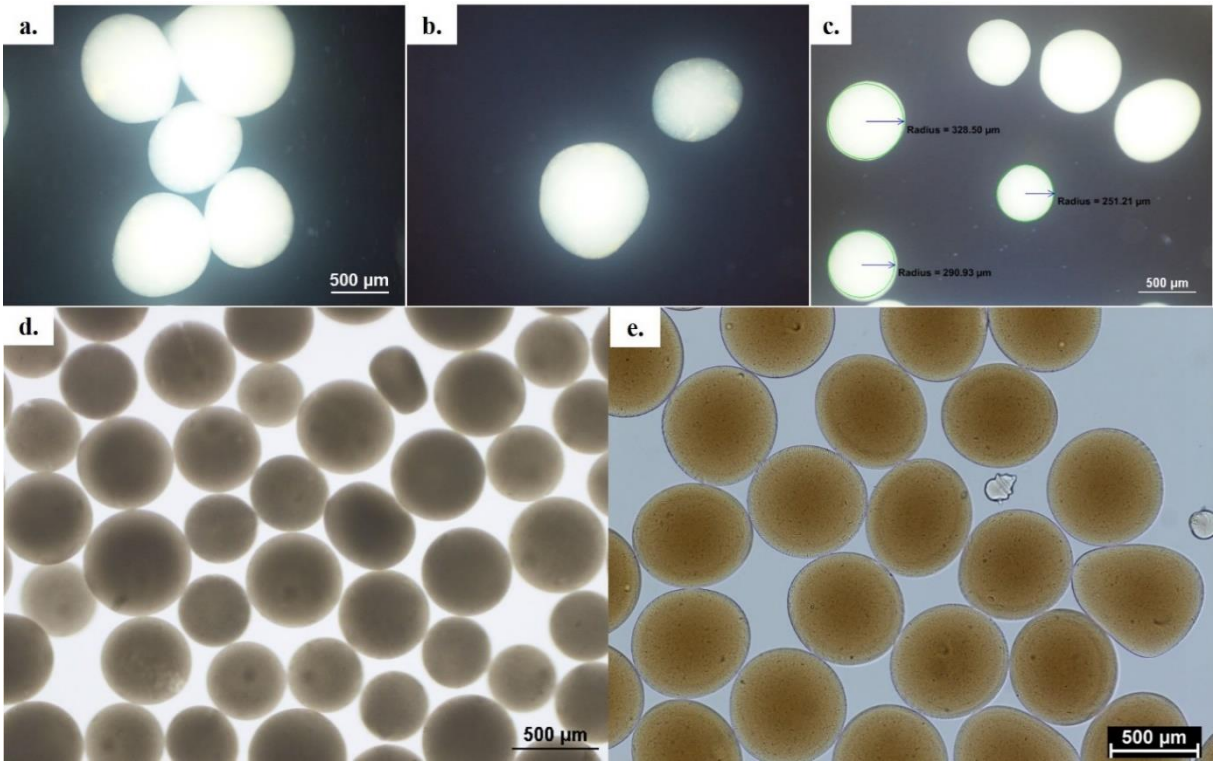


Figure 17: *Results of the different conditions listed in* ***Table 5*** *for the production of microparticles of alginate and shellac.*

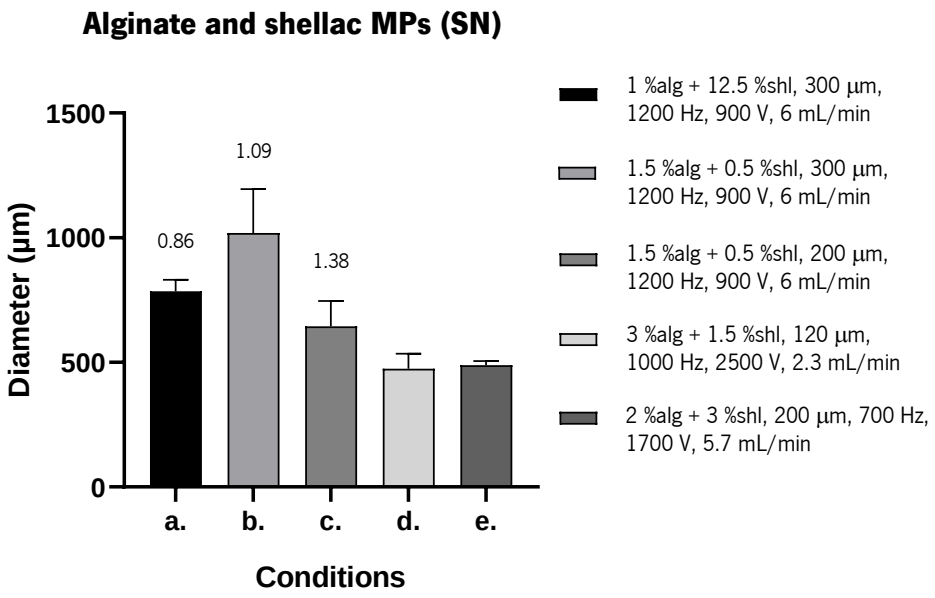


***Figure 18**: Mean diameter and density of the microparticles of alginate and shellac produced according to the parameters presented in **Table 5**.*

4.3.1.3 Optimization of the gelling bath pH

The pH of all working solutions was measured using a pH meter in order to verify if it might be influencing the MPs production. The 10 % shellac solution has a pH of 7.25, the 2 % alginate solution was 6.81, the 3 % alginate + 1.5 % shellac mixture was 6.71 and finally, the 3.2 % $CaCl_2$ gelling solution pH was 10.52. We decided to decrease the pH of the gelling solution because it was very alkaline, relatively to the other solutions, also because the two polymers present solubility at pH above 7. So, we tested three different pH for the gelling solution to evaluate which one was the most suitable for the production of the MPs. We tested the pH 3, 5, and 7, the first two according to Messaoud et al. 2016. The MPs were produced as described in section 3.3.1.2 (condition **d.**) and collected into the different gelling solutions and the results were analyzed using Wide-Field Upright Microscope. The mean diameters were calculated for all the conditions and there were no significant differences between the three experiments (**Figure 19**). The pH 7 seemed to be a better option since the neutral pH represents a better environment for phages during gelation time; additionally, the results looked better compared to the other pH values; MPs were more regular in shape and more homogeneous in size (**Figure 20**). Aditionally, a new test was performed in which the MPs were collected in pH 7 and after 30 min were transferred to pH 3 to evaluate whether there was a significant size change from one pH to another, but the morphology and size remained the same, as we can see in **Figure 20** (**d.** and **e.**).

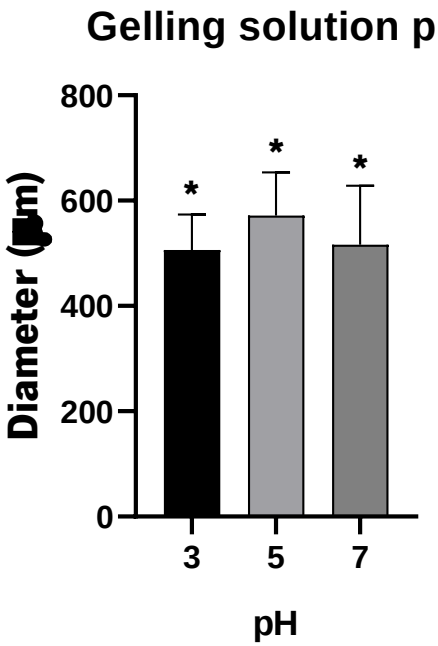


Figure 19: *Mean diameter of MPs collected in different pH (7,5 and 3) gelling solutions.*

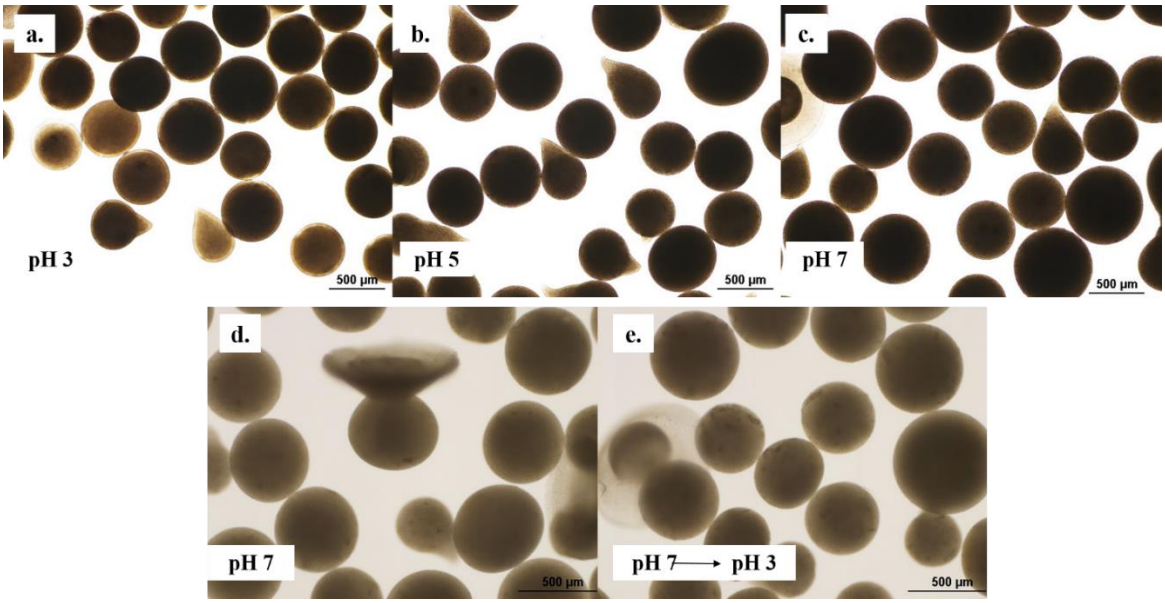


Figure 20: *Microparticles of alginate and shellac collected at gelling solutions of $CaCl_2$ with different pH (3, 5, and7),* ***a. b.*** *and* ***c.****.*

*Also, some microparticles were collected in a pH 7 gelling solution then transferred to a pH 3 gelling solution (****d.*** *and* ***e.****).*

4.3.1.4 Alginate and shellac microcapsules produced using Flow Vibration Nozzle

To achieve a smaller mean diameter, the FVN pulsation chamber was used. It consists of two concentric nozzles, where WM passes through the inner nozzle (under vibration) and a controlled flow of air passes through the outer nozzle that might be at least twice the diameter of the inner nozzle. The air flows around the liquid emerging from the tip of the inner nozzle, immediately removing the polymer liquid from the tip of the nozzle and allowing a thinner liquid jet to be produced. Therefore, like the SN, the size of the beads produced with FVN is a function of the nozzle size, the vibration frequency, the liquid flow rate, and liquid viscosity. However, it is also a function of the airflow rate employed, with higher airflow rates leading to smaller beads. This way, this nozzle enables more viscous solutions to be processed and it is expected to produce up to 40 % smaller beads in comparison to SN when the same nozzle size is being used. Here the rule is that the final bead size is slightly larger than the inner nozzle size.

Different polymer flow rates and air pressures were tested according to a Full Factorial design with two central points (Box et al. 2005), as summarized in **Table 6**. All other parameters were kept constant for all conditions. The general parameters were: hardening solution composed of 1.5 % (w/v) $CaCl_2$ + 0.1 % Tween 80 pH 7; 3 % alginate and 1.5 % shellac polymer solution as WM; inner and outer nozzle of 200 µm and 400 µm respectively; VF= 1400 Hz; Amplitude= 8; EV= 1000 V. The FR (inner nozzle) values were 2.00, 1.60 and 1.80 mL/min and the AP (outer nozzle) values were 0.40, 0.30 and 0.35 bar. These parameters were chosen taking as a reference the recommended values in the FVN technical manual for highly viscous alginate solutions (4 % w/v).

The obtained results are shown in

Figure 21, and **Table 6**. As we can see, conditions **b**. (+1/-1), **d**. (-1/-1), **e**. (0/0), and **f.** (0/0) were the ones that resulted in MPs with more homogeneous size and also with mean diameters above 200 µm, 250 ± 49 µm, 286 ± 56 µm, 259 ± 49, 241 ± 47, respectively. Condition **d.** (-1/-1) was the one with the highest yield, representing that approximately 97.6 % of the MPs produced with this methodology were between the range of 200-400 µm.

Table 6: *Experimental domain, codification of independent variables in the full factorial design, and experimental values obtained for the production of alginate-shellac microparticles using the Flow Vibration Nozzle.*

(MPs diameter and Yield, as the percentage of particles in the range 200–400 µm).

Condition	Coded Values		Natural Values		Responses	
	FR	AP	FR (ml/min)	AP (bar)	Mean diameter ± St. Dev. (µm)	Yield (%)

a.	1	1	2	0.4	127 ± 25	88.6
b.	1	-1	2	0.3	250 ± 49	88.6
c.	-1	1	1.6	0.4	186 ± 35	40.3
d.	-1	-1	1.6	0.3	286 ± 56	97.6
e.	0	0	1.8	0.35	259 ± 49	89.4
f.	0	0	1.8	0.35	241 ± 47	83.5

Codification: $V_c = (V_n - V_0) / \Delta V_n$; *Decodification:* $V_n = V_0 + (\Delta V_n \times V_c)$

V_c = *coded value;* V_n = *natural value;* V_0 = *natural value in the centre of the domain;*

ΔV_n = *increment of* V_n *per unit of* V_c.

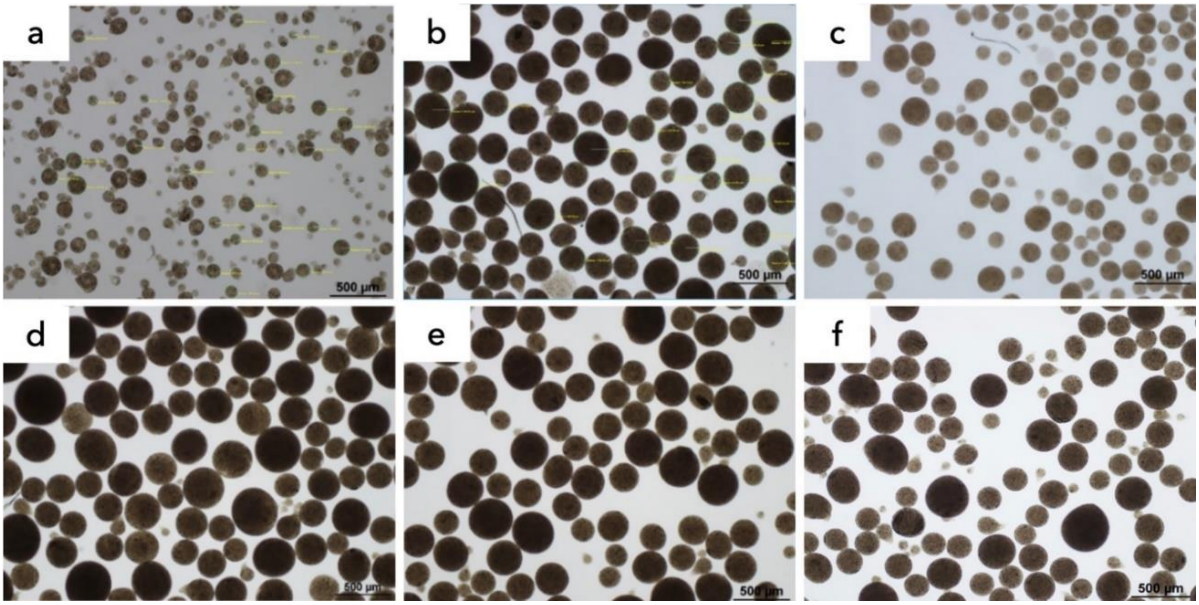


Figure 21: *Results of the different conditions listed in* ***Table 6*** *for the production of microparticles of alginate and shellac with FVN.*

To better understand the effect of the variables (flow rate and air pressure), the experimental data were analysed according to the Design of Experiments methodology as described in section 3.3.1.4. After initial fitting, the following equation was obtained:

MPs Size (µm) = 224.83 - 23.25 FR - 55.75 AP - 5.75 FR AP (**Equation 3**)

with a correlation coefficient R2 = 87.81%. However, after the statistical analysis of the adjustment parameters, it was verified that in the range of values tested, only the *Air Pressure variable (AP)* has a significant effect ($p<0.05$) on the particle size (see **Figure 33** and **Figure 34** in **Annex 3** – Analysis of the Factorial Desing for optimization of MPs produced with FVN). Thus, we eliminated the non-significant parameters from the theoretical model (*FR* and the *FR AP* interaction term) and refitted the model, obtaining the following equation:

$$MPs\ Size\ (\mu m) = 224.83 - 55.75\ AP \quad \textbf{(Equation 4)}$$

The model obtained shows an inversely proportional relationship between air pressure (*AP*) and MPs size (**Figure 22**), with MPs size decreasing strongly with increasing pressure. The model shows a reasonably good fit to the experimental data (R^2 = 73.64%), especially considering the complexity of the system, and it is acceptable from a statistical point of view (the regression model is consistent and the lack of fit is not significant, according to the criteria described in Section 3.3.1.4).

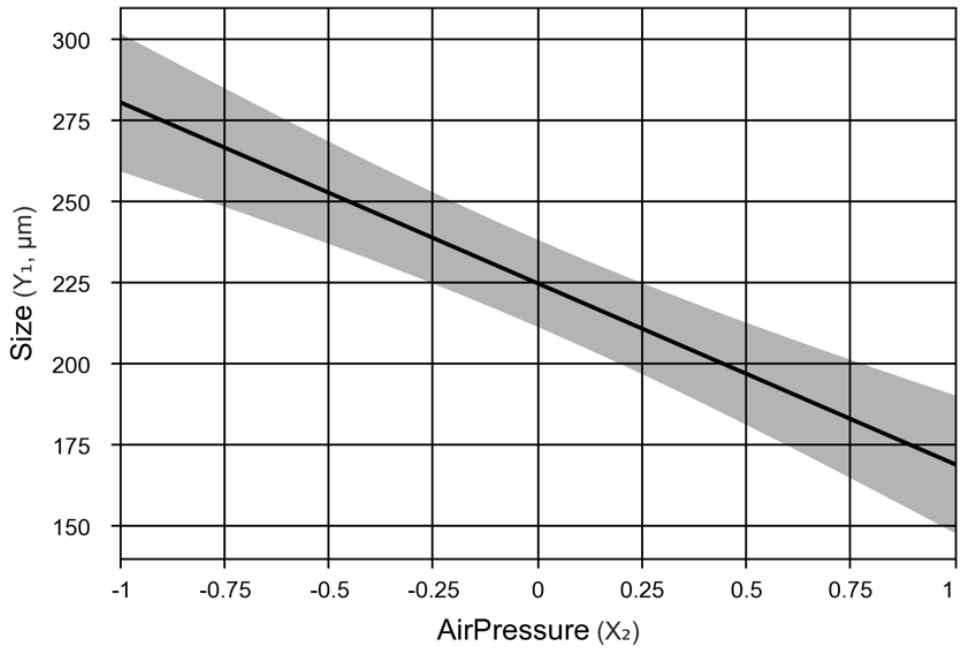


Figure 22: *Predicted effect of air pressure (AP) on MPs size according to the model obtained (Equation 4) for the FVN system. Gray area represents a 95% confidence for the prediction.*

4.3.2 Coated matrix type system

4.3.2.1 Alginate and shellac core-shell microcapsules produced with Concentric Nozzle

As contemplated in the Project aims, the development of a coated matrix type system was also tested, using the CN that enables the co-extrusion of the core material through the inner nozzle and the shell material through the outer nozzle, here the vibration is applied to the shell material. Different combinations of core and shell materials, two gelling solutions, and several parameters were used in this optimization process (**Table 7**). The MPs produced with the first condition (**Figure 23a**) were constituted by a 2 % alginate core coated with a 2 % alginate + 2 % shellac outer layer and were produced with a set of nozzles of 200 and 500 µm. They had a mean diameter of 899 ± 27 µm and density of 1.06 ± 0.01 g/cm^3 and as shown in **Figure 24a.**, the polymeric matrix was unstable. This instability leads to the collapse of the particles, so we increased the percentage of alginate and decreased the percentage of shellac in the shell material. We also tested a new gelling solution, described by Silva et al. 2016, eliminating the ethanol and Tween 80 of the gelling solution and increasing the percentage of $CaCl_2$ to 3.2, increasing the ionic strength of the solution. It was observed that this new solution affected neither the morphology of the particles nor the matrix consistency, so it was used for the following methods. In the following methods (**b.-e.**), the MPs produced were constituted by a 2 % alginate core coated with a 3 % alginate + 1.5 % shellac outer layer, different sets of nozzles were used and the equipment parameters were optimized, to decrease the mean diameter. This goal was successfully achieved; as we can see in **Figure 24**, the smaller diameter achieved was 634 ± 8 µm with method **e.**, using the 120-200 µm nozzles set. The MPs obtained presented a regular spherical morphology and a narrow size distribution. At the last experiment (**f.**), the polymers' concentrations had to be decreased in order to obtain an even smaller mean diameter. A smaller mean diameter was obtained (417 ± 53 µm) also with the 120-200 µm nozzles set, but the MPs had an irregular morphology and a size variation slightly higher, possibly due to the lower polymer concentration (**Figure 23** and **Figure 24f**).

Table 7: *Conditions used to produce core-shell type alginate-shellac microparticles.*

The table presents the different parameters tested for the optimization of the encapsulation system.

COATED MATRIX TYPE (ALGINATE+SHELLAC)

Conditions	Gelling solution	Polymer concentrations (w/w)	CN sizes (µm)	Vibration frequency (Hz)	Amplitude	Eletrode voltage (V)	Flow rate (mL/min) [Core/Shell]
a.	H_2O/Ethanol (1:1 v/v), 0.1 M $CaCl_2$, 0.1 % (v/v) Tween 80	2 % Alginate **(Core)**	200	800	3	2100-1800	4/25
		2 % Alginate+2 % Shellac **(Shell)**	500				
b.	$CaCl_2$ 3.2 % (w/v)	2 % Alginate **(Core)**	200	200		1200	4/16
		3 % Alginate+1.5 % Shellac **(Shell)**	500				
c.		2 % Alginate **(Core)**	200				
		3 % Alginate+1.5 % Shellac **(Shell)**	400				
d.		2 % Alginate **(Core)**	150	600		2200	2.3/29
		3 % Alginate+1.5 % Shellac **(Shell)**	300				
e.		2 % Alginate **(Core)**	120	1000		1200	1.5/617 mbar *
		3 % Alginate+1.5 % Shellac **(Shell)**	200				
f.		1 % Alginate **(Core)**	120	600		1200	1.5/394 mbar *
		2 % Alginate+1 % Shellac **(Shell)**	200				

**Here, the flow rate was not previously adjusted; instead the air pressure was adjusted until a stable dispersion cone was achieved. After that, the MPs were collected into the gelling bath.*

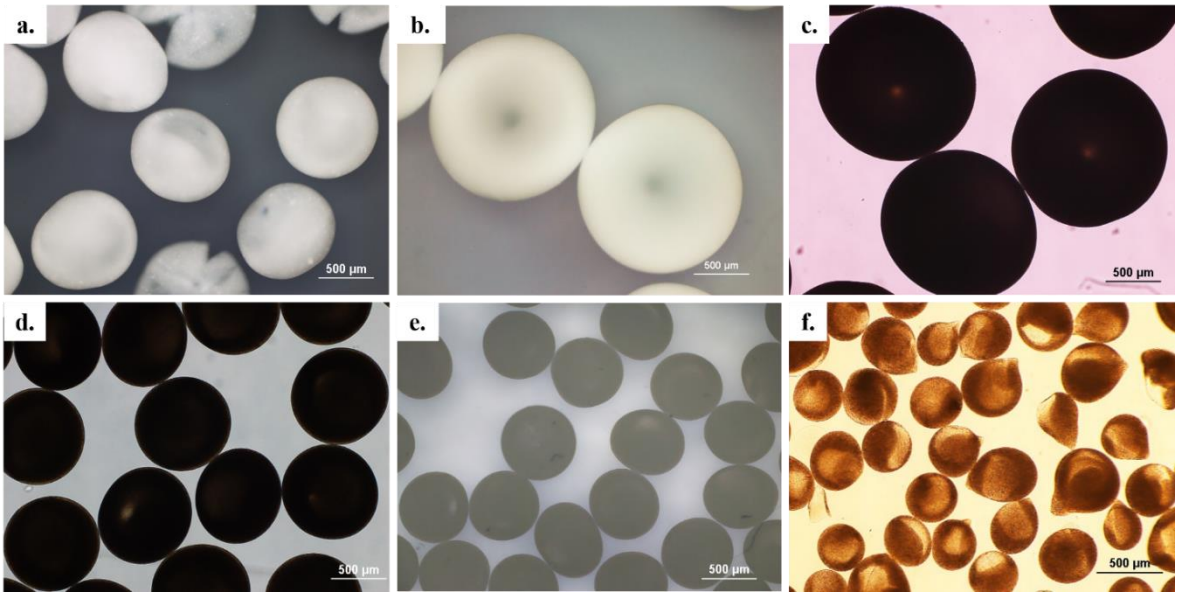


Figure 23: *Results of the different conditions listed in* ***Table 7****, for the production of microcapsules of alginate and shellac with concentric nozzle.*

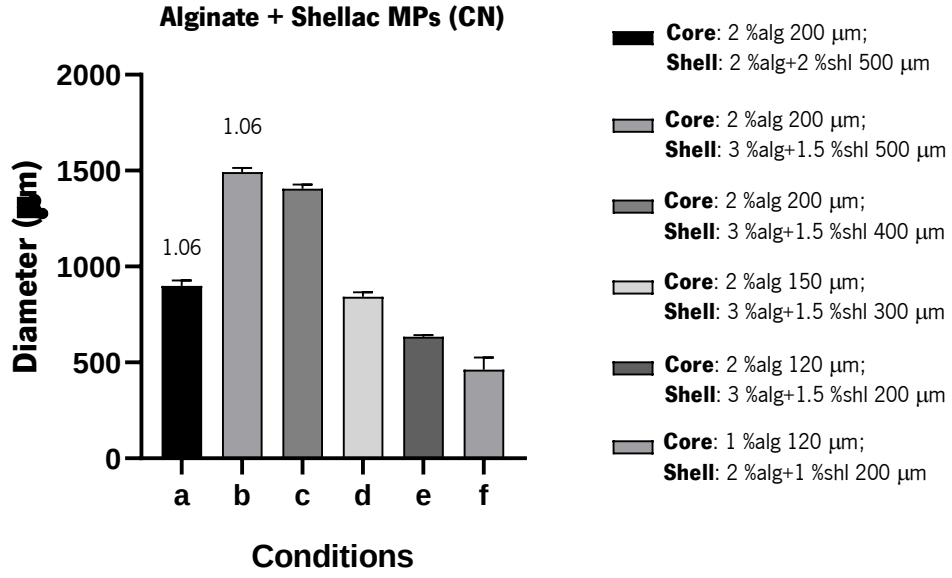


Figure 24: *Mean diameters of Concentric Nozzles optimization process.*

Note: *Densities, when calculated are presented above the bars*

4.4 Microparticles stability during *in vitro* gastrointestinal simulation

Condition **d.** of the FVN methodology (Section 4.3.1.4) produced particles with the most suitable particle size (286 ± 56 µm) for use in rumen bypass applications. However, the FVN methodology requires a complex setup and is difficult to scale up, so it was finally decided to select for the successive tests of stability, characterization, and encapsulation the particles produced in condition **e.** of the SN methodology (Section 4.3.1.2), with an average size of 488 ± 16 µm and composition of 2 % alginate and 3 % shellac (very close to the optimal size range for rumen bypass and with a very low standard deviation).

Thus, a new batch of the selected particles (produced as described in section 3.3.1.2) were produced and tested as described in **Table 8** on the buffers. As we can see in **Figure 25**, the MPs remained intact at the rumen and abomasum pH and dissolved at intestine pH. The three pH values tested for rumen are between the range of values stipulated for this compartment and these values depend on the feeding and the health of the animal. Since the healthy values are between 6 and 7, the pH= 5.8 is rare to occur and pH below 6 are a signal of subacute ruminal acidosis (SARA), a condition that can culminate in the death of the animal (Humer et al. 2018; Nagata et al. 2018; Zhao et al. 2018).

Table 8: *Conditions of the incubations realized for the simulation of gastrointestinal compartments environment.*

Gastrointestinal compartment	Buffer	pH	Volume of buffer (mL)	Mass of MPs (g)	Time of Incubation (h)	Temperature (°C)	Orbital agitation (rpm)
Simulated Rumen Fluid	0.1 M MES	5.8 6.5 7	15	0.5	5	38.5	100
Simulated Abomasum Fluid	0.1 M citrate	3			3		
Simulated Intestine Fluid	0.1 M TRIS	7.5			-		

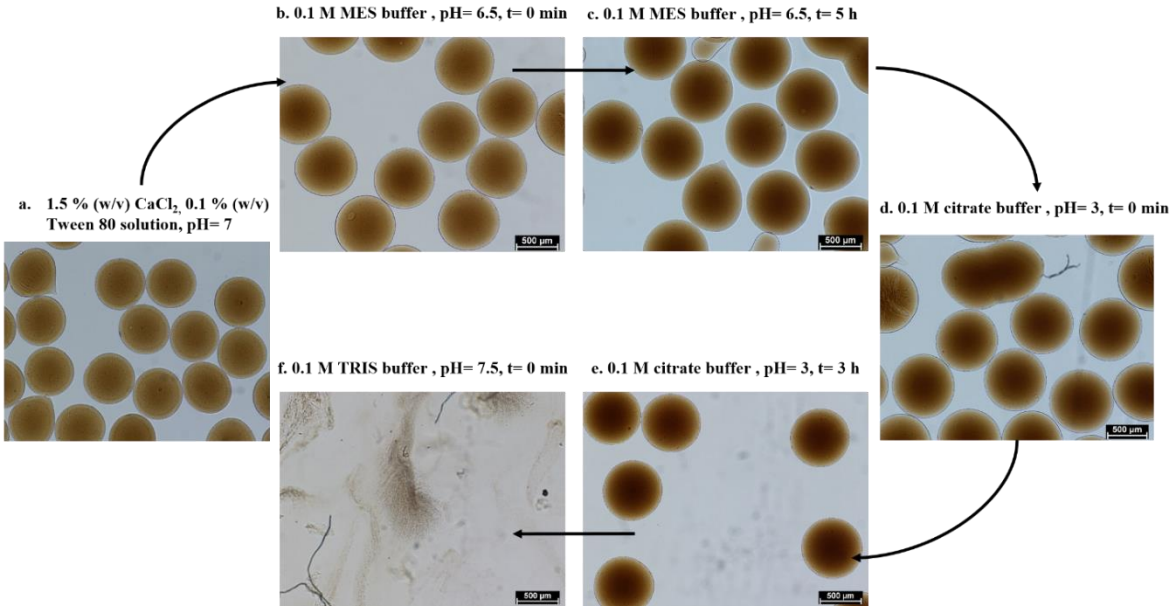


Figure 25: *Schematic representation of the results of the gastrointestinal pH simulation, described in* ***Table 8.***

4.5 Scanning Electron Microscopy

The last MPs optimized were dried in a desiccator overnight and were observed under SEM at different magnifications (**Figure 26**: **A.** -50x; **B.** -100x; **C.** -300x; **D.** -1000x). It was possible to verify that after the drying process, the MPs diameter decreased significantly as they have lost their high water

content. In **Figure 26C**, it is possible to observe that the bead surfaces are smooth, with some striations on the surface of the MPs resulting from the drying process, similar to the results demonstrated by (Morales et al. 2017). It is also possible to observe pores, which confirm the porous nature of the polymeric matrix of alginate and shellac. **Figure 26D** shows an inner section of a MP, where it is possible to see some pores again and some white spots that resulted from $CaCl_2$ salts deposition.

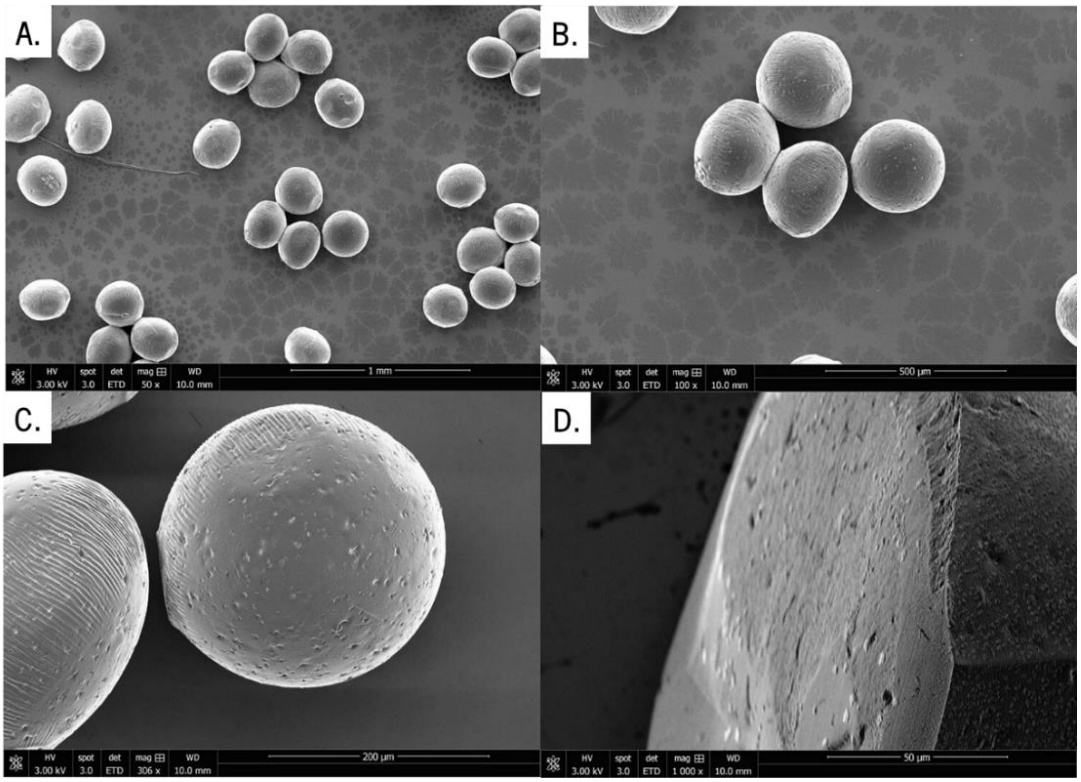


***Figure 26**: Microparticles analyzed under Scanning Electron Microscopy*

4.6 Fluorescent nano-spheres encapsulation and Confocal microscopy analysis

The fluorescent nano-spheres were encapsulated as previously described in section 3.5. The nano-spheres with a diameter of 0.2 µm (200 nm) were chosen to simulate the phage size since T4 and CBA120 are approximately 200 nm long (Kutter et al. 2011; Plattner et al. 2019; Yap and Rossmann 2014). The CM analyzes showed that the fluorescent nano-spheres remained inside the MPs after three days of storage (**Figure 27a**). However, when MPs were put into 0.1 M TRIS pH 7.5 buffer, they soon began to disintegrate and release the nano-spheres. **Figure 27b** represents the MPs after approximately

1 min and **Figure 27c** after 5 min. This proved that our encapsulation system would be able to encapsulate and release phages, so we moved to the task of encapsulating the phages.

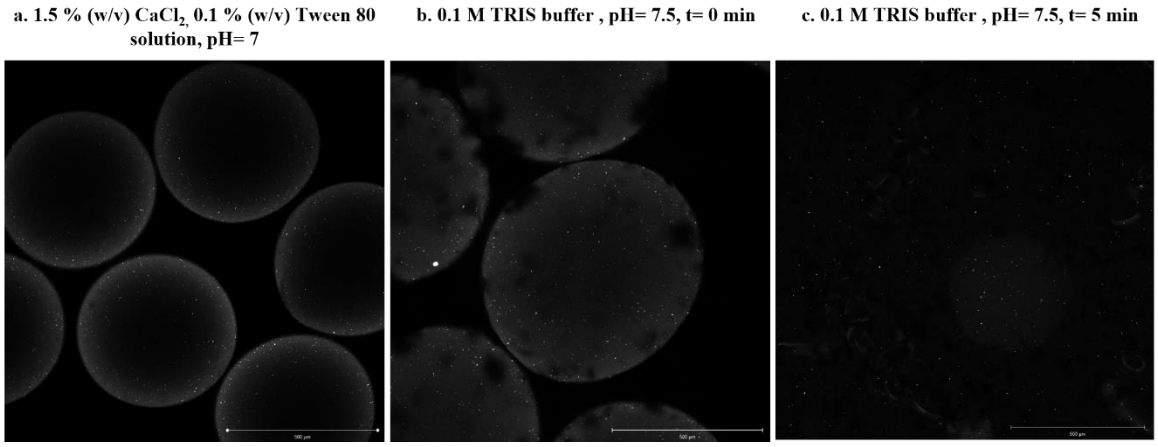


Figure 27: *Confocal microscope images of the fluorescent nano-spheres of diameter equal to 200 nm, encapsulated within the alginate and shellac microparticles.*

4.7 Phage encapsulation and *in vitro* gastrointestinal simulation

Both phages were encapsulated in the selected MPs and submitted to *in vitro* GIT simulation, as previously described. After GIT simulation, the phage titer in each solution was determined and results are shown in **Figure 28**.

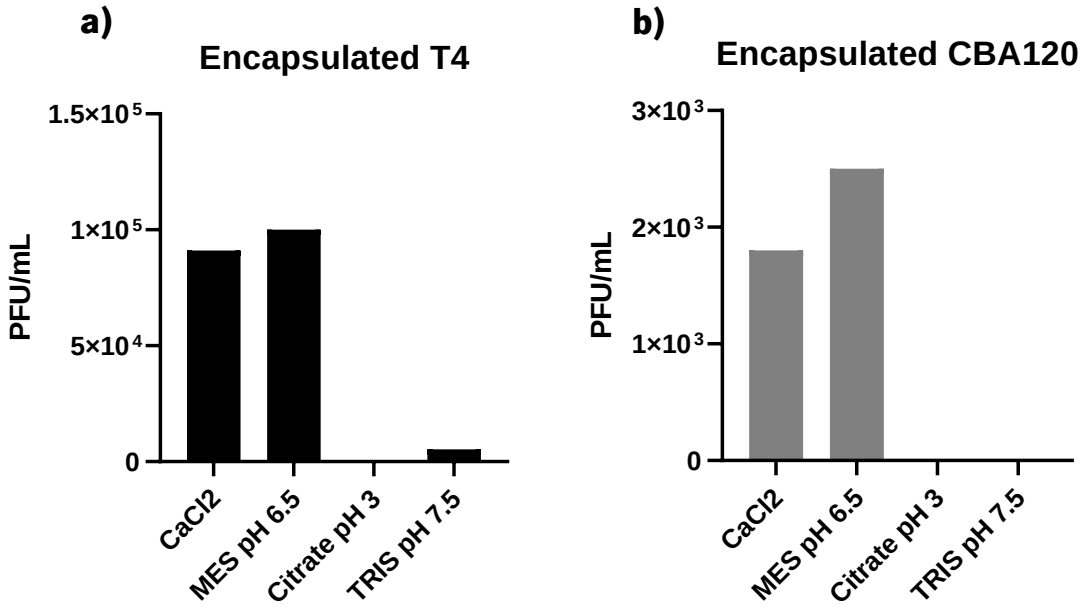


Figure 28: *Release tests of encapsulated phages during in vitro simulation.*

Both encapsulated phages were submitted to the in vitro gastrointestinal simulation and the phage titer was calculated at each step and in the gelling bath.

Results showed that phages T4 and CBA120 were detected at the gelling bath (9.1×10^4 and 1.8×10^3 PFU/mL, respectively). There are two possible explanations for this: **1)** the porosity of this polymeric matrix is not suitable for the total entrapment of the phages and they are being released throughout the time (Mancini et al. 1999; Messaoud et al. 2016); or **2)** the phages detected were those that remained attached to the outer surface of the particles during the encapsulation process, as described by Cinquerrui et al. 2018. The latter hypothesis could only be confirmed by confocal microscopy, using phages previously labeled with a fluorescence dye (e.g. FITC) (Gitis et al. 2002). A slightly higher concentration of phages was also detected at the 0.1 M MES buffer pH 6.5 (T4 - 1.0×10^5 and CBA120 - 2.5×10^3 PFU/mL), which is consistent with the first hypothesis, once the MPs were rinsed with distilled water after the gelling bath, non-encapsulated phages would have been removed from the MPs surface. At 0.1 M citrate buffer pH 3, as expected, no phages were detected since it was proved that they do not survive in such an acidic environment **Figure 14**.

Considering hypothesis **1)** to be correct, the reduced concentration of phage T4 (5.2×10^3 PFU/mL) and the absence of phage CBA120 in the 0.1 M TRIS buffer pH 7.5 (where the MPs dissolved completely) is justifiable, due to the losses that occurred along the way. On the other hand, the detection of T4 phage after 3 hours of incubation at pH 3 proves that the MPs protected the phages from these

acidic conditions. Also, in the assay performed with the MPs dissolved in 0.1 M phosphate buffer pH 7 right after the gelation bath, phages were detected at higher titers (6.0×10^5 for T4 and 2.9×10^6 PFU/mL for CBA120). This proves that the phages are indeed being encapsulated, but escape from inside the MPs during the GIT simulation, which was also described by Colom et al. during gastric conditions incubation of alginate/ $CaCO_3$-encapsulated phages.

These results led us to the next steps to improve this phage encapsulation system: **i)** decrease the porosity of the MPs, achieving better phage retention and **ii)** increase the titer of the encapsulated phage, since in the literature encapsulated phage titers are described above 10^9 PFU per grams of MPs (Vinner et al. 2019) or per animal (Ma et al. 2016) in orally administered dosage, in order to effective amouts of phages reach the intestine. The results of the GIT simulation with free CBA120 (**Figure 29**) showed that the phage survived a 5 hours incubation at 0.1 M MES pH 6.5 (1.4×10^5 PFU/mL), but it was not detected after incubation at simulated abomasum fluid. The results of the second experiment, where the incubations at pH 3 and pH 7.5 were performed individually, proved that the inactivation occurs at 0.1 M citrate pH 3, and at 0.1 M MES pH 7.5 the phage titer was 1×10^8 PFU/mL.

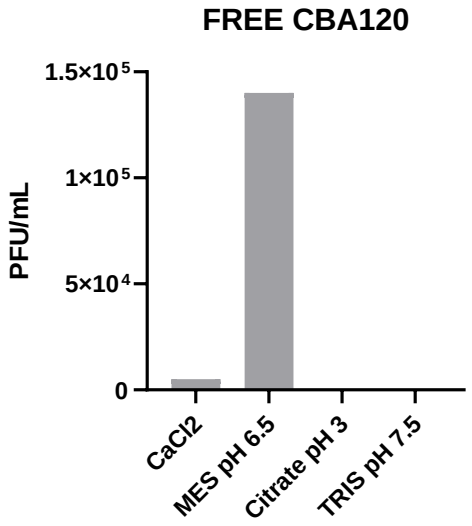


Figure 29: *In vitro GIT simulation with free CBA120.*

Chapter 5: Conclusions and Future perspectives

Chapter 5: Conclusions and Future perspectives

5.1 Conclusions

In conclusion, it is important to note that three different processes were optimized for the production of microparticles composed of two natural polymers, alginate and shellac (SN, FVN and CN). Although only one of the processes was applied for phage encapsulation and subsequent *in vitro* testing, the others can still be adapted for a variety of applications in the future. The project aim of developing a biodegradable and biocompatible encapsulation system, with intestinal phage release was almost fulfilled, but additional studies to increase phage retention until arrival in the intestine are lacking. Microparticles with an average diameter of 488 ± 16 μm and composition of 2 % alginate and 3 % shellac were produced by a practical, fast and reproducible extrusion process and under favorable conditions to preserve phage viability. The structure of the microparticles was analyzed by scanning electron microscopy that showed a spherical structure and a smooth and porous surface. Additionally, the MPs remained intact at rumen and abomasum pH, resisting GIT simulation for about 8 hours, but rapidly disintegrated at intestine pH as desired. Taking into consideration that free phages showed an activity loss at pH values similar to abomasum pH, the results of this study allow us to conclude that the encapsulation system here developed may offer protection against GIT pH conditions.

5.2 Future perspectives

As future work, it would be interesting to perform further characterization of the MPs, improving the retention capacity, to decrease or even eliminate the phage titer losses observed during *in vitro* GIT simulation, as well as subsequent release studies *ex vivo* and *in vivo*:

1) Fourier Transform Infrared (FTIR) spectroscopy to analyze the chemical properties of the MPs and Thermogravimetric (TGA) to monitor the MPs mass variations at different temperatures are relevant analyses that must be performed;

2) The retention capacity of the MPs can be improved with the addition of a chitosan external layer or alterations on the encapsulation process parameters (polymer concentrations or gelling bath composition);

3) Release studies using animal digestive fluids (*ex vivo*) and oral administration to live animals (*in vivo*) are also necessary in order to evaluate the efficiency of the developed encapsulation system, regarding the elimination of foodborne pathogens from ruminants intestines.

Chapter 6: References

Chapter 6: References

Adams, J. C., J. A. Gazaway, M. D. Brailsford, P. A. Hartman, and N. L. Jacobson. 1966. "Isolation of Bacteriophages from the Bovine Rumen." *Experientia*. doi: 10.1007/bf01901335.

Ahmed, Mahmoud, and M. S. Youssef. 2012. "Characteristics of Mean Droplet Size Produced by Spinning Disk Atomizers." *Journal of Fluids Engineering, Transactions of the ASME*. doi: 10.1115/1.4006819.

Akhtar, Mahmood, Brent S. Murray, and Sifiso Dowu. 2014. "A Novel Continuous Process for Making Mayonnaise and Salad Cream Using the Spinning Disc Reactor: Effect of Heat Treatment." *Food Hydrocolloids*. doi: 10.1016/j.foodhyd.2014.06.007.

Al-Gousous, J., M. Penning, and P. Langguth. 2015. "Molecular Insights into Shellac Film Coats from Different Aqueous Shellac Salt Solutions and Effect on Disintegration of Enteric-Coated Soft Gelatin Capsules." *International Journal of Pharmaceutics*. doi: 10.1016/j.ijpharm.2014.12.060.

Alex, Rainer, and Roland Bodmeier. 1990. "Encapsulation of Water-Soluble Drugs by a Modified Solvent Evaporation Method. I. Effect of Process and Formulation Variables on Drug Entrapment." *Journal of Microencapsulation*. doi: 10.3109/02652049009021845.

Alves, Diana, Arlete Marques, Catarina Milho, Maria José Costa, Lorenzo M. Pastrana, Miguel A. Cerqueira, and Sanna Maria Sillankorva. 2019. "Bacteriophage ΦIBB-PF7A Loaded on Sodium Alginate-Based Films to Prevent Microbial Meat Spoilage." *International Journal of Food Microbiology*. doi: 10.1016/j.ijfoodmicro.2018.11.026.

Arshady, Reza, and Maurice H. George. 1993. "Suspension, Dispersion, and Interfacial Polycondensation: A Methodological Survey." *Polymer Engineering & Science*. doi: 10.1002/pen.760331402.

Atterbury, Robert J., Phillippa L. Connerton, Christine E. R. Dodd, Catherine E. D. Rees, and Ian F. Connerton. 2003. "Application of Host-Specific Bacteriophages to the Surface of Chicken Skin Leads to a Reduction in Recovery of Campylobacter Jejuni." *Applied and Environmental Microbiology*. doi: 10.1128/AEM.69.10.6302-6306.2003.

Azeredo, Henriette Monteiro Cordeiro de. 2005. "Encapsulação: Aplicação à Tecnologia de Alimentos." *Alimentos e Nutrição Araraquara* 16(1):89–97.

Bach, Susan J., Tim A. McAllister, Doug M. Veira, Victor P. J. Gannon, and Rick A. Holley. 2003. " Effect of Bacteriophage DC22 on Escherichia Coli O157:H7 in an Artificial Rumen System (Rusitec) and Inoculated Sheep ." *Animal Research*. doi: 10.1051/animres:2003009.

De Beer, M. M., L. Pezzi Martins Loane, J. T. F. Keurentjes, J. C. Schouten, and J. van der Schaaf. 2014. "Single Phase Fluid-Stator Heat Transfer in a Rotor-Stator Spinning Disc Reactor." *Chemical Engineering Science*. doi: 10.1016/j.ces.2014.08.008.

Belverdy, Mohsen Sahraei, Ali A. Alamouti, and Mohammad Hossein Azizi. 2019. "Microencapsulation in the Ruminant Feed Industry." *Dairy Knowledge Center* (1).

Benczedi, Daniel (Firmenich SA), and Pierre-Etienne (Firmenich SA) Bouquerand. 2003. "Process for the Preparation of Granules for the Controlled Release of Volatile Compounds." 2(12).

Benichou, Axel, and Nissim Garti. 2001. "Double Emulsions for Controlled-Release Applications- Progress and Trends." Pp. 377–407 in *Encyclopedic Handbook of Emulsion Technology*.

Benoy, C. J., L. A. Elson, and R. Schneider. 1972. "Multiple Emulsions, a Suitable Vehicle to Provide Sustained Release of Cancer Chemotherapeutic Agents." *British Journal of Pharmacology* 45(1):135P-136P. doi: 10.1111/j.1476-5381.1972.tb09587.x.

Bhanvase, B. A., D. V. Pinjari, P. R. Gogate, S. H. Sonawane, and A. B. Pandit. 2011. "Process Intensification of Encapsulation of Functionalized CaCO3 Nanoparticles Using Ultrasound Assisted Emulsion Polymerization." *Chemical Engineering and Processing: Process Intensification*. doi: 10.1016/j.cep.2011.09.002.

Bibbal, Delphine, Estelle Loukiadis, Monique Kérourédan, Franck Ferré, Françoise Dilasser, Carine Peytavin de Garam, Philippe Cartier, Eric Oswald, Emilie Gay, Frédéric Auvray, and Hubert Brugère. 2015. "Prevalence of Carriage of Shiga Toxin-Producing Escherichia Coli Serotypes O157:H7, O26:H11, O103:H2, O111:H8, and O145:H28 among Slaughtered Adult Cattle in France." *Applied and Environmental Microbiology* 81(4):1397–1405. doi: 10.1128/AEM.03315-14.

Box, George E. P., J. Suart Hunter, and William G. Hunter. 2005. "Statistics for Experimenters: Design, Innovation, and Discovery." *John Wiley & Sons.*

Brandenberger, H., D. Nüssli, V. Piëch, and F. Widmer. 1999. "Monodisperse Particle Production: A Method to Prevent Drop Coalescence Using Electrostatic Forces." *Journal of Electrostatics*. doi: 10.1016/S0304-3886(98)00052-7.

Buch, Karl, Manfred Penning, Eva Wchtersbach, Michael Maskos, and Peter Langguth. 2009. "Investigation of Various Shellac Grades: Additional Analysis for Identity." *Drug Development and Industrial Pharmacy*. doi: 10.1080/03639040802563253.

Callaway, T. R., R. C. Anderson, T. S. Edrington, R. O. Elder, K. J. Genovese, K. M. Bischoff, T. L. Poole, Y. S. Jung, R. B. Harvey, and D. J. Nisbet. 2003. "Preslaughter Interventions Strategies to Reduce Food-Borne Pathogens in Food Animals." *American Society of Animal Science* 1–7.

Callaway, T. R., T. S. Edrington, R. C. Anderson, R. B. Harvey, K. J. Genovese, C. N. Kennedy, D. W. Venn, and D. J. Nisbet. 2008. "Probiotics, Prebiotics and Competitive Exclusion for Prophylaxis against Bacterial Disease." *Animal Health Research Reviews / Conference of Research Workers in Animal Diseases* 9(2):217–25. doi: 10.1017/S1466252308001540.

Callaway, Todd R., Robin C. Anderson, Tom S. Edrington, Kenneth J. Genovese, Roger B. Harvey, Toni L. Poole, and David J. Nisbet. 2013. "Novel Methods for Pathogen Control in Livestock Pre-Harvest: An Update." in *Advances in Microbial Food Safety*.

Castro, Natalia, Vanessa Durrieu, Christine Raynaud, Antoine Rouilly, Luc Rigal, and Christian Quellet. 2016. "Melt Extrusion Encapsulation of Flavors: A Review." *Polymer Reviews*. doi: 10.1080/15583724.2015.1091776.

Ceballos, Adela María, Gloria Inés Giraldo, and Carlos Eduardo Orrego. 2012. "Effect of Freezing Rate on Quality Parameters of Freeze Dried Soursop Fruit Pulp." *Journal of Food Engineering*. doi: 10.1016/j.jfoodeng.2012.02.010.

Chandy, Thomas, and Chandra P. Sharma'. 1990. "Chitosan - as a Biomaterial." *Biomaterials, Artificial Cells and Artificial Organs*. doi: 10.3109/10731199009117286.

Chaucheyras-Durand, Frédérique, Fahima Faqir, Aurélie Ameilbonne, Christine Rozand, and Christine Martin. 2010. "Fates of

Acid-Resistant and Non-Acid-Resistant Shiga Toxin-Producing Escherichia Coli Strains in Ruminant Digestive Contents in the Absence and Presence of Probiotics." *Applied and Environmental Microbiology* 76(3):940–47. doi: 10.1128/AEM.02054-09.

Chávarri, María, Izaskun Marañón, Raquel Ares, Francisco C. Ibáñez, Florencio Marzo, and María del Carmen Villarán. 2010. "Microencapsulation of a Probiotic and Prebiotic in Alginate-Chitosan Capsules Improves Survival in Simulated Gastro-Intestinal Conditions." *International Journal of Food Microbiology*. doi: 10.1016/j.ijfoodmicro.2010.06.022.

Chemat, Farid, Zill-E-Huma, and Muhammed Kamran Khan. 2011. "Applications of Ultrasound in Food Technology: Processing, Preservation and Extraction." in *Ultrasonics Sonochemistry*.

Chiang, Chia Ying, Ming Hui Chang, Hwai Shen Liu, Clifford Y. Tai, and Sheryl Ehrman. 2012. "Process Intensification in the Production of Photocatalysts for Solar Hydrogen Generation." *Industrial and Engineering Chemistry Research*. doi: 10.1021/ie202522g.

Chin-fatt, Adam, Ed Topp, and Rima Menassa. 2018. *Prospects of Plant-Based Vaccines in Veterinary Medicine*. Springer International Publishing.

Cinquerrui, Salvatore, Francesco Mancuso, Goran T. Vladisavljevic, Saskia E. Bakker, and Danish J. Malik. 2018. "Nanoencapsulation of Bacteriophages in Liposomes Prepared Using Microfluidic Hydrodynamic Flow Focusing." *Frontiers in Microbiology*. doi: 10.3389/fmicb.2018.02172.

Clokie, Martha R. J., Andrew D. Millard, Andrey V. Letarov, and Shaun Heaphy. 2011. "Phages in Nature." *Bacteriophage*. doi: 10.4161/bact.1.1.14942.

Cobián Güemes, Ana Georgina, Merry Youle, Vito Adrian Cantú, Ben Felts, James Nulton, and Forest Rohwer. 2016. "Viruses as Winners in the Game of Life." *Annual Review of Virology*. doi: 10.1146/annurev-virology-100114-054952.

Cole, Graham, John Hogan, and Michael Aulton. 1995. *Pharmaceutical Coating Technology*.

Colom, Joan, Mary Cano-Sarabia, Jennifer Otero, Javier Aríñez-Soriano, Pilar Cortés, Daniel Maspoch, and Montserrat Llagostera. 2017. "Microencapsulation with Alginate/CaCO 3: A Strategy for Improved Phage Therapy." *Scientific Reports*. doi: 10.1038/srep41441.

Coradin, T., N. Nassif, and J. Livage. 2003. "Silica-Alginate Composites for Microencapsulation." *Applied Microbiology and Biotechnology*.

Costa-Pinto, Ana Rita, Rui L. Reis, and Nuno M. Neves. 2011. "Scaffolds Based Bone Tissue Engineering: The Role of Chitosan." *Tissue Engineering - Part B: Reviews*. doi: 10.1089/ten.teb.2010.0704.

Degen, Patrick, Elena Zwar, Imke Schulz, and Heinz Rehage. 2015. "Magneto-Responsive Alginate Capsules." in *Journal of Physics Condensed Matter*.

Dehkordi, Asghar Molaei, and Alireza Vafaeimanesh. 2009. "Synthesis of Barium Sulfate Nanoparticles Using a Spinning Disk Reactor: Effects of Supersaturation, Disk Rotation Speed, Free Ion Ratio, and Disk Diameter." *Industrial and Engineering Chemistry Research*. doi: 10.1021/ie801799v.

Desai, Kashappa Goud H., and Hyun Jin Park. 2005. "Recent Developments in Microencapsulation of Food Ingredients." *Drying Technology*.

Dhama, K., S. Rajagunalan, S. Chakraborty, A. K. Verma, A. Kumar, R. Tiwari, and S. Kapoor. 2013. "Food-Borne Pathogens of Animal Origin-Diagnosis, Prevention, Control and Their Zoonotic Significance: A Review." *Pakistan Journal of Biological*

Sciences. doi: 10.3923/pjbs.2013.1076.1085.

Ding, W. K., and N. P. Shah. 2008. "Survival of Free and Microencapsulated Probiotic Bacteria in Orange and Apple Juices." *International Food Research Journal*.

Dini, Cecilia, Germán A. Islan, Patricio J. de Urraza, and Guillermo R. Castro. 2012. "Novel Biopolymer Matrices for Microencapsulation of Phages: Enhanced Protection against Acidity and Protease Activity." *Macromolecular Bioscience*. doi: 10.1002/mabi.201200109.

Duan, Wenjun, Qingbo Yu, Kun Wang, Qin Qin, Limin Hou, Xin Yao, and Tianwei Wu. 2015. "ASPEN Plus Simulation of Coal Integrated Gasification Combined Blast Furnace Slag Waste Heat Recovery System." *Energy Conversion and Management*. doi: 10.1016/j.enconman.2015.04.066.

EFSA. 2020. "Food-Borne Zoonotic Diseases | European Food Safety Authority." Retrieved May 20, 2020 (https://www.efsa.europa.eu/en/topics/topic/food-borne-zoonotic-diseases).

Elder, R. O., J. E. Keen, T. E. Wittum, T. R. Callaway, T. S. Edrington, R. C. Anderson, and D. J. Nisbet. 2002. "Intervention to Reduce Fecal Shedding of Enterohemorrhagic Escherichia Coli O157H7 in Naturally Infected Cattle Using Neomycin Sulfate." *Amer. Soc. Anim. Sci.* 602.

Emin, M. A., and H. P. Schuchmann. 2013a. "Analysis of the Dispersive Mixing Efficiency in a Twin-Screw Extrusion Processing of Starch Based Matrix." *Journal of Food Engineering*. doi: 10.1016/j.jfoodeng.2012.10.008.

Emin, M. A., and H. P. Schuchmann. 2013b. "Droplet Breakup and Coalescence in a Twin-Screw Extrusion Processing of Starch Based Matrix." *Journal of Food Engineering*. doi: 10.1016/j.jfoodeng.2012.12.010.

Estevinho, Berta Nogueiro, Fernando Rocha, Lúcia Santos, and Arminda Alves. 2013. "Microencapsulation with Chitosan by Spray Drying for Industry Applications - A Review." *Trends in Food Science and Technology*.

Farooq, Fajar, Umer Ali, Shabu Shoukat, Adil Rasool Paray, Mudasir Ali, and Umar Rasool Parry. 2019. "Bacteriophages as Biocontrol Agents for Foodborne Diseases." 7(1):67–71.

Florence, A. T., A. W. Jenkins, and A. H. Loveless. 1976. "Effect of Formulation of Intramuscular Injections of Phenothiazines on Duration of Activity." *Journal of Pharmaceutical Sciences* 66(11):1665–68. doi: 10.1002/jps.2600651125.

Fuciños, Clara, Pablo Fuciños, Lorenzo M. Pastrana, and María L. Rúa. 2014. "Functional Characterization of Poly(N-Isopropylacrylamide) Nanohydrogels for the Controlled Release of Food Preservatives." *Food and Bioprocess Technology* 7(12):3429–41. doi: 10.1007/s11947-014-1351-5.

Gaitzsch, F., A. Gäbler, and M. Kraume. 2011. "Analysis of Droplet Expulsion in Stagnant Single Water-in-Oil-in-Water Double Emulsion Globules." *Chemical Engineering Science* 66(20):4663–69. doi: 10.1016/j.ces.2011.06.020.

Garti, Nissim. 1997. "Progress in Stabilization and Transport Phenomena of Double Emulsions in Food Applications." *LWT - Food Science and Technology* 30(3):222–35. doi: 10.1006/fstl.1996.0176.

Del Gaudio, Pasquale, Giulia Auriemma, Paola Russo, Teresa Mencherini, Pietro Campiglia, Mariateresa Stigliani, and Rita Patrizia Aquino. 2014. "Novel Co-Axial Prilling Technique for the Development of Core-Shell Particles as Delayed Drug Delivery Systems." *European Journal of Pharmaceutics and Biopharmaceutics*. doi: 10.1016/j.ejpb.2014.02.010.

Del Gaudio, Pasquale, Paolo Colombo, Gaia Colombo, Paola Russo, and Fabio Sonvico. 2005. "Mechanisms of Formation and Disintegration of Alginate Beads Obtained by Prilling." *International Journal of Pharmaceutics*. doi: 10.1016/j.ijpharm.2005.05.041.

Del Gaudio, Pasquale, Paola Russo, Maria Rosaria Lauro, Paolo Colombo, and Rita P. Aquino. 2009. "Encapsulation of Ketoprofen and Ketoprofen Lysinate by Prilling for Controlled Drug Release." *AAPS PharmSciTech*. doi: 10.1208/s12249-009-9309-z.

Gbassi, Gildas K., and Thierry Vandamme. 2012. "Probiotic Encapsulation Technology: From Microencapsulation to Release into the Gut." *Pharmaceutics*.

Gharsallaoui, Adem, Gaëlle Roudaut, Odile Chambin, Andrée Voilley, and Rémi Saurel. 2007. "Applications of Spray-Drying in Microencapsulation of Food Ingredients: An Overview." *Food Research International*.

Gibbs, Bernard F., Selim Kermasha, Inteaz Alli, and Catherine N. Mulligan. 1999. "Encapsulation in the Food Industry: A Review." *International Journal of Food Sciences and Nutrition*. doi: 10.1080/096374899101256.

Gitis, Vitaly, Avner Adin, Abed Nasser, Jenny Gun, and Ovadia Lev. 2002. "Fluorescent Dye Labeled Bacteriophages - A New Tracer for the Investigation of Viral Transport in Porous Media: 1. Introduction and Characterization." *Water Research* 36(17):4227–34. doi: 10.1016/S0043-1354(02)00163-X.

Goldberg, Barry B., Ji Bin Liu, and Flemming Forsberg. 1994. "Ultrasound Contrast Agents: A Review." *Ultrasound in Medicine and Biology*. doi: 10.1016/0301-5629(94)90001-9.

Goodridge, Lawrence D., and Bledar Bisha. 2011. "Phage-Based Biocontrol Strategies to Reduce Foodborne Pathogens in Foods." *Bacteriophage*. doi: 10.4161/bact.1.3.17629.

Gouin, Sébastien. 2004. "Microencapsulation: Industrial Appraisal of Existing Technologies and Trends." in *Trends in Food Science and Technology*.

Van Der Graaf, S., C. G. P. H. Schroën, and R. M. Boom. 2005. "Preparation of Double Emulsions by Membrane Emulsification - A Review." *Journal of Membrane Science* 251(1–2):7–15. doi: 10.1016/j.memsci.2004.12.013.

Gyles, C. L. 1998. "Vaccines and Shiga Toxin-Producing Escherichia Coli in Animals." Pp. 434–444 in *Escherichia coli O157:H7 and other shiga toxin-producing E. coli strains.*, edited by J. B. Kaper and D. O'Brien. Washington, DC: Amer. Soc. Microbiol. Press.

Haghighi, Hossein, Serge Kameni Leugoue, Frank Pfeifer, Heinz Wilhelm Siesler, Fabio Licciardello, Patrizia Fava, and Andrea Pulvirenti. 2020. "Development of Antimicrobial Films Based on Chitosan-Polyvinyl Alcohol Blend Enriched with Ethyl Lauroyl Arginate (LAE) for Food Packaging Applications." *Food Hydrocolloids*. doi: 10.1016/j.foodhyd.2019.105419.

Hamad, Shwan A., Simeon D. Stoyanov, and Vesselin N. Paunov. 2012. "Triggered Cell Release from Shellac-Cell Composite Microcapsules." *Soft Matter*. doi: 10.1039/c2sm07488e.

Hedberg, Craig W. 2011. "Foodborne Illness Acquired in the United States." *Emerging Infectious Diseases* 17(7):1338–40. doi: 10.3201/eid1707.110019.

Henning, Stefan, Sabine Leick, Maureen Kott, Heinz Rehage, and Dieter Suter. 2012. "Sealing Liquid-Filled Pectinate Capsules with a Shellac Coating." *Journal of Microencapsulation*. doi: 10.3109/02652048.2011.635220.

Heredia, Norma, and Santos García. 2018. "Animals as Sources of Food-Borne Pathogens: A Review." *Animal Nutrition* 4(3):250–55. doi: 10.1016/j.aninu.2018.04.006.

Hoemann, C. D., A. Chenite, J. Sun, M. Hurtig, A. Serreqi, Z. Lu, E. Rossomacha, and M. D. Buschmann. 2007. "Cytocompatible Gel Formation of Chitosan-Glycerol Phosphate Solutions Supplemented with Hydroxyl Ethyl Cellulose Is Due to the Presence of Glyoxal." *Journal of Biomedical Materials Research - Part A*. doi: 10.1002/jbm.a.31365.

House, J. K., M. M. Ontiveros, N. M. Blackmer, E. L. Dueger, J. B. Fitchhorn, G. R. McArthur, and B. P. Smith. 2001. "Evaluation of an Autogenous Salmonella Bacterin and a Modified Live Salmonella Serotype Choleraesuis Vaccine on a Commercial Dairy Farm." *American Journal of Veterinary Research*. doi: 10.2460/ajvr.2001.62.1897.

Hsu, F. C., Y. C. Caro. Shieh, and M. D. Sobsey. 2002. "Enteric Bacteriophages as Potential Fecal Indicators in Ground Beef and Poultry Meat." *Journal of Food Protection*. doi: 10.4315/0362-028X-65.1.93.

Hu, Shang Hsiu, Chia Hui Tsai, Chen Fu Liao, Dean Mo Liu, and San Yuan Chen. 2008. "Controlled Rupture of Magnetic Polyelectrolyte Microcapsules for Drug Delivery." *Langmuir*. doi: 10.1021/la801138e.

Huang, Zheng Ming, Chuang Long He, Aizhao Yang, Yanzhong Zhang, Xiao Jian Han, Junlin Yin, and Qingsheng Wu. 2006. "Encapsulating Drugs in Biodegradable Ultrafine Fibers through Co-Axial Electrospinning." *Journal of Biomedical Materials Research - Part A*. doi: 10.1002/jbm.a.30564.

Huang, Zheng Ming, Y. Z. Zhang, M. Kotaki, and S. Ramakrishna. 2003. "A Review on Polymer Nanofibers by Electrospinning and Their Applications in Nanocomposites." *Composites Science and Technology*. doi: 10.1016/S0266-3538(03)00178-7.

Humer, E., J. R. Aschenbach, V. Neubauer, I. Kröger, R. Khiaosa-ard, W. Baumgartner, and Q. Zebeli. 2018. "Signals for Identifying Cows at Risk of Subacute Ruminal Acidosis in Dairy Veterinary Practice." *Journal of Animal Physiology and Animal Nutrition*.

Hussain, Malik A., Huan Liu, Qi Wang, Fang Zhong, Qian Guo, and Sampathkumar Balamurugan. 2017. "Use of Encapsulated Bacteriophages to Enhance Farm to Fork Food Safety." *Critical Reviews in Food Science and Nutrition*. doi: 10.1080/10408398.2015.1069729.

Ignacio-Espinoza, Julio Cesar, and Jed A. Fuhrman. 2018. "A Non-Tailed Twist in the Viral Tale." *Nature*.

Iqbal, Muhammad, Nadiah Zafar, Hatem Fessi, and Abdelhamid Elaissari. 2015. "Double Emulsion Solvent Evaporation Techniques Used for Drug Encapsulation." *International Journal of Pharmaceutics*.

Islam, Golam S., Qi Wang, and Parviz M. Sabour. 2018. "Encapsulation Strategies of Bacteriophage (Felix O1) for Oral Therapeutic Application." in *Methods in Molecular Biology*.

Jacobsen, Nikolas C., and Olaf Hinrichsen. 2012. "Micromixing Efficiency of a Spinning Disk Reactor." *Industrial and Engineering Chemistry Research*. doi: 10.1021/ie300411b.

Kailasapathy, Kaila. 2002. "Microencapsulation of Probiotic Bacteria: Technology and Potential Applications." *Current Issues in Intestinal Microbiology*.

Kaper, James B. 1998. "Enterohemorrhagic Escherichia Coli." *Current Opinion in Microbiology*. doi: 10.1016/S1369-5274(98)80149-5.

Kaper, James B., James P. Nataro, and Harry L. T. Mobley. 2004. "Pathogenic Escherichia Coli." *Nature Reviews Microbiology*.

Kentish, S., T. J. Wooster, M. Ashokkumar, S. Balachandran, R. Mawson, and L. Simons. 2008. "The Use of Ultrasonics for Nanoemulsion Preparation." *Innovative Food Science and Emerging Technologies* 9:170–75. doi: 10.1016/j.ifset.2007.07.005.

Khanvilkar, Aparna M., Rahul C. Ranveer, and Akashya K. Sahoo. 2016. "Carrier Materials for Encapsulation of Bio-Active Components of Food." *International Journal of Pharmaceutical Sciences Review and Research*.

Kim, Kwangsok, Yen K. Luu, Charles Chang, Dufei Fang, Benjamin S. Hsiao, Benjamin Chu, and Michael Hadjiargyrou. 2004.

"Incorporation and Controlled Release of a Hydrophilic Antibiotic Using Poly(Lactide-Co-Glycolide)-Based Electrospun Nanofibrous Scaffolds." *Journal of Controlled Release*. doi: 10.1016/j.jconrel.2004.04.009.

Klieve, A. V., and T. Bauchop. 1988. "Morphological Diversity of Ruminal Bacteriophages from Sheep and Cattle." *Applied and Environmental Microbiology*.

Krasaekoopt, Wunwisa, Bhesh Bhandari, and Hilton Deeth. 2003. "Evaluation of Encapsulation Techniques of Probiotics for Yoghurt." *International Dairy Journal*.

Kumpugdee-Vollrath, M., M. Tabatabaeifar, and M. Helmis. 2014. "New Coating Materials Based on Mixtures of Shellac and Pectin for Pharmaceutical Products." *International Journal of Pharmacological and Pharmaceutical Sciencee*.

Kutter, Elizabeth M., Kyobi Skutt-Kakaria, Bob Blasdel, Ayman El-Shibiny, Anna Castano, Daniel Bryan, Andrew M. Kropinski, Andre Villegas, Hans Wolfgang Ackermann, Ana L. Toribio, Derek Pickard, Hany Anany, Todd Callaway, and Andrew D. Brabban. 2011. "Characterization of a Vil-like Phage Specific to Escherichia Coli O157:H7." *Virology Journal*. doi: 10.1186/1743-422X-8-430.

Lausecker, R., V. Badilita, U. Gleißner, and U. Wallrabe. 2016. "Introducing Natural Thermoplastic Shellac to Microfluidics: A Green Fabrication Method for Point-of-Care Devices." *Biomicrofluidics*. doi: 10.1063/1.4955062.

Lee, Hyunah, Chanmin Jeong, Kashif Ghafoor, Sungyeon Cho, and Jiyong Park. 2011. "Oral Delivery of Insulin Using Chitosan Capsules Cross-Linked with Phytic Acid." *Bio-Medical Materials and Engineering*. doi: 10.3233/BME-2011-0654.

Leick, Sabine, Maureen Kott, Patrick Degen, Stefan Henning, Tobias Päsler, Dieter Suter, and Heinz Rehage. 2011. "Mechanical Properties of Liquid-Filled Shellac Composite Capsules." *Physical Chemistry Chemical Physics*. doi: 10.1039/c0cp01803a.

Leighton, T. G., and Robert E. Apfel. 1994. " The Acoustic Bubble ." *The Journal of the Acoustical Society of America*. doi: 10.1121/1.410082.

Leong, Thomas S. H., Gregory J. O. Martin, and Muthupandian Ashokkumar. 2017. "Ultrasonic Encapsulation – A Review." *Ultrasonics Sonochemistry*.

Li, Dan, and Younan Xia. 2004. "Electrospinning of Nanofibers: Reinventing the Wheel?" *Advanced Materials* 16(14):1151–70. doi: 10.1002/adma.200400719.

Li, Ming, Olivier Rouaud, and Denis Poncelet. 2008. "Microencapsulation by Solvent Evaporation: State of the Art for Process Engineering Approaches." *International Journal of Pharmaceutics*.

Li, Xiao Yu, Li Ji Jin, Tim A. McAllister, Kim Stanford, Jing Yi Xu, Ya Nan Lu, Yu Hong Zhen, Yong Xin Sun, and Yong Ping Xu. 2007. "Chitosan-Alginate Microcapsules for Oral Delivery of Egg Yolk Immunoglobulin (IgY)." *Journal of Agricultural and Food Chemistry*. doi: 10.1021/jf062900q.

Li, Yuan, Grigori M. Sisoev, and Yulii D. Shikhmurzaev. 2018. "Spinning Disk Atomization: Theory of the Ligament Regime." *Physics of Fluids*. doi: 10.1063/1.5044429.

Lim, Franklin, and Anthony M. Sun. 1980. "Microencapsulated Islets as Bioartificial Endocrine Pancreas." *Science*. doi: 10.1126/science.6776628.

Lupo, Bryshila, Alicia Maestro, José M. Gutiérrez, and Carmen González. 2015. "Characterization of Alginate Beads with Encapsulated Cocoa Extract Toprepare Functional Food: Comparison of Two Gelation Mechanisms." *Food Hydrocolloids*. doi: 10.1016/j.foodhyd.2015.02.023.

Ma, Yin Hing, Golam S. Islam, Ying Wu, Parviz M. Sabour, James R. Chambers, Qi Wang, Shirley X. Y. Wu, and Mansel W. Griffiths. 2016. "Temporal Distribution of Encapsulated Bacteriophages during Passage through the Chick Gastrointestinal Tract." *Poultry Science*. doi: 10.3382/ps/pew260.

Madene, Atmane, Muriel Jacquot, Joël Scher, and Stéphane Desobry. 2006. "Flavour Encapsulation and Controlled Release - A Review." *International Journal of Food Science and Technology*. doi: 10.1111/j.1365-2621.2005.00980.x.

Mancini, Marco, Mauro Moresi, and Roberto Rancini. 1999. "Mechanical Properties of Alginate Gels: Empirical Characterisation." *Journal of Food Engineering*. doi: 10.1016/S0260-8774(99)00022-9.

Markowiak, Paulina, and Katarzyna Ślizewska. 2018. "The Role of Probiotics, Prebiotics and Synbiotics in Animal Nutrition." *Gut Pathogens*.

Martau, Gheorghe Adrian, Mihaela Mihai, and Dan Cristian Vodnar. 2019. "The Use of Chitosan, Alginate, and Pectin in the Biomedical and Food Sector-Biocompatibility, Bioadhesiveness, and Biodegradability." *Polymers*.

Marti-Mestres, Gilberte, and Françoise Nielloud. 2002. "Emulsions in Health Care Applications—An Overview." *Journal of Dispersion Science and Technology* 23(1–3):419–39. doi: 10.1080/01932690208984214.

Martinez, L., F. Agnely, R. Bettini, M. Besnard, P. Colombo, and G. Couarraze. 2004. "Preparation and Characterization of Chitosan Based Micro Networks: Transposition to a Prilling Process." *Journal of Applied Polymer Science*. doi: 10.1002/app.20785.

Mascarenhas, W. J., H. U. Akay, and M. J. Pikal. 1997. "A Computational Model for Finite Element Analysis of the Freeze-Drying Process." *Computer Methods in Applied Mechanics and Engineering*. doi: 10.1016/S0045-7825(96)00078-3.

Messaoud, Ghazi Ben, Laura Sánchez-González, Laurent Probst, Carole Jeandel, Elmira Arab-Tehrany, and Stéphane Desobry. 2016. "Physico-Chemical Properties of Alginate/Shellac Aqueous-Core Capsules: Influence of Membrane Architecture on Riboflavin Release." *Carbohydrate Polymers* 144:428–37. doi: 10.1016/j.carbpol.2016.02.081.

Miladi, K., S. Sfar, H. Fessi, and A. Elaissari. 2013. "Drug Carriers in Osteoporosis: Preparation, Drug Encapsulation and Applications." *International Journal of Pharmaceutics*.

Miller, Megge, and Amy Cawthorne. 2017. "Strengthening Surveillance of and Response to Foodborne Diseases: A Practical Manual. Introductory Module." Pp. 2–7 in *Using indicator- and event-based surveillance to detect foodborne events*, edited by M. Miller and A. Cawthorne. Geneva: World Health Organization.

Mirtič, Janja, Tomaž Rijavec, Špela Zupančič, Alenka Zvonar Pobirk, Aleš Lapanje, and Julijana Kristl. 2018. "Development of Probiotic-Loaded Microcapsules for Local Delivery: Physical Properties, Cell Release and Growth." *European Journal of Pharmaceutical Sciences*. doi: 10.1016/j.ejps.2018.05.022.

Mirzataheria, Mojgan, Amir Hossein Mahtabanib, and Mehrnaz Lotfalieic. 2014. "Emulsion Polymerization and Encapsulation of Micro and Nanoparticles within Polymer Droplets." *Jordan Journal of Chemistry*. doi: 10.12816/0025977.

Moghtader, Farzaneh, Sinan Eğri, and Erhan Piskin. 2017. "Phages in Modified Alginate Beads." *Artificial Cells, Nanomedicine and Biotechnology*. doi: 10.3109/21691401.2016.1153485.

Mohammadi, Somaieh, Adam Harvey, and Kamelia V. K. Boodhoo. 2014. "Synthesis of TiO2 Nanoparticles in a Spinning Disc Reactor." *Chemical Engineering Journal*. doi: 10.1016/j.cej.2014.07.042.

Morales, Eduardo, Mónica Rubilar, César Burgos-Díaz, Francisca Acevedo, Manfred Penning, and Carolina Shene. 2017. "Alginate/Shellac Beads Developed by External Gelation as a Highly Efficient Model System for Oil Encapsulation with

Intestinal Delivery." *Food Hydrocolloids* 70:321–28. doi: 10.1016/j.foodhyd.2017.04.012.

Nagata, Rie, Yo Han Kim, Akira Ohkubo, Shiro Kushibiki, Toshihiro Ichijo, and Shigeru Sato. 2018. "Effects of Repeated Subacute Ruminal Acidosis Challenges on the Adaptation of the Rumen Bacterial Community in Holstein Bulls." *Journal of Dairy Science*. doi: 10.3168/jds.2017-13859.

Nair, Lakshmi S., Subhabrata Bhattacharyya, and Cato T. Laurencin. 2004. "Development of Novel Tissue Engineering Scaffolds via Electrospinning." *Expert Opinion on Biological Therapy*.

Nguon, Olivier, François Lagugné-Labarthet, Frank A. Brandys, Jian Li, and Elizabeth R. Gillies. 2018. "Microencapsulation by in Situ Polymerization of Amino Resins." *Polymer Reviews*.

Nobrega, Franklin L., Marnix Vlot, Patrick A. de Jonge, Lisa L. Dreesens, Hubertus J. E. Beaumont, Rob Lavigne, Bas E. Dutilh, and Stan J. J. Brouns. 2018. "Targeting Mechanisms of Tailed Bacteriophages." *Nature Reviews Microbiology*.

Orpin, C. G., and E. A. Munn. 1974. "The Occurrence of Bacteriophages in the Rumen and Their Influence on Rumen Bacterial Populations." *Experientia*. doi: 10.1007/BF01938983.

Outokesh, Mohammad, Hitoshi Mimura, Yuichi Niibori, and Kouichi Tanaka. 2006. "Preparation of Stable Alginate Microcapsules Coated with Chitosan or Polyethyleneimine for Extraction of Heavy Metal Ions." *Journal of Microencapsulation*. doi: 10.1080/02652040500435188.

Ozkan, Gulay, Paola Franco, Iolanda De Marco, Jianbo Xiao, and Esra Capanoglu. 2019. "A Review of Microencapsulation Methods for Food Antioxidants: Principles, Advantages, Drawbacks and Applications." *Food Chemistry*. doi: 10.1016/j.foodchem.2018.07.205.

Pandey, Abhijeet P., Saurabh S. Singh, Ganesh B. Patil, Pravin O. Patil, Chetan J. Bhavsar, and Prashant K. Deshmukh. 2015. "Sonication-Assisted Drug Encapsulation in Layer-by-Layer Self-Assembled Gelatin-Poly (Styrenesulfonate) Polyelectrolyte Nanocapsules: Process Optimization." *Artificial Cells, Nanomedicine and Biotechnology* 43(6):413–24. doi: 10.3109/21691401.2014.898646.

Partridge, L., D. C. Y. Wong, M. J. H. Simmons, E. I. Părău, and S. P. Decent. 2005. "Experimental and Theoretical Description of the Break-up of Curved Liquid Jets in the Prilling Process." *Chemical Engineering Research and Design*. doi: 10.1205/cherd.05090.

Patel, Ashok R., Caroline Remijn, Ana Isabel Mulet Cabero, Patricia C. M. Heussen, Jack W. M. Seije. Ten Hoorn, and Krassimir P. Velikov. 2013. "Novel All-Natural Microcapsules from Gelatin and Shellac for Biorelated Applications." *Advanced Functional Materials*. doi: 10.1002/adfm.201300320.

Patel, Ashok R., Domien Schatteman, Winnok H. De Vos, and Koen Dewettinck. 2013. "Shellac as a Natural Material to Structure a Liquid Oil-Based Thermo Reversible Soft Matter System." *RSC Advances*. doi: 10.1039/c3ra40934a.

Pawar, Siddhesh N., and Kevin J. Edgar. 2012. "Alginate Derivatization: A Review of Chemistry, Properties and Applications." *Biomaterials*.

Peniche, C., I. Howland, O. Carrillo, C. Zaldívar, and W. Argüelles-Monal. 2004. "Formation and Stability of Shark Liver Oil Loaded Chitosan/Calcium Alginate Capsules." *Food Hydrocolloids*. doi: 10.1016/j.foodhyd.2004.02.001.

Penning, Manfred. 1996. "Aqueous Shellac Solutions for Controlled Release Coatings." Pp. 146–54 in *Chemical aspects of drug delivery systems*.

Perignon, Carole, Gisèle Ongmayeb, Ronald Neufeld, Yves Frere, and Denis Poncelet. 2015. "Microencapsulation by Interfacial

Polymerisation: Membrane Formation and Structure." *Journal of Microencapsulation*. doi: 10.3109/02652048.2014.950711.

Phan The, D., F. Debeaufort, D. Luu, and A. Voilley. 2008. "Moisture Barrier, Wetting and Mechanical Properties of Shellac/Agar or Shellac/Cassava Starch Bilayer Bio-Membrane for Food Applications." *Journal of Membrane Science*. doi: 10.1016/j.memsci.2008.07.052.

Pinto, Graça, Carina Almeida, and Joana Azeredo. 2020. "Bacteriophages to Control Shiga Toxin-Producing E. Coli–Safety and Regulatory Challenges." *Critical Reviews in Biotechnology*.

Pisani, Emilia, Elias Fattal, Juliane Paris, Catherine Ringard, Véronique Rosilio, and Nicolas Tsapis. 2008. "Surfactant Dependent Morphology of Polymeric Capsules of Perfluorooctyl Bromide: Influence of Polymer Adsorption at the Dichloromethane-Water Interface." *Journal of Colloid and Interface Science*. doi: 10.1016/j.jcis.2008.07.013.

Pitigraisorn, Panithan, Kantaphong Srichaisupakit, Nutnicha Wongpadungkiat, and Saowakon Wongsasulak. 2017. "Encapsulation of Lactobacillus Acidophilus in Moist-Heat-Resistant Multilayered Microcapsules." *Journal of Food Engineering*. doi: 10.1016/j.jfoodeng.2016.07.022.

Plattner, Michel, Mikhail M. Shneider, Nikolay P. Arbatsky, Alexander S. Shashkov, Alexander O. Chizhov, Sergey Nazarov, Nikolai S. Prokhorov, Nicholas M. I. Taylor, Sergey A. Buth, Michela Gambino, Yilmaz Emre Gencay, Lone Brøndsted, Elizabeth M. Kutter, Yuriy A. Knirel, and Petr G. Leiman. 2019. "Structure and Function of the Branched Receptor-Binding Complex of Bacteriophage CBA120." *Journal of Molecular Biology*. doi: 10.1016/j.jmb.2019.07.022.

Pollet, Bruno G. 2010. "The Use of Ultrasound for the Fabrication of Fuel Cell Materials." *International Journal of Hydrogen Energy*.

Preneuf, Flore de, and Xenia Zia Morales. 2018. "Food-Borne Illnesses Cost US$ 110 Billion Per Year in Low- and Middle-Income Countries." *The World Bank*. Retrieved February 12, 2021 (https://www.worldbank.org/en/news/press-release/2018/10/23/food-borne-illnesses-cost-us-110-billion-per-year-in-low-and-middle-income-countries).

Pu, Jianing, J. David Bankston, and Subramaniam Sathivel. 2011. "Developing Microencapsulated Flaxseed Oil Containing Shrimp (Litopenaeus Setiferus) Astaxanthin Using a Pilot Scale Spray Dryer." *Biosystems Engineering*. doi: 10.1016/j.biosystemseng.2010.11.005.

Qussi, Basel, and Wolfgang G. Suess. 2005. "Investigation of the Effect of Various Shellac Coating Compositions Containing Different Water-Soluble Polymers on in Vitro Drug Release." *Drug Development and Industrial Pharmacy*. doi: 10.1081/DDC-44226.

Radzikowski, Daniel. 2017. "Effect of Probiotics, Prebiotics and Synbiotics on the Productivity and Health of Dairy Cows and Calves." *WSN*.

Rattes, Alysson Leandro Ribeiro, and Wanderley Pereira Oliveira. 2007. "Spray Drying Conditions and Encapsulating Composition Effects on Formation and Properties of Sodium Diclofenac Microparticles." *Powder Technology*. doi: 10.1016/j.powtec.2006.09.007.

Raya, Raul R., Rebecca A. Oot, Ben Moore-Maley, Serena Wieland, Todd R. Callaway, Elizabeth Martin Kutter, and Andrew D. Brabban. 2011. " Naturally Resident and Exogenously Applied T4-like and T5-like Bacteriophages Can Reduce Escherichia Coli O157 ." *Bacteriophage*. doi: 10.4161/bact.1.1.14175.

Raya, Raul R., Peter Varey, Rebecca A. Oot, Michael R. Dyen, Todd R. Callaway, Tom S. Edrington, Elizabeth M. Kutter, and

Andrew D. Brabban. 2006. "Isolation and Characterization of a New T-Even Bacteriophage, CEV1, and Determination of Its Potential to Reduce Escherichia Coli O157:H7 Levels in Sheep." *Applied and Environmental Microbiology*. doi: 10.1128/AEM.03011-05.

Ren, Lili, Xiaoxia Yan, Jiang Zhou, Jin Tong, and Xingguang Su. 2017. "Influence of Chitosan Concentration on Mechanical and Barrier Properties of Corn Starch/Chitosan Films." *International Journal of Biological Macromolecules*. doi: 10.1016/j.ijbiomac.2017.02.008.

Rinaudo, Marguerite. 2008. "Main Properties and Current Applications of Some Polysaccharides as Biomaterials." *Polymer International* 397–430.

Risch, Sara J. 1995. "Encapsulation: Overview of Uses and Techniques." (7):2–7. doi: 10.1021/bk-1995-0590.ch001.

Rivas, Lucia, Brid Coffey, Olivia McAuliffe, Mary J. McDonnell, Catherine M. Burgess, Aidan Coffey, R. Paul Ross, and Geraldine Duffy. 2010. "In Vivo and Ex Vivo Evaluations of Bacteriophages E11/2 and E4/1c for Use in the Control of Escherichia Coli O157:H7." *Applied and Environmental Microbiology*. doi: 10.1128/AEM.01530-10.

Roberfroid, Marcel. 2018. "Prebiotics: The Concept Revisited." *The Journal of Nutrition*. doi: 10.1093/jn/137.3.830s.

Rodríguez-Dorado, Rosalia, Mariana Landín, Ayça Altai, Paola Russo, Rita P. Aquino, and Pasquale Del Gaudio. 2018. "A Novel Method for the Production of Core-Shell Microparticles by Inverse Gelation Optimized with Artificial Intelligent Tools." *International Journal of Pharmaceutics*. doi: 10.1016/j.ijpharm.2018.01.023.

da Rosa, Cleonice Gonçalves, Caroline Dellinghausen Borges, Rui Carlos Zambiazi, Michael Ramos Nunes, Edilson Valmir Benvenutti, Suzane Rickes da Luz, Roseane Farias D'Avila, and Josiane Kuhn Rutz. 2013. "Microencapsulation of Gallic Acid in Chitosan, β-Cyclodextrin and Xanthan." *Industrial Crops and Products*. doi: 10.1016/j.indcrop.2012.12.053.

Rozema, Erica A., Tyler P. Stephens, Susan J. Bach, Erasmus K. Okine, Roger P. Johnson, Klim Stanford, and Tim A. McAllister. 2009. "Oral and Rectal Administration of Bacteriophages for Control of Escherichia Coli O157:H7 in Feedlot Cattle." *Journal of Food Protection* 72(2):241–50. doi: 10.4315/0362-028x-72.2.241.

Schafroth, Nina, Cordin Arpagaus, Umesh Y. Jadhav, Sushil Makne, and Dennis Douroumis. 2012. "Nano and Microparticle Engineering of Water Insoluble Drugs Using a Novel Spray-Drying Process." *Colloids and Surfaces B: Biointerfaces*. doi: 10.1016/j.colsurfb.2011.09.038.

Schell, Daniel, and Christopher Beermann. 2014. "Fluidized Bed Microencapsulation of Lactobacillus Reuteri with Sweet Whey and Shellac for Improved Acid Resistance and In-Vitro Gastro-Intestinal Survival." *Food Research International*. doi: 10.1016/j.foodres.2014.03.016.

Schuch, Anna, Philipp Deiters, Julius Henne, Karsten Köhler, and Heike P. Schuchmann. 2013. "Production of W/O/W (Water-in-Oil-in-Water) Multiple Emulsions: Droplet Breakup and Release of Water." *Journal of Colloid and Interface Science*. doi: 10.1016/j.jcis.2013.03.066.

Serp, D., E. Cantana, C. Heinzen, U. Von Stockar, and I. W. Marison. 2000. "Characterization of an Encapsulation Device for the Production of Monodisperse Alginate Beads for Cell Immobilization." *Biotechnology and Bioengineering*.

Shahidi, Fereidoon, Janak Kamil Vidana Arachchi, and You Jin Jeon. 1999. "Food Applications of Chitin and Chitosans." *Trends in Food Science and Technology*.

Sheng, Haiqing, Hannah J. Knecht, Indira T. Kudva, and Carolyn J. Hovde. 2006. "Application of Bacteriophages to Control Intestinal Escherichia Coli O157:H7 Levels in Ruminants." *Applied and Environmental Microbiology*. doi:

10.1128/AEM.00099-06.

Shilpa, Anu, S. S. Agrawal, and Alok R. Ray. 2003. "Controlled Delivery of Drugs from Alginate Matrix." *Journal of Macromolecular Science - Polymer Reviews*.

Sillankorva, Sanna M., Hugo Oliveira, and Joana Azeredo. 2012. "Bacteriophages and Their Role in Food Safety." *International Journal of Microbiology* 2012:1–13. doi: 10.1155/2012/863945.

Silva, Marluci P., Fabrício L. Tulini, Evandro Martins, Manfred Penning, Carmen S. Fávaro-Trindade, and Denis Poncelet. 2018. "Comparison of Extrusion and Co-Extrusion Encapsulation Techniques to Protect Lactobacillus Acidophilus LA3 in Simulated Gastrointestinal Fluids." *LWT - Food Science and Technology*. doi: 10.1016/j.lwt.2017.11.008.

Silva, Marluci P., Fabricio L. Tulini, Marcela M. Ribas, Manfred Penning, Carmen S. Fávaro-Trindade, and Denis Poncelet. 2016. "Microcapsules Loaded with the Probiotic Lactobacillus Paracasei BGP-1 Produced by Co-Extrusion Technology Using Alginate/Shellac as Wall Material: Characterization and Evaluation of Drying Processes." *Food Research International*. doi: 10.1016/j.foodres.2016.09.008.

Smith, H. W., M. B. Huggins, and K. M. Shaw. 2009. "Factors Influencing the Survival and Multiplication of Bacteriophages in Calves and in Their Environment." *Microbiology*. doi: 10.1099/00221287-133-5-1127.

Solans, Conxita, Daniel Morales, and Maria Homs. 2016. "Spontaneous Emulsification." *Current Opinion in Colloid and Interface Science*.

Soto, María José, Julio Retamales, Humberto Palza, and Roberto Bastías. 2018. "Encapsulation of Specific Salmonella Enteritidis Phage F3αSE on Alginate-Spheres as a Method for Protection and Dosification." *Electronic Journal of Biotechnology*. doi: 10.1016/j.ejbt.2017.11.006.

Specht, Felix, Marianne Saugestad, Tor Waaler, and Bernd W. Müller. 1999. "The Application of Shellac as an Acidic Polymer for Enteric Coating." *Pharmaceutical Technology*.

Stanford, K., T. A. McAllister, Y. D. Niu, T. P. Stephens, A. Mazzocco, T. E. Waddell, and R. P. Johnson. 2010. "Oral Delivery Systems for Encapsulated Bacteriophages Targeted at Escherichia Coli O157:H7 in Feedlot Cattle." *Journal of Food Protection* 73(7):1304–12. doi: 10.4315/0362-028x-73.7.1304.

Stroeve, Pieter, and Padma Prabodh Varanasi. 1982. "Transport Processes in Liquid Membranes: Double Emulsion Separation Systems." *Separation & Purification Reviews* 11(1):29–69. doi: 10.1080/03602548208066016.

Stroeve, Pieter, and Padma Prabodh Varanasi. 1984. "An Experimental Study on Double Emulsion Drop Breakup in Uniform Shear Flow." *Journal of Colloid And Interface Science* 99(2):360–73. doi: 10.1016/0021-9797(84)90123-1.

Stummer, Stefanie, Sharareh Salar-Behzadi, Frank M. Unger, Silvester Oelzant, Manfred Penning, and Helmut Viernstein. 2010. "Application of Shellac for the Development of Probiotic Formulations." *Food Research International*. doi: 10.1016/j.foodres.2010.03.017.

Sun, Yongqi, Zuotai Zhang, Lili Liu, and Xidong Wang. 2015. "Heat Recovery from High Temperature Slags: A Review of Chemical Methods." *Energies*.

Suslick, Kenneth S., and Gareth J. Price. 1999. *Applications of Ultrasound to Materials Chemistry*.

Taj, M. K., J. X. Ling, L. L. Bing, Z. Qi, I. Taj, T. M. Hassani, Z. Samreen, and W. Yunlin. 2014. "Effect of Dilution, Temperature and PH on the Lysis Activity of T4 Phage against E.Coli BL21." *Journal of Animal and Plant Sciences*.

Tan, Lay Hui, Lai Wah Chan, and Paul Wan Sia Heng. 2009. "Alginate/Starch Composites as Wall Material to Achieve

Microencapsulation with High Oil Loading." *Journal of Microencapsulation*. doi: 10.1080/02652040802305519.

Tozaki, Hideyuki, Tomokazu Odoriba, Naoki Okada, Takuya Fujita, Akira Terabe, Tsutomu Suzuki, Susumu Okabe, Shozo Muranishi, and Akira Yamamoto. 2002. "Chitosan Capsules for Colon-Specific Drug Delivery: Enhanced Localization of 5-Aminosalicylic Acid in the Large Intestine Accelerates Healing of TNBS-Induced Colitis in Rats." *Journal of Controlled Release*. doi: 10.1016/S0168-3659(02)00084-6.

Uyeno, Yutaka, Suguru Shigemori, and Takeshi Shimosato. 2015. "Effect of Probiotics/Prebiotics on Cattle Health and Productivity." *Microbes and EnvironmentsMicrobes and Environments* 30(2):126–32. doi: 10.1264/jsme2.me14176.

De Vaux, Albane, Mark Morrison, and Robert W. Hutkins. 2002. "Displacement of Escherichia Coli O157:H7 from Rumen Medium Containing Prebiotic Sugars." *Applied and Environmental Microbiology*. doi: 10.1128/AEM.68.2.519-524.2002.

Verreck, Geert, Iksoo Chun, Joel Rosenblatt, Jef Peeters, Alex Van Dijck, Jurgen Mensch, Marc Noppe, and Marcus E. Brewster. 2003. "Incorporation of Drugs in an Amorphous State into Electrospun Nanofibers Composed of a Water-Insoluble, Nonbiodegradable Polymer." *Journal of Controlled Release*. doi: 10.1016/S0168-3659(03)00342-0.

Vinner, Gurinder K., Zahra Rezaie-Yazdi, Miika Leppanen, Andrew G. F. Stapley, Mark C. Leaper, and Danish J. Malik. 2019. "Microencapsulation of Salmonella-Specific Bacteriophage Felix O1 Using Spray-Drying in a Ph-Responsive Formulation and Direct Compression Tableting of Powders into a Solid Oral Dosage Form." *Pharmaceuticals*. doi: 10.3390/ph12010043.

de Vos, Paul, Marijke M. Faas, Milica Spasojevic, and Jan Sikkema. 2010. "Encapsulation for Preservation of Functionality and Targeted Delivery of Bioactive Food Components." *International Dairy Journal*.

Walker, W. Allan, and Linda C. Duffy. 1998. "Diet and Bacterial Colonization: Role of Probiotics and Prebiotics." *Journal of Nutritional Biochemistry*.

Wang, Qi Zhao, Xi Guang Chen, Nan Liu, Shi Xi Wang, Cheng Sheng Liu, Xiang Hong Meng, and Chen Guang Liu. 2006. "Protonation Constants of Chitosan with Different Molecular Weight and Degree of Deacetylation." *Carbohydrate Polymers*. doi: 10.1016/j.carbpol.2006.01.001.

Wen, Peng, Min Hua Zong, Robert J. Linhardt, Kun Feng, and Hong Wu. 2017. "Electrospinning: A Novel Nano-Encapsulation Approach for Bioactive Compounds." *Trends in Food Science and Technology*.

Whelehan, Micheal, and Ian W. Marison. 2011. "Microencapsulation Using Vibrating Technology." *Journal of Microencapsulation*.

WHO. 2015. *WHO Estimates of the Global Burden of Foodborne Diseases*. Switzerland.

WHO. 2020. "Zoonoses and the Environment." *World Health Organization*. Retrieved December 11, 2020 (https://www.who.int/foodsafety/areas_work/zoonose/en/).

Witte, Wolfgang. 2000. "Selective Pressure by Antibiotic Use in Livestock." *International Journal of Antimicrobial Agents*. doi: 10.1016/s0924-8579(00)00301-0.

Woodward, Martin J., Angus Best, Katherine A. Sprigings, Geoffrey R. Pearson, Andrew M. Skuse, Andrew Wales, Christine M. Hayes, John M. Roe, J. Chris Low, and Roberto M. La Ragione. 2003. "Non-Toxigenic Escherichia Coli O157:H7 Strain NCTC12900 Causes Attaching-Effacing Lesions and Eae-Dependent Persistence in Weaned Sheep." *International Journal of Medical Microbiology*. doi: 10.1078/1438-4221-00264.

Wu, Chunhua, Yang Zhu, Tiantian Wu, Lin Wang, Yi Yuan, Jicheng Chen, Yaqin Hu, and Jie Pang. 2019. "Enhanced Functional Properties of Biopolymer Film Incorporated with Curcurmin-Loaded Mesoporous Silica Nanoparticles for Food Packaging." *Food Chemistry*. doi: 10.1016/j.foodchem.2019.03.010.

Wu, H., Yanlin Lei, J. Lu, Rui Zhu, Di Xiao, Chun Jiao, Rui Xia, Zhiqing Zhang, Guanghui Shen, Yuntao Liu, Shanshan Li, and Meiliang Li. 2019. "Effect of Citric Acid Induced Crosslinking on the Structure and Properties of Potato Starch/Chitosan Composite Films." *Food Hydrocolloids*. doi: 10.1016/j.foodhyd.2019.105208.

Xue, Jing, and Zhibing Zhang. 2008. "Preparation and Characterization of Calcium-Shellac Spheres as a Carrier of Carbamide Peroxide." *Journal of Microencapsulation*. doi: 10.1080/02652040802049638.

Xue, Jing, and Zhibing Zhang. 2009. "Physical, Structural, and Mechanical Characterization of Calcium-Shellac Microspheres as a Carrier of Carbamide Peroxide." *Journal of Applied Polymer Science*. doi: 10.1002/app.30090.

Yap, Moh Lan, and Michael G. Rossmann. 2014. "Structure and Function of Bacteriophage T4." *Future Microbiology* 9(12):1319–37. doi: 10.2217/fmb.14.91.

Yeo, Yoon, Namjin Baek, and Kinam Park. 2001. "Microencapsulation Methods for Delivery of Protein Drugs." *Biotechnology and Bioprocess Engineering*. doi: 10.1007/BF02931982.

Yongsheng, Ma, Jennifer C. Pacan, Qi Wang, Yongping Xu, Xiaoqing Huang, Anton Korenevsky, and Parviz M. Sabour. 2008. "Microencapsulation of Bacteriophage Felix O1 into Chitosan-Alginate Microspheres for Oral Delivery." *Applied and Environmental Microbiology*. doi: 10.1128/AEM.00246-08.

Young, S. L., X. Sarda, and M. Rosenberg. 1993. "Microencapsulating Properties of Whey Proteins. 2. Combination of Whey Proteins with Carbohydrates." *Journal of Dairy Science*. doi: 10.3168/jds.S0022-0302(93)77626-2.

Yuan, Yi, Xia Zhang, Ziran Pan, Qingrong Xue, Yongtai Wu, Yuting Li, Bing Li, and Lin Li. 2021. "Improving the Properties of Chitosan Films by Incorporating Shellac Nanoparticles." *Food Hydrocolloids*. doi: 10.1016/j.foodhyd.2020.106164.

Zhang, Hui, Hong Wang, Xun Zhu, Yong Jun Qiu, Kai Li, Rong Chen, and Qiang Liao. 2013. "A Review of Waste Heat Recovery Technologies towards Molten Slag in Steel Industry." *Applied Energy*. doi: 10.1016/j.apenergy.2013.02.019.

Zhang, Yanzhong, Teck Lim Chwee, Seeram Ramakrishna, and Zheng Ming Huang. 2005. "Recent Development of Polymer Nanofibers for Biomedical and Biotechnological Applications." *Journal of Materials Science: Materials in Medicine*.

Zhao, Chenxu, Guowen Liu, Xiaobing Li, Yuan Guan, Yazhou Wang, Xue Yuan, Guoquan Sun, Zhe Wang, and Xinwei Li. 2018. "Inflammatory Mechanism of Rumenitis in Dairy Cows with Subacute Ruminal Acidosis." *BMC Veterinary Research*. doi: 10.1186/s12917-018-1463-7.

Zhao, Qing Sheng, Qiu Xia Ji, Xiao Jie Cheng, Gang Zheng Sun, Chao Ran, Bing Zhao, and Xi Guang Chen. 2010. "Preparation of Alginate Coated Chitosan Hydrogel Beads by Thermosensitive Internal Gelation Technique." *Journal of Sol-Gel Science and Technology*. doi: 10.1007/s10971-010-2187-8.

Zuidam, Nicolaas Jan, and Eyal Shimoni. 2010. "Overview of Microencapsulates for Use in Food Products or Processes and Methods to Make Them." in *Encapsulation Technologies for Active Food Ingredients and Food Processing*.

Chapter 7: Annexes

Chapter 7: Annexes

7.1 Annex 1 - BUCHI Encapsulator model B-395 Pro

Figure 30: *The standard setup of the BUCHI Encapsulator model B-395 Pro.*

(The presence of the reaction vessel enables the operator to perform the encapsulation process under fully sterile conditions.)

Figure 31: *Internal (a) and external (b) nozzle settings, SN (c), CN (d), FVN (e), and pulsation chambers of the BUCHI Encapsulator model B-395 Pro.*

7.2 Annex 2 - Optimization parameters

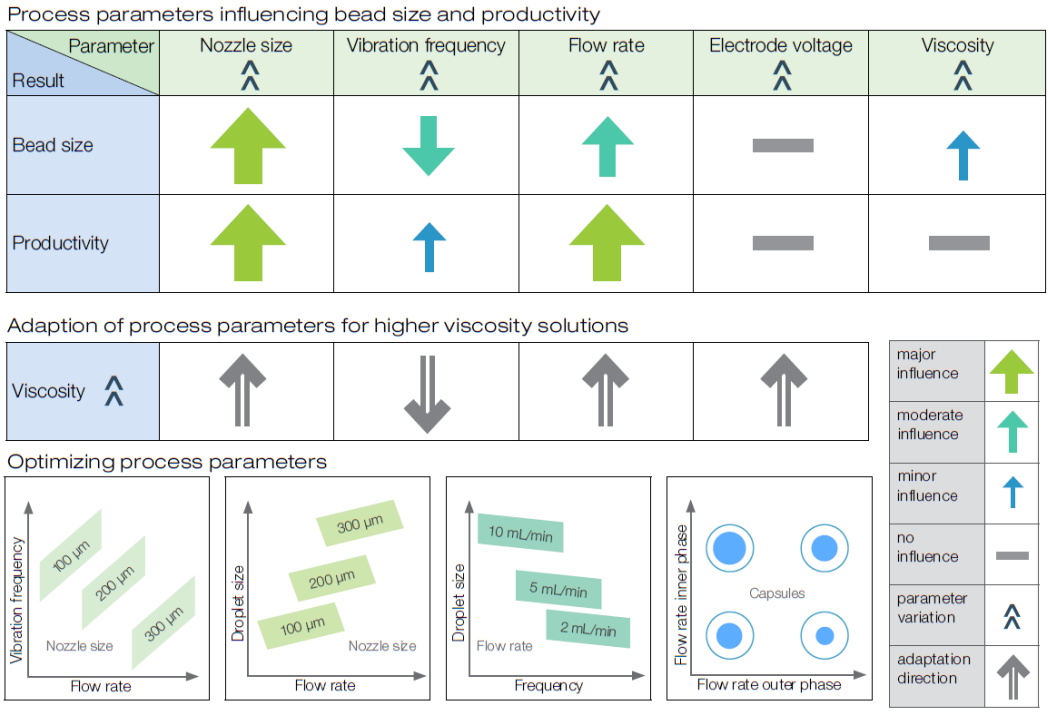


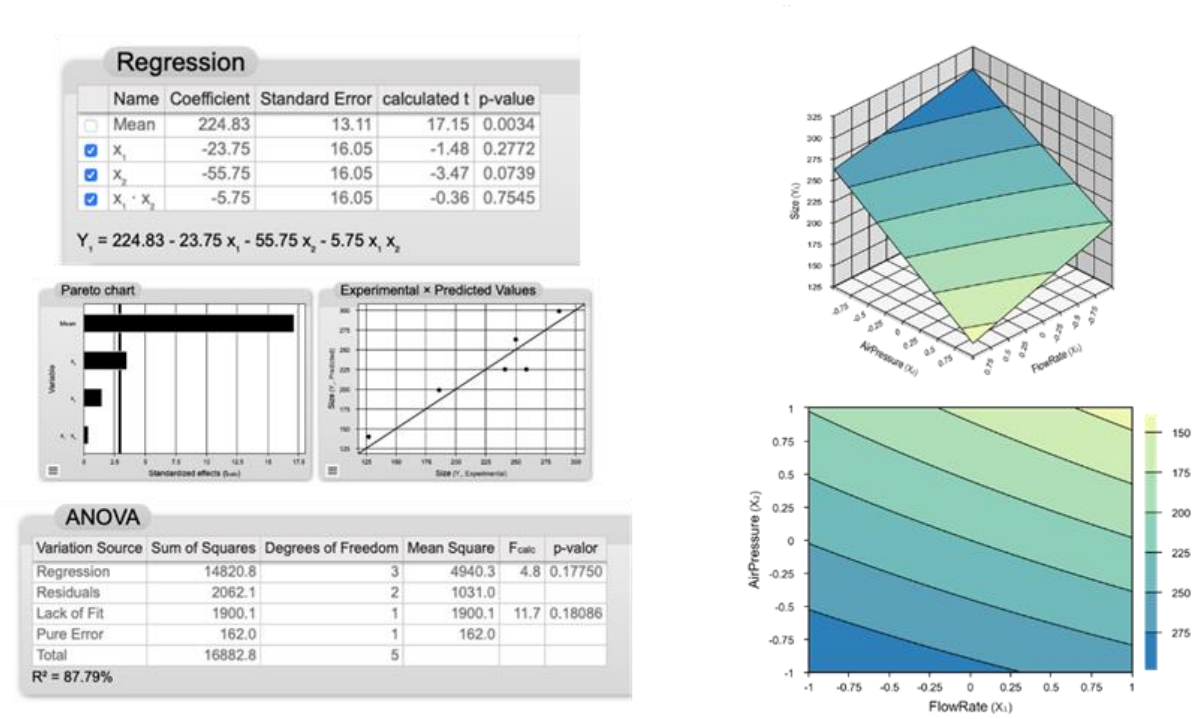
Figure 32: *Relation between the encapsulator settings and their influence on the MPs productivity. Source: "Buchi Encapsulator B-390/B-395 Pro -Laboratory Guide".*

Table 9: *Parameter range to produce Ca-alginate beads (in the sizes ranges outlined) when using the single nozzle system on the Encapsulator B-390 and B-395 Pro. Source: "Buchi Encapsulator B-390/B-395 Pro -Laboratory Guide".*

Nozzle (µm)	Flow rate range (ml/min)* (Production)	Air pressure (bar)	Optimal frequency range (Hz)**	Amplitude	Size range of produced beads (µm)
80	1.1	0.5 - 0.7	1300 - 3000	1 - 4	120 - 200
120	1.5 - 1.8	0.5 - 0.7	1000 - 2500	1 - 4	200 - 300
150	2.3 - 2.8	0.4 - 0.6	800 - 1800	1 - 3	260 - 350
200	3.5 - 4.5	0.4 - 0.6	600 - 1200	1 - 3	350 - 450
300	6.0 - 8.0	0.3 - 0.5	400 - 800	1 - 3	550 - 700
450	11 - 15	0.3 - 0.5	200 - 500	1 - 4	700 - 1150
750	19 - 25	0.3 - 0.5	40 - 300	6-9	1150 - 1800
1000	30 - 40	0.3 - 0.6	40 - 220	6-9	1600 - 2400

* This flow rate value can be obtained using the high precision syringe pump which comes separately or is mounted onto the Encapsulator B-395 Pro.**Frequency range was determined when using the 2% low viscosity grade alginate solution for the 750 and 1000 µm nozzle sizes, 1.5% alginate solution for 150 – 450 µm nozzle sizes and 1.2% alginate solution for the 80 and 120 µm nozzle sizes.

7.3 Annex 3 – Analysis of the Factorial Desing for optimization of MPs produced with FVN



Regression

	Name	Coefficient	Standard Error	calculated t	p-value
☐	Mean	224.83	13.11	17.15	0.0034
☑	x_1	-23.75	16.05	-1.48	0.2772
☑	x_2	-55.75	16.05	-3.47	0.0739
☑	$x_1 \cdot x_2$	-5.75	16.05	-0.36	0.7545

$Y_1 = 224.83 - 23.75\ x_1 - 55.75\ x_2 - 5.75\ x_1\ x_2$

ANOVA

Variation Source	Sum of Squares	Degrees of Freedom	Mean Square	F_{calc}	p-valor
Regression	14820.8	3	4940.3	4.8	0.17750
Residuals	2062.1	2	1031.0		
Lack of Fit	1900.1	1	1900.1	11.7	0.18086
Pure Error	162.0	1	162.0		
Total	16882.8	5			

$R^2 = 87.79\%$

Figure 33: *Initial Factorial Design analysis performed for the FVN optimization process as described in Section 3.3.1.4*

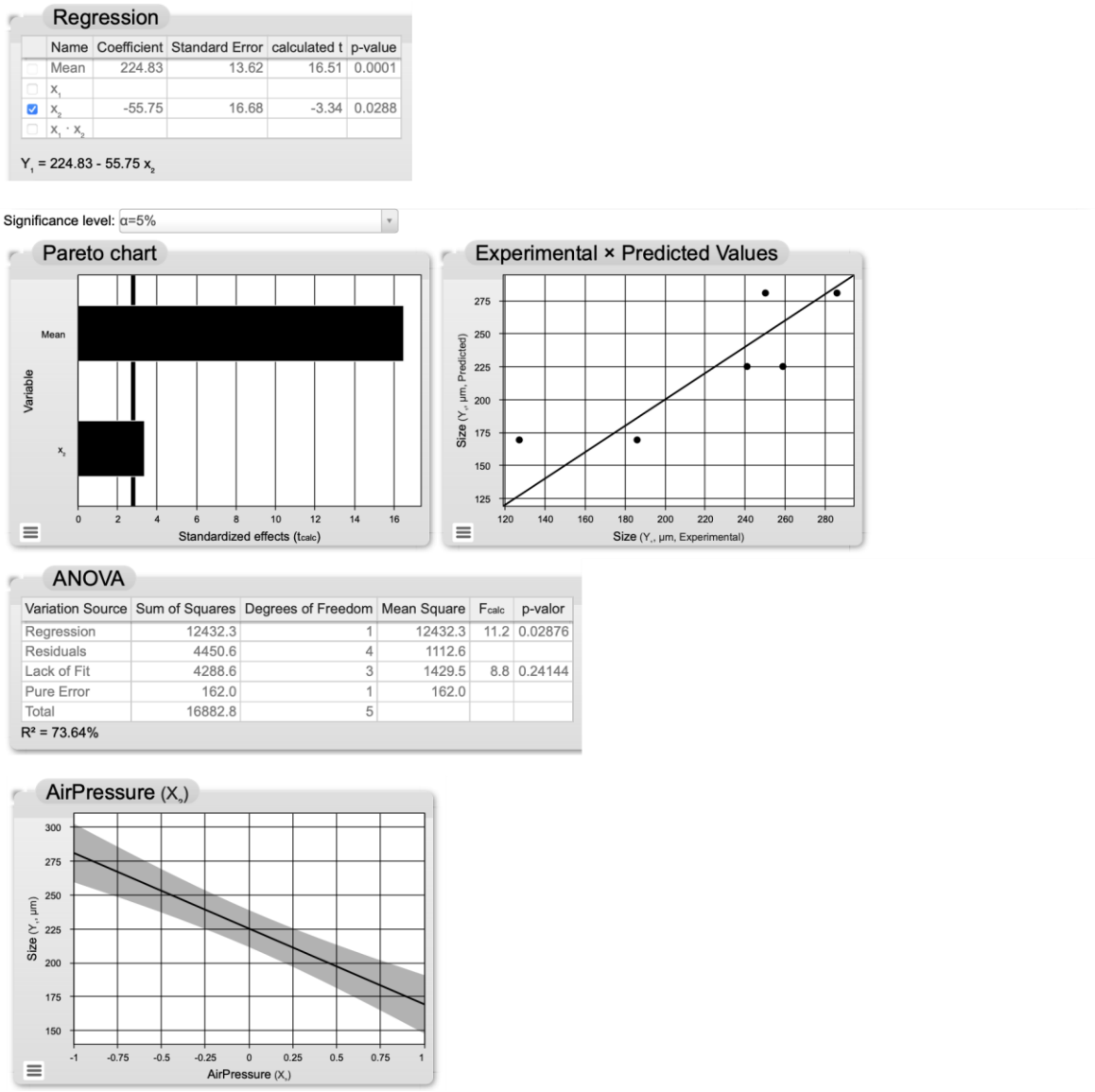


Figure 34: *Analysis of the Factorial Design after removing non-significant parameters (p-value>0.05 in the regression) for the FVN optimization process as described in Section 4.3.1.4.*

7.4 Annex 4 - Fluoresbrite® YG Microspheres calibration curves

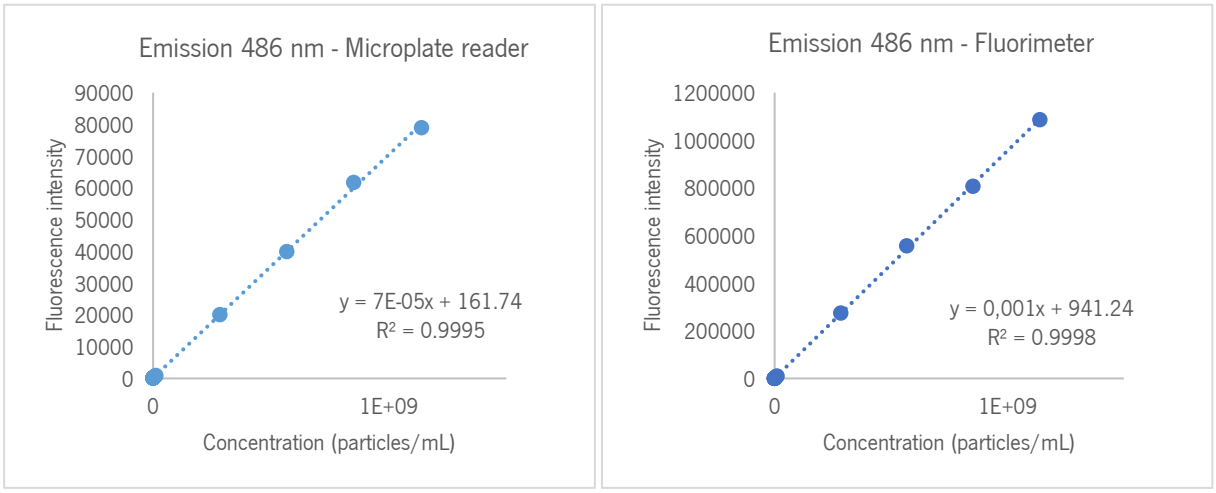


Figure 35: *Calibration curves (fluorescence intensity in order of particles concentration) for the 0.20 µm fluorescent nano-spheres using the microplate reader and the fluorimeter*

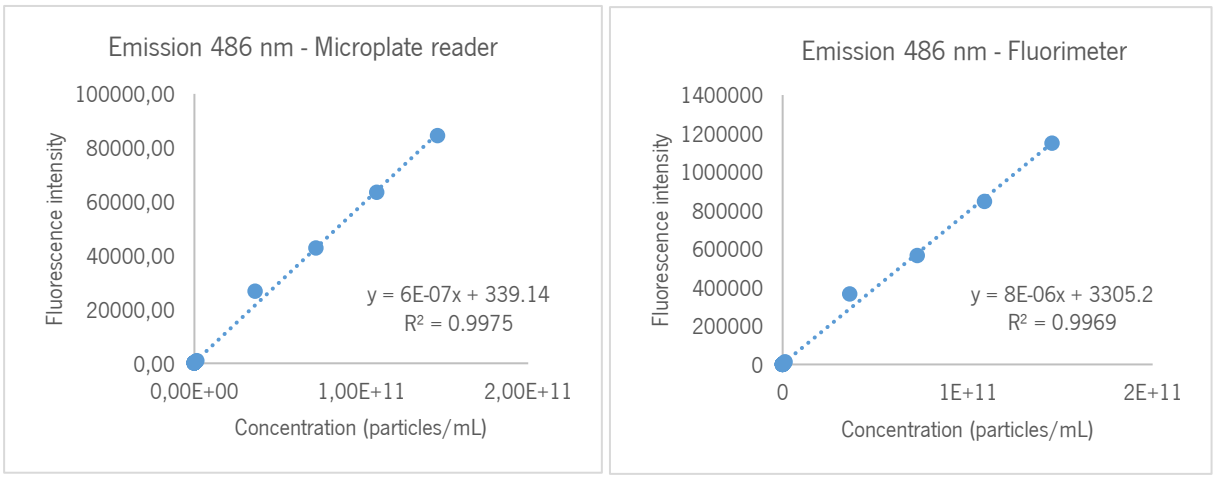


Figure 36: *Calibration curves (fluorescence intensity in order of particles concentration) for the 0.05 µm fluorescent nano-spheres using the microplate reader and the fluorimeter*