UNIVERSITÉ DU QUÉBEC À TROIS-RIVIÈRES

ENCAPSULATION DES BACTÉRIOPHAGES DANS DES BIOPOLYMÈRES POUR AMÉLIORER LEUR STABILITÉ DANS DES EMBALLAGES ALIMENTAIRES BIOACTIFS

MÉMOIRE PRÉSENTÉ
COMME EXIGENCE PARTIELLE
DE LA MAÎTRISE EN SCIENCES DE L'ENVIRONNEMENT

PAR
MEHDI HARB

SEPTEMBRE 2022

Avertissement

REMERCIEMENTS

En premier lieu, je tiens à remercier mon Directeur Professeur Simon Barnabé et mon Codirecteur, Dr. Tarik Jabrane. Grâce à votre support, votre encadrement et votre enthousiasme envers ce projet, le processus fut très agréable et enrichissant. Je tiens à remercier le professeur François Brouillette, membre de mon comité d'orientation, Docteur Hany Anany de l'Université de Guelph pour ta présence, ta bonne humeur et ton aide constante, le professeur Sylvain Moineau de l'Université Laval.

Merci aux membres de laboratoire de Simon Barnabé à mes collègues étudiants, Docteure Thi Thanh Ha Pham, Docteur Thanh Tung Lai, nos discussions et vos conseils ont fait progresser ma réflexion et m'ont permis d'approfondir mes connaissances sur mon projet.

Je remercie Dr. Agnès Lejeune, Dr. Benoit Bideau et M. Fréderic Richard pour leur aide ainsi que tout le personnel de I2E3 et Innofibre.

Je remercie particulièrement mon épouse Fatima et mes petits Nala et Noah pour leur support moral et intellectuel tout au long de ma maitrise

Le projet n'aurait jamais pu voir le jour sans le support financier de nombreux collaborateurs : le Conseil de recherches en sciences naturelles et en génie du Canada (CRSNG) et notre partenaire Kruger et le Consortium de recherche et innovations en bioprocédés industriels au Québec (CRIBIQ). Merci!

Avant-Propos

Ce mémoire a été réalisé dans le cadre du programme de maîtrise en sciences de l'environnement sous la direction de Simon Barnabé professeur à l'Université du Québec à Trois-Rivières au Département de chimie, biochimie et physique, et la codirection de Tarik Jabrane chercheur chez Innofibre.

Le principal objectif de mon projet était de trouver une combinaison de biopolymères optimale pour encapsuler le *Listex P100* pour qu'il résiste aux conditions industrielles et permettant le relâchement des bactériophages en présence des bactéries, plus spécifiquement *Listeria monocytogenes.*

Ce mémoire comporte quatre chapitres. Le premier chapitre est un résumé substantiel du projet de recherche rédigé en français. Le second chapitre est l'article; celui-ci est rédigé en anglais et a pour titre: « *Encapsulation of Listeria monocytogene Phage P100 by Different Polymer Combinations to Improve Phage Thermal Stability and Shelf life* ». Le troisième chapitre est un article rédigé en anglais et a pour titre « *Novel procedure for obtaining bioactive base paper to reduce listeriosis impact in food packaging* ». Ce chapitre est un résumé de mes principaux résultats de l'application des *Listex P100* sur papier. Le dernier chapitre est la conclusion générale du projet ainsi que les recommandations pour la suite, rédigé en français. Les articles présentés au chapitre II et III, dont je suis le premier auteur, seront soumis dans la revue scientifique « Food Microbiology».

RÉSUMÉ

Les maladies d'origine alimentaire causent au Canada des pertes économiques graves aux industries alimentaires surtout celles des viandes prête-à-manger. Les bactéries à l'origine de ces maladies deviennent de plus en plus résistantes aux antibiotiques. Cette résistance presse les chercheurs à utiliser d'autres moyens de lutte pour éviter l'utilisation des antibiotiques. Selon l'Organisation mondiale de la santé, les bactériophages sont la plus solide alternative pour lutter contre les infections alimentaires. À cause de leur spécificité, ces virus sont utilisés comme agent bioactif pouvant améliorer la qualité des emballages alimentaires en limitant la propagation des bactéries pathogènes pour l'humain. Mais l'utilisation des bactériophages doit être accompagnée d'une méthode qui garantit l'activité de ces derniers sous des conditions extrêmes comme la température de séchage et autres conditions qui peuvent être rencontrées dans plusieurs procédés industriels de fabrication d'emballage alimentaire. Pour répondre à ce besoin, notre projet vise à trouver un biopolymère ou une combinaison adéquate de biopolymères pour l'encapsulation des bactériophages en vue d'augmenter leur stabilité dans des conditions extrêmes.

Au cours de cette étude, on s'intéresse à une bactérie, *Listeria monocytogenes*, qui malgré sa rareté fait partie des éclosions les plus mortelles pour l'humain. Les bactériophages testés spécifiquement contre cette bactérie sont des bactériophages de type *Listex P100*.

Plusieurs candidats ont été testés pour les biopolymères, tel l'alginate, la gomme gellane, gélatine, la gomme arabique, la nanocellulose phosphorylée et bien d'autres. Les capsules et les films formés à partir de biopolymères différents ont été testés pour leur effet lytique sur la croissance de *Listeria monocytogenes*, leur stabilité à des températures élevées, et les conditions de stockage à court et long terme.

Une combinaison spécifique composée respectivement de 2,9-0,3-1m1 % en masse d'alginate de sodium, de gomme gellane et de gélatine a montré des propriétés améliorées, en comparaison avec les combinaisons retrouvées dans la littérature, pour la préservation de l'activité des bactériophages dans les conditions de température et d'entreposage testées. De même, cette composition a aussi été étalé sur papier de base avec des phages pour tester la possibilité d'avoir un papier bioactif fonctionnel, ce qui a bel et bien mené à l'obtention du produit mais qui nécessite une optimisation des conditions d'entreposage du produit pour une meilleure stabilité de l'élément bioactif.

Les résultats de ce projet permettront une validation à l'échelle pilote puis une application à l'échelle industrielle des bactériophages comme agent actif des emballages alimentaires pour pouvoir répondre aux problèmes des maladies liées aux infections alimentaires.

Mots-clés : Bactériophages, Biopolymères, Emballages bioactifs, Encapsulation, *Listeria monocytogenes*

TABLE DES MATIÈRES

LISTE DES FIGURES

Figure **Page**

LISTE DES TABLEAUX

Tableau **Page**

LISTE DES ABRÉVIATIONS

ADN	Acide désoxyribonucléique
Ala	Alanine
Arg	Arginine
ARN	Acide ribonucléique
EDTA	Ethylenediaminetetraacetic acid
EU	États unis d'amérique
FDA	Food and drug act
Gly	Glycine
Glu	Acide glutamique
Hyp	Hydroxyproline
PAM	Prêt-à-manger
UE	Union européenne

CHAPITRE 1

RÉSUMÉ SUBSTANTIEL

1. INTRODUCTION

1.1 Les maladies d'origine alimentaire

Les infections d'origine alimentaire, définies par leur mode de transmission, regroupent des infections virales, néomadienne, ou bactériennes causées par plus de deux cents bactéries, etc. (Sarfraz et al., 2017). Ces infections peuvent être transmises par l'ingestion d'aliments infectés, par contact direct avec des animaux, l'eau, de la femme enceinte à son fœtus, de personne à personne ou par d'autres voies. Les symptômes dépendent de l'agent pathogène causal de l'infection (*Salmonella spp., Campylobacter spp., Listeria monocytogenes, Yersinia enterocolitica*, Norovirus, etc.) ou bien de l'action des toxines excrétées dans l'aliment (*Bacillus cereus, Staphylococcus aureus, Clostridium botulinum, Clostridium perfringens,* etc.). Ces infections touchent surtout le système digestif. Elles peuvent être graves, voire mortelles, comme dans le cas de la méningo-encéphalite à *Listeria monocytogenes* (Chlebicz & Śliżewska, 2018).

Malgré l'évolution des pratiques industrielles pour une meilleure salubrité alimentaire, les éclosions des infections d'origine alimentaire continuent à présenter une préoccupation autant sur le plan économique que celui de la santé publique. Au Canada, plus de 4 millions de Canadiens sont victimes d'une intoxication alimentaire chaque année (H. C. Government of Canada, 2021). De plus, la confiance des Canadiens corrèle négativement avec la récurrence des rappels alimentaires selon une étude récente (Morton et al., 2019).

Malgré sa rareté, la listériose demeure une des maladies d'origine alimentaire les plus dangereuses au Canada. Son agent causal, *Listeria monocytogenes*, fait le sujet de plusieurs rappels alimentaires dans les dernières années. Son incidence est plus élevée dans les fromages à pâtes molles et les viandes prêtes-à-manger comme les pâtés, les viandes froides et le saumon fumé.

1.2 Mise en contexte

Pour éviter les rappels alimentaires, certaines industries essaient de développer des moyens de luttes préventifs contre la propagation des agents causant les éclosions. Avec l'inefficacité des moyens de luttes traditionnelles, l'utilisation des éléments bioactifs dans les emballages alimentaires commencent à émerger comme moyen pouvant limiter la propagation des bactéries dans les aliments emballés (Lone et al., 2016a). Ces emballages pourront contenir des bactériophages spécifiques aux agents causant des infections alimentaires puisque l'action des bactériophages et leur pouvoir à limiter la propagation des bactéries ont déjà été prouvés pour plusieurs types alimentaires (Moye et al., 2018)

Le géant Canadien de pâtes et papiers Kruger a donc décidé donc d'investir dans un projet portant sur le papier bioactif avec un investissement de plus de 120 millions de dollars canadiens pour deux usines spécialisées en emballages alimentaires flexibles et bioactifs à Bromptonville et Trois-Rivières. Cet investissement couvre aussi une collaboration avec l'Université de Québec à Trois-Rivières (UQTR) et le centre collégial de transfert de technologie Innofibre du CÉGEP de Trois-Rivières. La collaboration compte trois axes, la conception de phages microencapsulés, l'application de ces derniers sur du papier d'emballages alimentaires et les essais à l'échelle pilote. Dans le laboratoire du Professeur Simon Barnabé, l'équipe se charge des deux premiers axes, et le troisième axe concernant l'essai pilote se fera en collaboration avec Innofibre.

Pour mon projet, je m'intéresse à trouver une combinaison optimale pour encapsuler les phages dans un polymère résistant aux conditions industrielles et permettant le relâchement des bactériophages en présence des bactéries, plus spécifiquement *Listeria monocytogenes.*

1.3 Problématique

L'utilisation des bactériophages dans les emballages alimentaires commencent à devenir une solution presque unique pour lutter contre les infections alimentaires, surtout après l'émergence des différentes résistances aux antibiotiques par les bactéries responsables des infections alimentaires, notamment *Salmonella* (Ndoboli et al., 2018), *E. coli* (Coeffic et al., 2018) et *Listeria monocytogenes* (Komora et al., 2017(Olaimat et al., 2018a). Les phages sont très spécifiques aux

sérotypes bactériens, ce qui est un avantage pour leur utilisation de façon sécuritaire dans les emballages alimentaires sans soucis d'affecter la flore microbienne bénigne pour la santé humaine. Mais ces bactériophages ne peuvent être utilisé dans leurs formes libres directement dans les emballages à cause de leur sensibilité thermique et mécanique de ces phages (Ahmadi et al., 2018). D'où la problématique traitée dans ce sujet de maîtrise, soit l'optimisation d'une méthode de l'encapsulation des phages en vue de maintenir leur pouvoir antibactérien dans les conditions industrielles.

1.4 Revue de littérature

1.4.1 Incidences de la listériose au Canada

Les éclosions de listériose ont causé 23,000 individus affectés et presque 5500 morts en 2010 autour du globe (de Noordhout et al., 2014). Au Canada, environ 134 cas de listériose invasive par année ont été déclarés au cours des dernières années au Canada (C. F. I. A. Government of Canada, 2019). La majorité des cas sont liés à la consommation des viandes prêtes-à-manger mais aussi une des éclosions de listériose invasive concernait du lait au chocolat pasteurisé. Cette dernière était reportée en Ontario et avait touché environ 50 individus (0,4 cas/ 100000 personnes) par an depuis 2005 (Canada, 2016). Des éclosions plus récentes étaient liées à la consommation des viandes froides ou des fromages produits à partir de lait cru (Heymann et al., 2015). Le plus désastreux des événements en termes de perte économique liée aux éclosions de listériose a eu lieu en 2008, l'origine de l'infection était définie plus tard comme de la viande froide provenant d'une même usine. Suite à cette éclosion, il y eu 24 morts sur 57 individus atteints et le total des pertes économiques liées au rappel du produit, aux suivis de justice et aux coûts médicaux s'est élevé à 242 millions de dollars canadiens (Thomas et al., 2015).

En août 2018, un produit de poulet cuit de Rosemont fait le sujet d'un rappel par Santé Canada pour une contamination par *L. monocytogenes*, mais les dégâts au niveau des nombres de personnes affectés fut moins élevé à cause de la rapidité du rappel qui a pu être lié à une éclosion aux États-Unis quelques semaines d'avance n(C. F. I. A. Government of Canada, 2019).

Malgré toutes les précautions prises contre la COVID19, plusieurs rappels d'aliment infectés par la *Listeria monocytogenes* ont été issus par l'agence canadienne d'inspection des aliments en 2021. Le plus récent des rappels au Québec et en Ontario, est celui du 25 octobre 2021, concernant champignons blancs tranchés en raison d'une contamination par *L. monocytogenes*.

Au Canada, comme partout sur le globe, les éclosions de *Listeria monocytogenes* sont rares mais dangereuses. À chaque cas, les publications scientifiques sont là pour documenter ces éclosions ou comparer les sérotypes en question. Dans une étude récente, la plupart des éclosions de listériose au monde sont causés par des viandes prêtes-à-manger (Tableau 1, (Olaimat et al., 2018a).

Tableau 1: Récapitulatif des éclosions à base de listériose faisant sujet de publications scientifiques entre 2005 et 2018. PAM: prêt à manger. Adapté de Olaimat et al., 2018

Pays	Prévalence (%)	Aliments	Références
Turquie	9/146 (6.16%)	Viandes crues et cuites	Yucel et al. (2005)
ÉU	91/3063 (3.0%)	Viandes PAM	Shen et al. (2006)
Canada	124/800 (15.5%)	Viandes PAM	Bohaychuck et al. (2006)
Liban	30/160 (18.8%)	Produits laitiers	Harakeh et al. (2009)
Iran	5/290 (1.7%)	Yogourt iranien	Rahimi et al. (2010)
Jordanie	51/280 (18.2%)	Poulet PAM	Osaili et al. (2011)
Grèce	38/100 (38%)	Poulet	Sakaridis et al. (2011)
UE	310/2994 (10.4%)	Saumon fume	EFSA, (2013)
Canada	24/57 (42%)	Charcuterie	Currie et al. (2015)
Uruguay	71/635 (11.2%)	Viandes PAM	Braga et al., (2017)
ÉU	102/27389 (0.4%)	Aliments PAM	Luchansky et al. (2017)
Brésil	35/195 (17.9%)	Poulet	Oliveira et al. (2018)

1.4.2 Effet de *Listeria monocytogenes* sur la santé humaine

Listeria monocytogenes est un bacille Gram positif, seule du genre *Listeria monocytogenes* classé comme pathogène humain. Ce pathogène a été décrit pour la première fois en 1926 suite à une éclosion dans une population de lapin. *L. monocytogenes* est aérobie-anaérobie facultative, ayant une activité catalase et mobile à 20°C grâce à sa ciliature péritriche, mais est immobile à 37°C car la bactérie est incapable de synthétiser les composants du flagelle à cette température. Le bacille mesure 0,5 µm de large et 1-2 µm de long, son génome est d'environ 3 Mpb et a un faible contenu en GC (Liu, 2006).

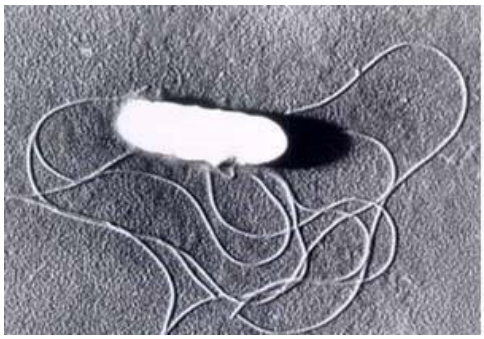


Figure 1 : Image en microscopie électronique à balayage de *Listeria monocytogenes* montrant les flagelles péritriches. (Source : Bureau des affaires sociales et de la santé publique, Gouvernement métropolitain de Tokyo, 2019)

Listeria monocytogenes est capable de pousser dans des conditions extrêmes de température, de salinité et de pH (Liu et al., 2005). Une listériose est considérée comme infection opportuniste, qui même si est infectieuse pour toutes les populations humaines, peut se propager davantage chez les personnes susceptibles, telles que les femmes enceintes, les nouveau-nés et les personnes âgées. Durant les premiers phases d'une listériose, les symptômes ressemblent plutôt à une grippe avec des frissons, de la fatigue et des maux de tête. Si une listériose demeure sans traitement, les symptômes s'aggravent pour atteindre une méningite, une encéphalite, des fausses-couches et parfois la mort (Vázquez-Boland et al., 2001). Ces complications font que le taux de mortalité suite à une listériose dépasse 30%, ce qui est le plus élevé parmi les infections d'origine alimentaire, comme *Salmonella enteiridis* qui cause 0,38% de mortalité, ou les espèces de Vibrio

0,01% (Liu, 2006). À la suite de l'ingestion d'aliments contaminé, *Listeria monocytogenes* survie grâce à sa résistance aux enzymes protéolytiques de l'hôte, ainsi que l'acidité de l'estomac (pH 2), les sels biliaires et les réponses inflammatoires non-spécifiques et ceci suite à l'expression de plusieurs gènes de réponses aux stresses (opuCA, lmo1421 and bsh) (Sleator et al., 2003).

Arrivée à l'épithélium intestinal, *L. monocytogenes* traverse ensuite la barrière digestive vers les vaisseaux sanguins et lymphatiques pour se diriger vers les organes cibles, tel le foie et la rate. La bactérie est aussi capable d'Atteindre la barrière entre le sang et le cerveau pour causer des encéphalites. La bactérie est capable de se multiplier à l'intérieur des cellules hôtes (ex-macrophages) et d'utiliser sa machinerie pour remodeler la chromatine et changer l'expression génique comme le montre la figure 2 (Radoshevich & Cossart, 2018)

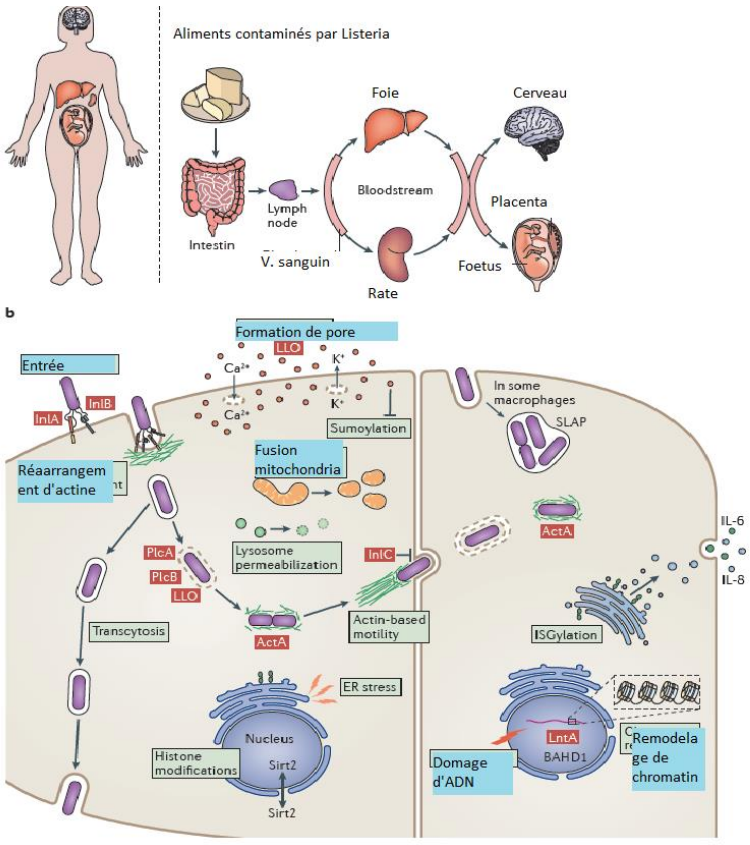


Figure 2 : Cycle infectieux de *Listeria monocytogenes* dans les cellules humaines (Radoshevich & Cossart, 2018)

1.4.3 Problèmes liés à l'utilisation des antibiotiques

Depuis quelques décennies, les infections de *L. monocytogenes* sont traitées avec des antibiotiques de la famille des β-lactames comme la pénicilline, l'ampicilline, la tétracycline et la gentamicine. Mais une comparaison entre l'effet de ces antibiotiques entre 1990 et 2008 montre une résistance dans quelques sérotypes de *L. monocytogenes*. Même si cette résistance concerne quelques sérotypes, mais ce qui est inquiétant est la grande diversité des antibiotiques qui s'avèrent sans effet avec le temps comme le montre le tableau 2 qui montre que plus que 95% des souches de *L. monocytogenes* sont devenus résistantes à des antibiotiques comme l'oxacilline, l'acide fusidique et la fosfomycine (Conter et al., 2009).

Tableau 2 : Résistance de *L. monocytogenes* aux différents antibiotiques (Conter et al., 2009)

Agent antimicrobien	Concentration(µg/ml)	Pourcentage des isolats susceptibles[a]		
		R	I	S
Penicillin	0.03–0.5	0	0	100
Ampicillin	2–32	1.6	0	98.4
Ampicillin/sulbactam	2–32	0	0	100
Oxacillin	0.25–4	97.6	0	2.4
Imipenem	1–16	0	0	100
Gentamicin	0.5–16	0	0	100
Ciprofloxacin	0.5–8	1.6	1.6	96.8
Moxifloxacin	0.25–8	0.8	69.7	29.5
Erythromycin	0.25–8	0	2.4	97.6
Clindamycin	0.25–8	3.2	4.8	92.0
Quinopristin/dalfopristin	0.25–16	0	0.8	99.2
Linezolid	0.5–8	3.2	0	96.8
Teicoplanin	0.5–32	0	0	100
Vancomycin	1–32	0.8	0	99.2
Tetracycline	1–16	0.8	0.8	98.4
Fosfomycin	8–128	96.8	0	3.2
Fusidic acid	0.5–32	96.8	0	3.2
Rifampicin	0.5–32	1.6	0	98.4
TMP–SMX[b]	10–320	1.6	0	98.4

[a] R: résistant, I: Intermédiaire, S: Susceptible

[b] Trimethoprim/sulfamethoxazole.

De même, l'usage excessive des antibiotiques dans l'alimentation animale pour assurer une croissance non-interrompue, ainsi que l'utilisation des antibiotiques comme pesticides sur la surface des fruits pour repousser les ravageurs ont mené à une résistance aux antibiotiques de plus en plus importante chez *L. monocytogenes* (Figure 3; (Olaimat et al., 2018a)

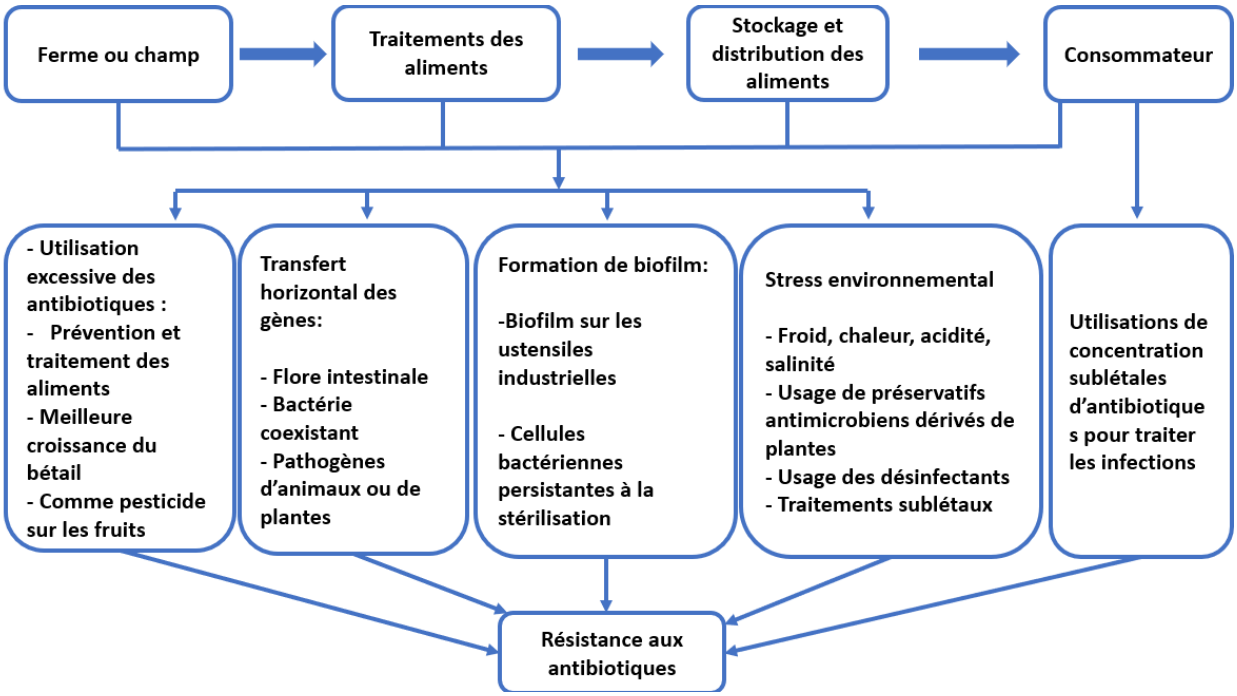


Figure 3 : Facteurs influençant la résistance de *L. monocytogenes* aux antibiotiques durant les deux dernières décennies. (Olaimat et al., 2018a)

1.4.4 Utilisation des bactériophages comme alternative

Depuis le début du 21^{ième} siècle, l'usage des bactériophages a commencé à être une option plus réaliste pour la lutte contre les bactéries que les antibiotiques. Les bactériophages sont des virus qui infectent les bactéries. Ces organismes ont été découvert, il y a un siècle, par un microbiologiste canadien, Félix d'Hérelle et un chercheur anglais Fredrick Twort (Salmond & Fineran, 2015). Un bactériophage est spécifique à une souche bactérienne et peut la reconnaître grâce à la reconnaissance d'un récepteur à la surface bactérienne. À la suite de cette reconnaissance, les bactériophages injectent leurs matériels génétiques dans la bactérie et détournent sa machinerie pour synthétiser les protéines de la capside et du corps des virus ainsi que dédoubler son génome.

Ensuite la progéniture peut s'accumuler dans la bactérie avant de la lyser pour se libérer dans l'environnement et recommencer le cycle comme l'explique la figure 3 (Doss et al., 2017).

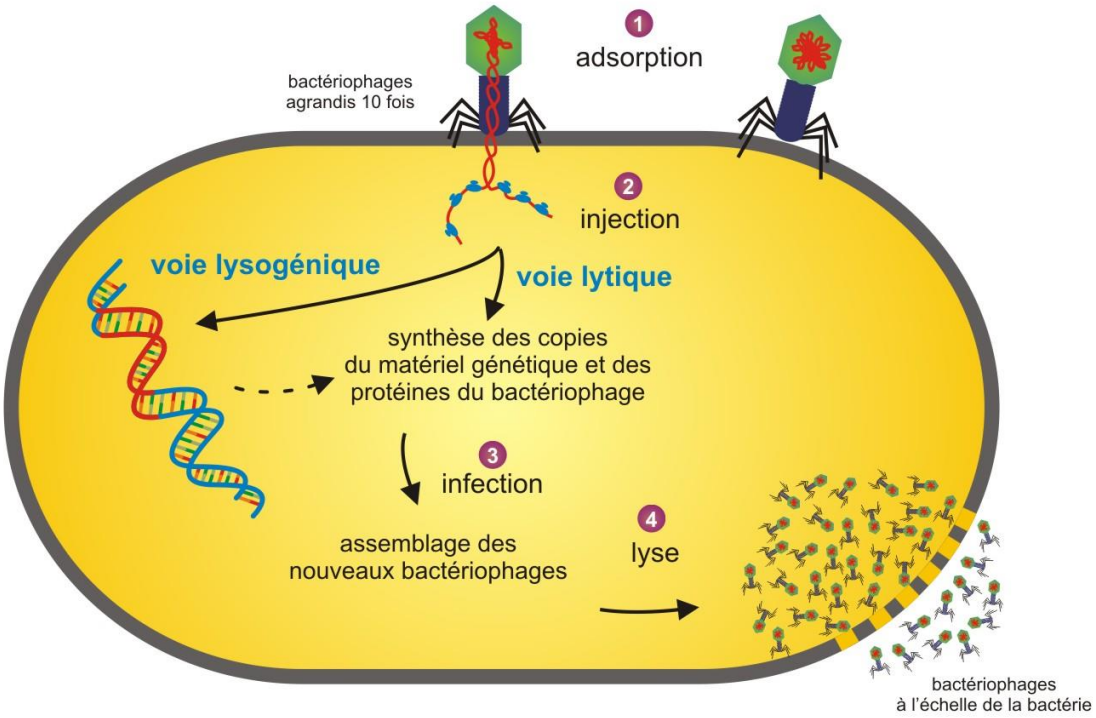


Figure 4 : Schéma montrant le cycle d'un bactériophage à la suite du contact avec la bactérie cible (Dufour et al., 2019)

Les bactériophages offrent un traitement avec un risque minime d'infections secondaires retrouvées durant les traitements par antibiotique qui compromettent la flore bactérienne bénéfique, et cela due à leurs spécificités et la limitation de la résistance bactérienne (Chernomordik, 1989).

1.4.5 Les bactériophages commerciaux *Listex P100 (Micreos)*

Les bactériophages sont généralement formés pas une capside contenant le génome ARN ou ADN et une queue et sont munis d'enzymes, les endolysines, capables de dégrader la paroi de la bactérie hôte. La taille d'un phage varie de 24 à 400 nm (Akhverdyan et al., 2011; Loessner, 2005). La morphologie de *Listeria P100* qui a une longueur d'environ 300 nm, formée d'une queue

contractile et d'une capside icosaédrique, cette morphologie est cohérente avec la classification antérieure de la famille des Myoviridae dans l'ordre des Caudovirales (Zink & Loessner, 1992). Ce phage est bien caractérisé et les génomes ont été séquencés. La dimension du diamètre de la tête mesure 89,6 nm, avec des longueurs de queue de 198,24 nm, et de diamètre de queue de 19 nm (Carlton et al., 2005; Klumpp et al., 2008). Ils ont un ADN double brin. La propagation des particules virales implique la fixation à la cellule hôte, l'injection de l'ADN double brin, puis l'hôte le transcrit et le traduit en de nouvelles particules de virion. Les ADN polymérases de la cellule hôte sont nécessaires pour répliquer le contenu génétique du phage.

C'est en 1921 que l'action des bactériophages contre une infection bactérienne a été prouvée pour la première fois. Depuis, les bactériophages ont été utilisés pour traiter des maladies humaines, des infections d'*Escherichia coli* du bétail et même des maladies bactériennes de plantes (Jamal et al., 2019).

Listex P100 a été approuvé par la FDA ('Food & Drug act') et l'USDA Département de l'agriculture des États-Unis) comme additif alimentaire pour lutter contre *Listeria monocytogenes*. Dans plusieurs applications agroalimentaires, partant des traitements des surfaces industrielles jusqu'à la désinfection des fruits, des bactériophages *Listex P100* ont commencé à être employé seuls ou avec des bactériocines (Guenther et al., 2009; Leverentz et al., 2003). De même, 21 souches différentes de *L. monocytogenes* appartenant à 13 sérotypes distincts ont été réduites de 5 log après un traitement avec de *Listex P100* (Soni & Nannapaneni, 2010). Pour cette raison, le *Listex P100* a été sélectionné comme agent bioactif des emballages dans ce projet.

1.4.6 Encapsulation des bactériophages par les biopolymères

L'encapsulation est une méthode polyvalente pour stabiliser les agents bioactifs dans des matrices polymères solides, liquides ou semi-solides. L'encapsulation de cellules microbiennes dans une matrice d'hydrogel est une méthode efficace pour améliorer leur stabilité et leur protection. La recherche sur l'encapsulation de bactériophages dans l'hydrogel s'est concentrée principalement sur les infections gastro-intestinales. Dans ce cas, le matériel d'encapsulation protège le bactériophage contre l'environnement acide sévère de l'estomac (Ma et al., 2008). Dans le cadre de ce projet, l'hydrogel ou les biopolymères comme alginate, gomme gellane, gomme arabique,

gélatine et autres, devraient conférer une protection aux bactériophages contre la température et les autres facteurs rencontrés dans les conditions industrielles. Et comme le produit issu de cette étude est prévu pour être utilisé avec les aliments prêt-à manger (PAM), il faut que le biopolymère le constituant soit sans goût, sans odeur et approuvé par la FDA comme additif alimentaire.

Plusieurs biopolymères ont été approuvés précédemment par la FDA pour l'utilisation dans le secteur agroalimentaire. Dans le cadre de ce mémoire, seuls les polymères utilisés dans les essais finaux sont décrits :

L'alginate de sodium ou polymannuronate sodique est une longue molécule synthétisée à partir de l'acide alginique, extraite d'algues brunes, composée de diverses proportions d'acide D-mannuronique et d'acide L-guluronique (King & Zall, 1983; Salvia-Trujillo et al., 2013). Le polysaccharide anionique a une liaison sucre linéaire 1-4 avec l'acide β-D-mannuronique (M) et l'acide α-L-guluronique (G). Le pKa du groupe carboxyle dans l'alginate est compris entre 3,3 et 3,7 selon les teneurs en acide mannuronique et guluronique. L'alginate a été largement utilisé pour les applications d'encapsulation, en raison de sa faible toxicité, de sa rentabilité et de sa facilité de gélification. Pour préparer des hydrogels réticulés, le polymère d'alginate de sodium est combiné avec une solution contenant des cations divalents comme le chlorure de calcium. Les ions Ca^{2+} se lient aux cavités électronégatives des structures d'alginate par interaction électrostatique entre le groupe carboxylate chargé négativement de l'alginate. Ce phénomène chimique a pour conséquence la formation d'un gel (Paredes Juárez et al., 2014).

Quant à elle, la gomme géllane est produite, entre autre, par la bactérie *Sphingomonas elodea.* C'est un hétéropolyoside linéaire anionique basé sur des unités d'oligoside composé de 4 oses (tétra-oside) (Vartak et al., 1995). Le D-glucose, le L-rhamnose et l'acide D-glucuronique en proportions 2:1:1 sont présents dans la gomme gellane sous forme d'éléments monomères. Le pKa de groupe carboxyle dans la gomme gellane est 3.5.

La gomme arabique ou la gomme d'acacia, est extrait des tiges des espèces d'acacia lorsque les tissus de la plante sont blessés. Il s'agit d'un polysaccharide chargé négativement qui est hautement ramifié constitué d'unités de squelette (acide 1→ β,D-galactopyranosyl uronique avec des chaînes latérales L-arabinofuranosyl, L-rhamnopyranosyl, D-galactopyranosyl et D-glucopyranosyl) (Nayak et al., 2012). Le pKa de groupe carboxyle dans la gomme arabique est 3,6.

Contrairement aux trois premiers polymères de type polysaccharidique, le dernier polymère utilisé est de nature peptidique. La gélatine est fréquemment appliquée dans les applications alimentaires et pharmaceutiques. Il s'agit d'un mélange de peptides et de protéines produits par hydrolyse partielle de collagène isolé à partir de peaux et d'os d'animaux/poissons, avec de l'acide. Sa composition chimique est similaire à celle de son collagène parent. La gélatine a un point isoélectrique (PI) de 4,7 et contient de nombreux résidus d'acides aminés de glycine (presque 1 sur 3), de proline et de 4-hydroxyproline. Une structure typique est -Ala-Gly-Pro-Arg-Gly-Glu-4Hyp-Gly-Pro- (Bagheri-Khoulenjani et al., 2016). Le pKa de groupe carboxyle dans la gélatine type A est entre 7 et 9,5 type B est entre 4,7 et 5,3.

Quant au choix des combinaison des polymères, l'alginate est un polysaccharide anionique, la gélatine est un excellent candidat pour l'incorporation avec des polysaccharides anioniques tels que l'alginate en raison de sa nature amphotère. L'ajout de polysaccharides, tels que le gomme gellane et la gomme arabique dans la production de billes de gel a été signalé comme une bonne stratégie pour augmenter l'efficacité d'encapsulation dans les billes de gel (Aguirre Calvo & Santagapita, 2016). La gomme gellane est un polymère hautement ramifié et lorsque ce polysaccharide est ajouté à un polymère plutôt linéaire tel l'alginate de sodium, il augmente le piégeage des particules dans la matrice alginate-gomme gellane, et augmente ainsi la thermostabilité de la combinaison (Solanki et al., 2013)

2. BUT ET OBJECTIFS

2.1 But

Le but général de ce projet est d'encapsuler des bactériophages par des biopolymères pour améliorer leur stabilité dans des emballages alimentaires bioactifs.

2.2 Objectifs

Les objectifs spécifiques de cette étude sont :

- Étudier une combinaison de biopolymères capable d'assurer la rétention et le relâchement des bactériophages;

- Étudier la stabilité des bactériophages encapsulés après un stress thermique et mécanique;
- Étudier la stabilité des bactériophages encapsulés dans le temps à différentes températures d'entreposage ;
- Appliquer les bactériophages encapsulés sur les papiers de base du partenaire (Kruger) sous forme d'un film et étudier leur stabilité après le séchage et dans le temps à différentes températures d'entreposage.

CHAPITRE II

Encapsulation of *Listeria monocytogenes* Phage P100 by Different Polymer Combinations to Improve Phage Thermal Stability and Shelf life.

Article en attente de soumission à
la revue scientifique Journal of Food Microbiology.

Mehdi Harb[1], Lai Thanh Tung[1], Ha Thanh Pham[1], Sylvain Moineau[2], Tarik Jabrane[1,3], Simon Barnabé[1]

[1]Institut d'innovations en Écomatériaux, Écoproduits et Écoénergies, Université du Québec à Trois-Rivières, 3351, boul. des Forges, C.P. 500, Trois-Rivières, QC, Canada G9A 5H

[2] Université de Laval, Québec, QC, Canada

[3] Innofibre, Cégep de Trois-Rivières, Trois-Rivières, QC, Canada

Corresponding author
simon.barnabe@uqtr.ca (S. Barnabé)

Highlights

- *Listeria* phages P100 have been microencapsulated with sodium alginate and other polymers (gellan gum, Arabic gum, and gelatin) and gained stability during storage at room temperature.
- A combination of alginate-gellan gum-gelatine and alginate-Arabic gum-gelatine in respective proportions of 2.9-0.3-1.1 and 2.9-1.2-1.1 % (w/v) proved to confer maximal protection against high temperature.

ABSTRACT

Bacteriophages are effective agents for control of foodborne pathogens. However, phages are difficult to apply to food packages because of the thermal processing related destruction. Microencapsulation is used for enhancing the stability of phages under harsh conditions, such as high temperature in food processing. Previous works showed that encapsulating bacteriophages in food-grade polymer confers some protection. Here an improved encapsulation of *Listeria phages P100* using a novel combination of sodium alginate, gellan gum, and gelatine is described. The aim is to ensure maximal thermostability and enhance shelf life of encapsulated bacteriophages by studying the antimicrobial activity after application of different stresses. A combination of alginate-gellan gum-gelatine at 2.9-0.3-1.1 % w/v was found to protect *Listeria phages P100* after exposure to 110°C up to 5 minutes. The same combination insured the minimal active phage loss after 3-months storage at room temperature and at 4°C. This proves the efficiency and suitability for food industrial application of the obtained alginate-gellan gum-gelatine formula as phage encapsulating solution.

Keywords

Sodium alginate, gellan gum, microencapsulation, *Listeria monocytogenes*, bacteriophages, thermal stability.

Abbreviations

Alg Sodium Alginate

CFU Colony Forming Unit

Gg Gellan Gum

Ga Arabic Gum

Gel Gelatin

OD Optical Density

PFU Phage Forming Unit

EB Encapsulated bacteriophage

1. Introduction

Foodborne diseases are caused by pathogens present in food and represent a burden in food industry as well as health sector. More than two hundred causative bacteria are responsible of foodborne infections, affecting more than 4 million Canadians each year. The symptoms depend on the causative pathogen of the infection (*Salmonella* spp., *Campylobacter* spp., *Listeria monocytogenes*, *Yersinia enterocolitica*, Norovirus, etc.) or on the action of toxins excreted in the food (*Bacillus cereus, Staphylococcus aureus, Clostridium botulinum, Clostridium perfringens*, etc.). These infections mainly affect the digestive system. They can be serious or even fatal, such as the example of meningo-encephalitis caused by *Listeria monocytogenes* (Coeffic et al., 2018; Morton et al., 2019).

Despite its rarity, listeriosis remains one of the most dangerous foodborne illnesses in Canada. Its causative agent, *L. monocytogenes*, has been responsible for several dietary recalls in recent years. Its incidence is the highest in soft cheeses and ready-to-eat meats such as pâtés, cold meats and smoked salmon (Komora et al., 2017). Several industries are developing preventive controls against the spread of the agents causing the outbreaks. Hence, several antibiotics were first administrated (Charpentier et al., 1995; Olaimat et al., 2018b; Walsh et al., 2001) to overcome listeriosis, but antibiotics resistance in Listeria appeared shortly after treatments. With the recurrence of antibiotic resistance, the use of other control strategies such as bioactive elements like bacteriophages in food packaging begun to emerge to limit the spread of bacteria in packaged food (Lone et al., 2016a). Bacteriophages are viruses that infect bacteria specifically by recognizing a receptor on the bacterial surface. Following this recognition, the phages inject their genetic materials into the bacteria and seize its machinery to synthesize the proteins of the capsid and the body of viruses as well as to duplicate its genome (Doss et al., 2017). Multiple approaches introduced phage usage in food industry to reduce foodborne pathogens such *L. monocytogenes*, but shortly limitations such as pH sensitivity and temperature inactivation (Ahmadi et al., 2018; Lone et al., 2016a). In the last decade, many studies started implementing bioactive compounds and phages in food applications for improving biocontrol (Lone et al., 2016a).

Bioactive packages may contain bacteriophages specific to the causative agents of food infections since the action of bacteriophages and their capacity to limit the spread of bacteria has already been proven for several food types (Lone et al., 2016a; Moye et al., 2018). Phages are specific to bacterial serotypes, an advantage for their safe use in food packaging without affecting benign microbial flora. In several agri-food applications, from industrial surface treatments to fruit disinfection, the bacteriophages specific to *L. monocytogenes* such as P100 and A511 have been used alone or along with bacteriocins (Leverentz et al., 2003). Likewise, 21 different strains of *L. monocytogenes* belonging to 13 distinct serotypes were reduced by 5 logs after treatment with P100 phages (Soni & Nannapaneni, 2010). If bacteriophages were to be introduced in food packaging, it will undergo high heat, physical pressure and some other extreme conditions due to the process of making the packaging in paper industry. However, bacteriophages cannot be used in their free forms directly under these conditions because of their sensitivity to temperature, pH and mechanical pressure was observed previously (Ahmadi et al., 2018). The researchers started investigating coating and encapsulating phages to preserve its bioactivity (Ahmadi et al., 2018) . Hence the problem addressed in this study is to optimize *Listeria* P100 phage encapsulation for enhanced protection of phage bioactivity under extreme thermal conditions.

Encapsulating hydrogel matrix protects phages against industrial conditions such as pH, high temperature and pressure as well as storage duration (Gonzalez-Menendez et al., 2018). Phage microencapsulation consists of coating phages particles in a hydrogel formed by polysaccharides w/o gelatine and applying extrusion in a calcium carbonate solution to ensure polymerization. Encapsulation of phages in polysaccharides such as alginate protects phages against different conditions by reducing the flow through the material (Soto et al., 2018). The high content of guluronic acid present in alginate results in a porous hydrogel, and size of the pores are a function of the remaining components of the encapsulating materiel such as other polysaccharides or polypeptides (Gombotz & Wee, 1998). Previously, *Listeria* bacteriophage A511 gained protection against temperature up to 70°C after encapsulation in 3% w/v sodium alginate, 1% arabic gum and 1% gelatine (Ahmadi et al., 2018). In this present work, the effect of encapsulation using biopolymers on the thermal stability and shelf life of the bacteriophages were investigated in regarding to the application of the phage on paper-based packaging. The parameters studied are:

1) high temperature in the dryer section of the paper-making process; 2) storage conditions (temperature and storage duration) of paper-based packaging containing phages.

2. Materials and methods

2.1 Bacteria and phage preparation

L. monocytogenes strain ATCC 19115 serotype 4b was used in all experiments described in the paper. *L. monocytogenes* culture was prepared by transferring a single colony to 5 mL tryptic soy broth (TSB; BD Diagnostics, San Jose, CA, United States) in a 50 mL screw cap, sterile Falcon tubes (Fisher Scientific) then incubated for 18 h at 30°C and 120 rpm in an orbital shaker incubator (Infors HT Ecotron). This resulted in cell concentration of 10^9 CFU/mL. *Listeria* bacteriophage Listex P100 (Carlton et al., 2005) was purchased from Micreos (Wageningen, The Netherlands) and this industrial stock of phages was used in all experiments to insure reproducibility between assays regarding bacteriophage concentration and population homogeneity. Bacteriophage stock is conserved at 4°C.

Phage activity on *Listeria* inhibition was determined by agar overlay plating method (Kropinski et al., 2009a) and a measure of optical density at 600 nm of *Listeria* culture using Varian Cary® 50 UV-Vis Spectrophotometer from Agilent (Santa Clara, USA).

2.2 Bacteriophage encapsulation

Gellan gum (Phytagel), sodium alginate (from brown algae, 2% viscosity at 25°C), arabic gum (from acacia tree), and gelatine (type B, from bovine skin) were purchased from Sigma Aldrich (Steinheim, Germany).

Encapsulation solutions were prepared as described previously (Ma et al., 2008). Briefly. encapsulation solutions were prepared in deionized water on a percent basis (%w/w) heated in a water bath at 80°C and mixed with a magnetic stirrer plate for 3 h, and then left to cool down at

room temperature (around 22°C). Sodium alginate 2.7, 2.9 and 3% (w/v), arabic gum 1.2 % (w/v), gellan gum 0.3, 0.8 and 1% (w/v) and gelatine 1.1% were combined in different ratios and permutations to ensure testing maximal number of capsule combinations. Polymer percentage was identified from previous studies for sodium alginate and gelatine (Ahmadi et al., 2018), and for gellan gum, it was determined by trial and error by classifiying capsules spherical shape and phage release.

In sterile conditions, 1 mL of phage preparation (10^{11}PFU/mL) was resuspended in 19 ml of each encapsulation solution and mixed for 1 min. The encapsulation solution containing phages was then introduced into a 20 mL disposable syringe with 22 ½ gauge needle and placed 15 cm above a cold beaker containing 0.1 M $CaCl_2$ while continuously stirring with a magnetic stirrer at lowest speed. Minimal pressure was applied to the syringe in order to have single droplets reach the $CaCl_2$ solution. The resulting capsules were left to rest in the $CaCl_2$ solution for 1 h, rinsed three times in distilled water then strained and conserved in sterile 50 mL conical tubes containing 5ml of SM buffer (Fisherbrand, Fisher Scientific International, Inc. Pittsburgh, United States). Capsules were stored at 4°C for long-term storage and a fraction was stored at room temperature for thermal stability tests.

The particle sizes of capsules were determined by ImagJ2 software (Rueden et al., 2017). For each encapsulation formula, 50 capsules were photographed using an iPhone 10X camera with a fixed distance in a Petri dish with a scale.

2.3 Enumeration of bacteriophage P100 titers

To determine bacteriophage P100 titers from capsules, 1 g of capsules was aseptically added to 9 mL of SM buffer in stomacher bags (Stomacher® 80 bags, Seward Laboratory Systems, Inc. Bohemia, NY, United States) and homogenized using a stomacher (Stomacher® 400 lab blender, Seward Laboratory Systems, Inc. Bohemia, NY, United States) set at 150 rpm for 10 min. No-phage capsules and free phages solution were used as control. Samples were used to make serial dilutions in SM buffer and appropriate dilutions plated for enumeration of bacteriophage titers

using the agar overlay assay (Kropinski et al., 2009a). Plates were incubated at 30°C overnight and were counted and presented as PFU/g.

2.4 Thermal treatment of free and encapsulated phage

Encapsulated bacteriophage (EB) effect on bacterial growth inhibition was verified for all capsules by following the decrease of a bacterial solution optical density few hours after adding 5 capsules containing phages (5x 10^9 PFU) in a *L. monocytogenes* culture of OD_{600nm} 0.35 units. Optical density was measured using Varian Cary® 50 UV-Vis Spectrophotometer from Agilent (Santa Clara, USA), bacterial growth decrease was monitored by turbidity as well via a Den-1B densitometer from BioSan (Riga, Latvia). Moreover, control capsules of each polymer combination were made by encapsulating solution extrusion without any phages, the obtained capsules were tested to verify the absence of polymer bioactivity on *Listeria* growth during 24h.

Thermal stability of free and encapsulated phages was tested at different temperatures (70-80-90-100-110°C) for 1 and 5 min. These ranges of temperature and durations were applied based on the conditions of paper coating and drying processes. Longer heat exposure could impact capsule physical stability by drying the hydrogel thus impacting any observation on bacteriophage fate. Briefly, tubes containing sterile EB or free phage solutions were placed in a digital block heater (Fisherbrand™ Isotemp™ Digital Dry Baths/Block Heaters). Temperature was monitored using a thermometer in a control tube containing 300 µl of SM solution and 10 control capsules. Once the thermometer indicated the desired temperature, the tubes containing capsules and phages were placed for either 1 or 5 minutes. Samples and dilutions were then used for lytic activity monitoring.

2.5 Evaluation of antimicrobial activity of encapsulated bacteriophage

To monitor the thermal stability of EB compared to free phages, lytic activity was monitored in liquid culture of *L.monocytogenes.* Free or encapsulated phages were added to a bacterial culture of 10^8-10^9 CFU / ml at phage – bacteria concentration ratio of 10/1(Cooper et al., 2016). Lytic activity was monitored in sterile tubes containing TSB medium at 30 ° C and optical density was

read at 600 nm. For all tests, a version of each polymer without any phages was tested to ensure that lytic activity was due to bacteriophages and not to antibacterial proprieties of the polymers.

2.6 Statistical analysis

All experiments were conducted in triplicates (technical replicates) and analyzed with GraphPad Prism 8.0 software (GraphPad, San Diego, USA). For evaluating the effect of encapsulating material on phage thermal stability, experiments were repeated three independent times. All values were expressed as their means ± standard error (SE). Analysis of variance (ANOVA) was conducted at a confidence interval of 95%.

3. Results

3.1 Physical characterization of encapsulation formulas

To compare the effects of polymer type and amount on the encapsulated phages, twenty combinations were tested, seven combinations are described in this paper based on the spherical shape of the capsules (Table 1). The results showed that capsules composed of 3% sodium alginate (alg) had an opaque appearance and a larger diameter (3.2 ± 0.2 mm) compared to other capsules and allowed phage antimicrobial activity when in contact with *Listeria* culture. Concerning the capsules formed from gellan gum (gg) of different ratios (0.8-1%), they were transparent and of smaller size (1.8 ± 0.3mm) but did not allow the observation of any phage bioactivity in the growth media ($\Delta_{turbidity} > 0$). Arabic gum capsules (ga) were slightly larger than gellan gum capsules but did not allow phage antimicrobial activity as well ($\Delta_{turbidity} > 0$). Sodium alginate and gellan gum (alg-gg) and alginate-gelatine and gellan gum capsules were transparent and did allow phage bioactivity as well as alginate-gelatine and arabic gum capsules (alg-gel-ga; $\Delta_{turbidity} < 0$). Alg-gel-gg and alg-gel-ga are similar in phage antimicrobial activity observation ($\Delta_{turbidity} < 0$).

3.2 Phage retention as a function of capsule composition

Based on phage characterisation results, only capsules made from alg, alg-gel-ga and alg-gel-gg were tested for active phage retention inside the capsules. Each type of capsule was analyzed for the P100 content by capsule homogenisation and serial dilution to obtain a mean active phage content and to compare it to the encapsulation solution theoretical phage content (obtained by calculating phage titer in main dilution of phages without capsules) (Figure 1). The ratio is expressed as a percentage of yield between actual phage titer after capsule homogenization and theoretical phage titer. Among all capsules, those made from alg-gel-gg capsules were the capsules that retained most of the P100 active phages (96%) while alg capsules retained only 6 % of the active phages contained in the encapsulation solution (Figure 1).

3.3 Encapsulation effect on stability during storage

After three months of storage at room temperature, 50% of the original P100 phages activity was lost. Interestingly, alg and alg-gel-ga capsules lost most of the original encapsulated P100 lytic activity after 90 days of storage at room temperature, while alg-gel-gg capsules did not show a significant loss (Figure 2). It is important to note that cold storage (4°C) did not affect the active phage titer in any of the free or encapsulated phages for 90 days (F igure 2).

3.4 Effect of encapsulation on phage thermal stability

Alg-gel-gg and alg-gel-ga EB underwent a thermal treatment by heating capsules to different temperatures and times before studying the effect of this stress on the protection conferred by encapsulating matrix to the phages. *L. monocytogenes* was grown for 2h and then placed in co-culture with free or encapsulated P100. The absorbance was then observed to determine the loss or stability of P100 lytic effect in free or encapsulated forms. As shown in figure 3, free P100 phages can inhibit bacterial growth fast, while bacteria grown in presence of free P100 phages that undergone a heat treatment at 80°during 5 min showed no significant change in the growth curve than *Listeria* grown without phages. Alg, alg-gel-gg and alg-gel-ga encapsulated P00 phages were showing an inhibition of the growth after 8h in a similar tendency for both control and heat-treated

capsules. (Figure 3). Moreover, P100 phage titer was calculated for alg-gel-gg capsules and free P100 (Figure 4). Before heat treatment, free P100 showed 9 log value of phage titers that was reduced to zero after 80°C for 5 min. In contrast, alg-gel-gg encapsulated P100 phages titer did not significantly change after the thermal treatment (8 logs for control compared to 7.7 logs for heat-treated capsules).

Alg, alg-gel-gg and alg-gel-ga encapsulated phages were also tested at higher temperatures. Alg-gel-ga and alg-gel-gg were stable and kept lytic activity even when exposed for 110°C during 5 min (Table 2).

3.5 Effect of encapsulation on phage mechanical stability

In order to get reproducibility among treatments, free phages and encapsulated phages were homogenized for 10 min at 150 rpm before phage titre count by agar overlay method. However, it was observed that free phages were not fully active after such treatment. Homogenized free phages show a significant difference of 6 logs of activity loss compared to non-homogenized free phages (one-way Anova test) ; in contrast, encapsulated phages maintained an unchanged phage content after homogenization (figure 5).

4. Discussion

Bacteriophages have been successfully used as biocontrol agents when harsh conditions are not involved in the food applications (Moye et al., 2018). Phage stability decrease after high temperature treatments appeared to be a limitation to bacteriophage usage. Specifically, *Listeria* phages P100 and A511 were shown to be partially inactivated at 70° C (Ahmadi et al., 2018). To bypass this challenge, several research groups developed a method to insure phage protection from thermal stress by encapsulation in polysaccharides/gelatine polymers (Ahmadi et al., 2018; Solanki et al., 2013). Successful bacteriophage capsules require a maximal retention of phages inside the encapsulation solution leading to capsules as well as phage stability during storage and high temperature exposure (Lone et al., 2016a). EB were studied here by evaluating host-virus interaction. This overlaps with long-term purpose of this research by improving bacteriophage encapsulation formula for optimal antimicrobial activity by following bacterial growth decrease via absorbance and turbidity or by counting active phage titer via agar overlay method.

In this study, optimal composition of encapsulation solution for both features was found with alginate-gelatin-gellan gum which showed high P100 phage retention (96% ±2.1), resistance to up 110°C exposure during 5 min and stability over 3 months of storage at room temperature. Similar results were found for a similar polysaccharide/gelatine composition capsules of *Listeria* phages A511 with a thermostability to 60-70°C (Ahmadi et al., 2018). It is possible that the increased effectiveness of phage stability in alginate-gelatine-gellan gum capsules observed in this work compared to previous observations is due to the selected phage and *Listeria* strain (P100 and *L. monocytogenes* strain ATCC 19115 serotype 4b). The choice of the strain was based on its availability as well as P100 phages enhanced bioactivity against *L. monocytogenes* compared to other *Listeria* phages. It could be important to apply the same conditions and polymer combinations from this study to different phages, such as A511, and *Listeria* strains, for instance, serotype 1/2b; and observe if the effectiveness of the capsule is strain independent. The difference could also be related to the usage of different gauge for encapsulation solution extrusion (16½ gauges in Ahmadi et al., 2018 vs 22½ gauges in this study) and different incubation temperatures (37°C in Ahmadi et al., 2018 vs 30°C in this study).

Along with strain-phage choice, polymer combinations in each capsules type have certainly influenced their behavior for both phage retention and thermal stability. For instance, sodium alginate is an anionic polysaccharide composed of D-mannuronic and L-guluronic units linked in carbons 1-4 which results in distribution of negative charges along the backbone of the polysaccharides once dissolved in water (Thu et al., 1996). While gelatin, as an amphoteric species helps ionic bonds to anionic polysaccharides such as sodium alginate (Sun & Griffiths, 2000), thus contributing to the consolidation of the different components of the encapsulating solution and therefore higher performing capsules. The success reported by using gelatine and alginate for encapsulation have been reported by other authors (Aguirre Calvo & Santagapita, 2016). Similarly to sodium alginate, gellan gum is an anionic polysaccharide formed of glucose, glucuronic acid, and rhamnose (Solanki et al., 2013). In another study, a blend of 1% gellan gum, 2% sodium and peptides protected probiotic *Bifidobacterium bifidum* during an exposure to 75 °C for 1 min (Chen & Li, 2007). Similar protection was provided by alginate and whey protein making encapsulated Felix O *Salmonella* phages more resistant to pH (Ergin et al., 2021). One common aspect was

repeated in many studies, the correlation between higher alginate composition and capsule size, as well as content protection (Ahmadi et al., 2018; Ergin et al., 2021)

This study showed the effectiveness for *Listeria* phages P100 encapsulation in previously obtained polymer blend (Ahmadi et al., 2018) used to encapsulate *Listeria* phages A511 in similar and more extreme temperature (up to 110°C). Moreover, a new blend of polysaccharides and gelatine is proven to be more effective for P100 retention and thermostability. Even though both blends are similar for many aspects, it is important to note that the composition of Alginate-gelatin-arabic gum is done by mixing respectively 2.9%, 1.1% and 1.2% w/v of each component in aqueous solution, while the new combination presented in this article is formed by mixing respectively 2.9%, 1.1% and 0.3% w/v of alginate, gelatine and gellan gum. From an industrial point of view, the cost of the novel polymer is more appealing considering the comparable effect on phage thermostability. Beside thermal stability, encapsulation material used in this study allowed a protection against mechanical stress.

5. Conclusion

Optimal blend of sodium alginate, gellan gum and gelatine was determined using response to high temperature of encapsulated P100 phages. Polymer type and concentrations in each capsule type did not only affect the thermostability but also influenced the bead size and aspect. The newly formulated combination of sodium alginate, gellan gum and gelatine is found to be more efficient for phage retention inside capsules, phage release in presence of *Listeria*, stability of encapsulated phages during room temperature storage as well as thermal resistance at high temperature compared to previously obtained capsules. It is also important to note that this formula of encapsulation allows the use of encapsulated phages in food processing even in presence of ultra-high temperature and not only pasteurization. This provides new insights on novel bioactive packaging in the aim of reducing foodborne disease.

6. Declaration of competing interest

The Authors declare that they have no personal relationships or competing financial interests that could influence the work reported in this paper.

7. Acknowledgement

Authors would like to thank Kruger Inc. and Natural Sciences and Engineering Research Council of Canada (NSERC) for financing this project. Authors would also like to thank Guy Samson for his valuable input on the article and Francois Brouillette for his advices on the project.

References

Aguirre Calvo, T., & Santagapita, P. (2016). Physicochemical characterization of alginate beads containing sugars and biopolymers. *Journal of Quality and Reliability Engineering*, *2016*.

Ahmadi, H., Wang, Q., Lim, L.-T., & Balamurugan, S. (2018). Encapsulation of Listeria phage A511 by alginate to improve its thermal stability. In *Bacteriophages* (pp. 89–95). Springer.

Akhverdyan, V. Z., Gak, E. R., Tokmakova, I. L., Stoynova, N. V., Yomantas, Y. A., & Mashko, S. V. (2011). Application of the bacteriophage Mu-driven system for the integration/amplification of target genes in the chromosomes of engineered Gram-negative bacteria—Mini review. *Applied Microbiology and Biotechnology*, *91*(4), 857–871.

Authority: The European Union summary report on trends... - Google Scholar. (n.d.). Retrieved 15 February 2022, from https://scholar.google.com/scholar_lookup?title=The%20European%20Union%20summary%20report%20on%20trends%20and%20sources%20of%20zoonoses%2C%20zoonotic%20agents%20and%20food-borne%20outbreaks%20in%202015&publication_year=2016&author=EFSA

Bagheri-Khoulenjani, S., Mirzadeh, H., Etrati-Khosroshahi, M., & Shokrgozar, M. A. (2016). Development of a method for measuring and modeling the NH2 content and crosslinking density of chitosan/gelatin/nanohydroxyapatite based microspheres. *Polymer Testing*, *51*, 20–28.

Buruaga-Ramiro, C., Valenzuela, S. V., Valls, C., Roncero, M. B., Pastor, F. I. J., Díaz, P., & Martinez, J. (2020). Development of an antimicrobial bioactive paper made from bacterial cellulose. *International Journal of Biological Macromolecules*, *158*, 587–594. https://doi.org/10.1016/j.ijbiomac.2020.04.234

Canada, P. H. A. of. (2016, August 10). *Listeriosis (Listeria)* [Education and awareness]. https://www.canada.ca/en/public-health/services/diseases/listeriosis.html

Carlton, R. M., Noordman, W. H., Biswas, B., De Meester, E. D., & Loessner, M. J. (2005). Bacteriophage P100 for control of Listeria monocytogenes in foods: Genome sequence, bioinformatic analyses, oral toxicity study, and application. *Regulatory Toxicology and Pharmacology*, *43*(3), 301–312.

Charpentier, E., Gerbaud, G., Jacquet, C., Rocourt, J., & Courvalin, P. (1995). Incidence of antibiotic resistance in Listeria species. *Journal of Infectious Diseases*, *172*(1), 277–281.

Chen, Y.-L., & Li, Q.-Z. (2007). Prediction of apoptosis protein subcellular location using improved hybrid approach and pseudo-amino acid composition. *Journal of Theoretical Biology*, *248*(2), 377–381.

Chernomordik, A. B. (1989). Bacteriophages and their therapeutic-prophylactic use. *Meditsinskaia Sestra*, *48*(6), 44–47.

Chibeu, A., Agius, L., Gao, A., Sabour, P. M., Kropinski, A. M., & Balamurugan, S. (2013). Efficacy of bacteriophage LISTEX™ P100 combined with chemical antimicrobials in reducing Listeria monocytogenes in cooked turkey and roast beef. *International Journal of Food Microbiology*, *167*(2), 208–214.

Chlebicz, A., & Śliżewska, K. (2018). Campylobacteriosis, salmonellosis, yersiniosis, and listeriosis as zoonotic foodborne diseases: A review. *International Journal of Environmental Research and Public Health*, *15*(5), 863.

CHURCHILL, K. J., SARGEANT, J. M., FARBER, J. M., & O'CONNOR, A. M. (2019). Prevalence of Listeria monocytogenes in Select Ready-to-Eat Foods—Deli Meat, Soft Cheese, and Packaged Salad: A Systematic Review and Meta-Analysis. *Journal of Food Protection*, *82*(2), 344–357. https://doi.org/10.4315/0362-028X.JFP-18-158

Coeffic, T., Thibaut, S., Boutoille, D., & Caillon, J. (2018). Évolution de la résistance aux antibiotiques des souches d'Escherichia coli productrices de bêtalactamase à spectre étendu. *Médecine et Maladies Infectieuses*, *48*(4), S48.

Colom, J., Cano-Sarabia, M., Otero, J., Aríñez-Soriano, J., Cortés, P., Maspoch, D., & Llagostera, M. (2017). Microencapsulation with alginate/CaCO3: A strategy for improved phage therapy. *Scientific Reports*, *7*(1), 1–10.

Conter, M., Paludi, D., Zanardi, E., Ghidini, S., Vergara, A., & Ianieri, A. (2009). Characterization of antimicrobial resistance of foodborne Listeria monocytogenes. *International Journal of Food Microbiology*, *128*(3), 497–500.

Cooper, C. J., Khan Mirzaei, M., & Nilsson, A. S. (2016). Adapting drug approval pathways for bacteriophage-based therapeutics. *Frontiers in Microbiology*, *7*, 1209.

Das, M., & Giri, T. K. (2020). Hydrogels based on gellan gum in cell delivery and drug delivery. *Journal of Drug Delivery Science and Technology*, *56*, 101586. https://doi.org/10.1016/j.jddst.2020.101586

de Noordhout, C. M., Devleesschauwer, B., Angulo, F. J., Verbeke, G., Haagsma, J., Kirk, M., Havelaar, A., & Speybroeck, N. (2014). The global burden of listeriosis: A systematic review and meta-analysis. *The Lancet Infectious Diseases*, *14*(11), 1073–1082.

Deshwal, G. K., Panjagari, N. R., & Alam, T. (2019). An overview of paper and paper based food packaging materials: Health safety and environmental concerns. *Journal of Food Science and Technology*, *56*(10), 4391–4403.

Doss, J., Culbertson, K., Hahn, D., Camacho, J., & Barekzi, N. (2017). A review of phage therapy against bacterial pathogens of aquatic and terrestrial organisms. *Viruses*, *9*(3), 50.

Dufour, N., Delattre, R., Chevallereau, A., Ricard, J.-D., & Debarbieux, L. (2019). Phage therapy of pneumonia is not associated with an overstimulation of the inflammatory response compared to antibiotic treatment in mice. *Antimicrobial Agents and Chemotherapy*, *63*(8), e00379-19.

Ergin, F., Atamer, Z., Göcer, E. M. C., Demir, M., Hinrichs, J., & Kucukcetin, A. (2021). Optimization of Salmonella bacteriophage microencapsulation in alginate-caseinate formulation using vibrational nozzle technique. *Food Hydrocolloids*, *113*, 106456.

Gombotz, W. R., & Wee, S. (1998). Protein release from alginate matrices. *Advanced Drug Delivery Reviews*, *31*(3), 267–285.

Gonzalez-Menendez, E., Fernandez, L., Gutierrez, D., Rodriguez, A., Martinez, B., & Garcia, P. (2018). Comparative analysis of different preservation techniques for the storage of Staphylococcus phages aimed for the industrial development of phage-based antimicrobial products. *PLoS One*, *13*(10), e0205728.

Government of Canada, C. F. I. A. (2019, October 8). *Food safety investigation: Various brands of cooked, diced and shredded chicken products may contain Listeria monocytogenes* [Reference material]. https://inspection.canada.ca/about-cfia/transparency/regulatory-transparency-and-openness/food-safety-investigations/various-brands-of-chicken-products/eng/1570129428140/1570129428596#a5

Government of Canada, H. C. (2021, August 25). *Pinty's brand chicken breast products recalled due to Listeria—Recalls, advisories and safety alerts – Canada.ca*. Government of Canada, Health Canada, CFIA. https://recalls-rappels.canada.ca/en/alert-recall/pinty-s-brand-chicken-breast-products-recalled-due-listeria

Guenther, S., Huwyler, D., Richard, S., & Loessner, M. J. (2009). Virulent bacteriophage for efficient biocontrol of Listeria monocytogenes in ready-to-eat foods. *Applied and Environmental Microbiology*, *75*(1), 93–100.

Hazards (BIOHAZ), E. P. on B. (2016). Evaluation of the safety and efficacy of Listex^{TM} P100 for reduction of pathogens on different ready-to-eat (RTE) food products. *EFSA Journal*, *14*(8), e04565. https://doi.org/10.2903/j.efsa.2016.4565

Heymann, D. L., Chen, L., Takemi, K., Fidler, D. P., Tappero, J. W., Thomas, M. J., Kenyon, T. A., Frieden, T. R., Yach, D., & Nishtar, S. (2015). Global health security: The wider lessons from the west African Ebola virus disease epidemic. *The Lancet*, *385*(9980), 1884–1901.

Jabrane, T., Laloi, M., Dubé, M., & Mangin, P. (2010). Printing and coating of T4 phage based bioactive paper. *Advances in Printing and Media Technology*, *37*, 351–358.

Jamal, M., Bukhari, S. M., Andleeb, S., Ali, M., Raza, S., Nawaz, M. A., Hussain, T., Rahman, S. U., & Shah, S. S. (2019). Bacteriophages: An overview of the control strategies against multiple bacterial infections in different fields. *Journal of Basic Microbiology*, *59*(2), 123–133.

Khwaldia, K., Arab-Tehrany, E., & Desobry, S. (2010). Biopolymer coatings on paper packaging materials. *Comprehensive Reviews in Food Science and Food Safety*, *9*(1), 82–91.

King, V. A. E., & Zall, R. R. (1983). Ethanol fermentation of whey using calcium alginate entrapped yeasts. *Process Biochemistry*, *18*(6), 17–20.

Komora, N., Bruschi, C., Ferreira, V., Maciel, C., Brandão, T. R., Fernandes, R., Saraiva, J. A., Castro, S. M., & Teixeira, P. (2018). The protective effect of food matrices on Listeria lytic bacteriophage P100 application towards high pressure processing. *Food Microbiology*, *76*, 416–425.

Komora, N., Bruschi, C., Magalhães, R., Ferreira, V., & Teixeira, P. (2017). Survival of Listeria monocytogenes with different antibiotic resistance patterns to food-associated stresses. *International Journal of Food Microbiology*, *245*, 79–87.

Kopacic, S., Walzl, A., Zankel, A., Leitner, E., & Bauer, W. (2018). Alginate and chitosan as a functional barrier for paper-based packaging materials. *Coatings*, *8*(7), 235.

Kropinski, A. M., Mazzocco, A., Waddell, T. E., Lingohr, E., & Johnson, R. P. (2009a). Enumeration of bacteriophages by double agar overlay plaque assay. In *Bacteriophages* (pp. 69–76). Springer.

Kropinski, A. M., Mazzocco, A., Waddell, T. E., Lingohr, E., & Johnson, R. P. (2009b). Enumeration of Bacteriophages by Double Agar Overlay Plaque Assay. In M. R. J. Clokie & A. M. Kropinski (Eds.), *Bacteriophages: Methods and Protocols, Volume 1: Isolation, Characterization, and Interactions* (pp. 69–76). Humana Press. https://doi.org/10.1007/978-1-60327-164-6_7

Kuruwita, D. P., Jiang, X., Darby, D., Sharp, J. L., & Fraser, A. M. (2020). Persistence of Escherichia coli O157: H7 and Listeria monocytogenes on the exterior of three common food packaging materials. *Food Control*, *112*, 107153.

Leverentz, B., Conway, W. S., Camp, M. J., Janisiewicz, W. J., Abuladze, T., Yang, M., Saftner, R., & Sulakvelidze, A. (2003). Biocontrol of Listeria monocytogenes on fresh-cut produce by treatment with lytic bacteriophages and a bacteriocin. *Applied and Environmental Microbiology*, *69*(8), 4519–4526.

Liu, D. (2006). Identification, subtyping and virulence determination of Listeria monocytogenes, an important foodborne pathogen. *Journal of Medical Microbiology*, *55*(6), 645–659.

Liu, D., Lawrence, M. L., Ainsworth, A. J., & Austin, F. W. (2005). Comparative assessment of acid, alkali and salt tolerance in Listeria monocytogenes virulent and avirulent strains. *FEMS Microbiology Letters*, *243*(2), 373–378.

Loessner, M. J. (2005). Bacteriophage endolysins—Current state of research and applications. *Current Opinion in Microbiology*, *8*(4), 480–487. https://doi.org/10.1016/j.mib.2005.06.002

Lone, A., Anany, H., Hakeem, M., Aguis, L., Avdjian, A.-C., Bouget, M., Atashi, A., Brovko, L., Rochefort, D., & Griffiths, M. W. (2016a). Development of prototypes of bioactive packaging materials based on immobilized bacteriophages for control of growth of bacterial pathogens in foods. *International Journal of Food Microbiology*, *217*, 49–58.

Lone, A., Anany, H., Hakeem, M., Aguis, L., Avdjian, A.-C., Bouget, M., Atashi, A., Brovko, L., Rochefort, D., & Griffiths, M. W. (2016b). Development of prototypes of bioactive packaging materials based on immobilized bacteriophages for control of growth of bacterial pathogens in foods. *International Journal of Food Microbiology*, *217*, 49–58.

Ma, Y., Pacan, J. C., Wang, Q., Xu, Y., Huang, X., Korenevsky, A., & Sabour, P. M. (2008). Microencapsulation of bacteriophage Felix O1 into chitosan-alginate microspheres for oral delivery. *Applied and Environmental Microbiology*, *74*(15), 4799–4805.

Miguéis, S., Saraiva, C., & Esteves, A. (2017). Efficacy of LISTEX P100 at Different Concentrations for Reduction of Listeria monocytogenes Inoculated in Sashimi. *Journal of Food Protection*, *80*(12), 2094–2098. https://doi.org/10.4315/0362-028X.JFP-17-098

Morton, V. K., Thomas, M. K., Ciampa, N., Cutler, J., Hurst, M., & Currie, A. (2019). Comparison of 3-day and 7-day recall periods for food consumption reference values in foodborne disease outbreak investigations. *Epidemiology & Infection*, *147*.

Moye, Z. D., Woolston, J., & Sulakvelidze, A. (2018). Bacteriophage applications for food production and processing. *Viruses*, *10*(4), 205.

Nayak, A. K., Das, B., & Maji, R. (2012). Calcium alginate/gum Arabic beads containing glibenclamide: Development and in vitro characterization. *International Journal of Biological Macromolecules*, *51*(5), 1070–1078.

Ndoboli, D., Roesel, K., Heilmann, M., Alter, T., Clausen, P.-H., Wampande, E., Grace, D., & Huehn, S. (2018). Sérotypes et profils de résistance aux antibiotiques de Salmonella enterica subsp. Enterica présente dans le porc et les légumes frais servis dans les points de vente de Kampala, Ouganda. *Revue d'élevage et de Médecine Vétérinaire Des Pays Tropicaux*, *71*(1–2), 103–109.

Nemati, S., Rezabakhsh, A., Khoshfetrat, A. B., Nourazarian, A., Biray Avci, Ç., Goker Bagca, B., Alizadeh Sardroud, H., Khaksar, M., Ahmadi, M., Delkhosh, A., Sokullu, E., & Rahbarghazi, R. (2017). Alginate-gelatin encapsulation of human endothelial cells promoted angiogenesis in in vivo and in vitro milieu. *Biotechnology and Bioengineering*, *114*(12), 2920–2930. https://doi.org/10.1002/bit.26395

Olaimat, A. N., Al-Holy, M. A., Shahbaz, H. M., Al-Nabulsi, A. A., Abu Ghoush, M. H., Osaili, T. M., Ayyash, M. M., & Holley, R. A. (2018a). Emergence of antibiotic resistance in Listeria monocytogenes isolated from food products: A comprehensive review. *Comprehensive Reviews in Food Science and Food Safety*, *17*(5), 1277–1292.

Olaimat, A. N., Al-Holy, M. A., Shahbaz, H. M., Al-Nabulsi, A. A., Abu Ghoush, M. H., Osaili, T. M., Ayyash, M. M., & Holley, R. A. (2018b). Emergence of antibiotic resistance in Listeria monocytogenes isolated from food products: A comprehensive review. *Comprehensive Reviews in Food Science and Food Safety*, *17*(5), 1277–1292.

Osman, K. M., Kappell, A. D., Fox, E. M., Orabi, A., & Samir, A. (2020). Prevalence, Pathogenicity, Virulence, Antibiotic Resistance, and Phylogenetic Analysis of Biofilm-Producing Listeria monocytogenes Isolated from Different Ecological Niches in Egypt: Food, Humans, Animals, and Environment. *Pathogens*, *9*(1), 5.

Paredes Juárez, G. A., Spasojevic, M., Faas, M. M., & de Vos, P. (2014). Immunological and technical considerations in application of alginate-based microencapsulation systems. *Frontiers in Bioengineering and Biotechnology*, *2*, 26.

Park, M. S., Park, K. H., & Bahk, G. J. (2018). Combined influence of multiple climatic factors on the incidence of bacterial foodborne diseases. *Science of the Total Environment*, *610*, 10–16.

Prabhu, G., Husak, R., Peterson, R., Hagens, S., Iancu, C., Riemer, Y., & Engle, R. (2018). Validation of the Effect of Listex P100 Bacteriophage on Survival of Listeria Monocytogenes. *Meat and Muscle Biology*, *1*(2).

Radoshevich, L., & Cossart, P. (2018). Listeria monocytogenes: Towards a complete picture of its physiology and pathogenesis. *Nature Reviews Microbiology*, *16*(1), 32–46.

Ribeiro-Santos, R., Andrade, M., de Melo, N. R., & Sanches-Silva, A. (2017). Use of essential oils in active food packaging: Recent advances and future trends. *Trends in Food Science & Technology*, *61*, 132–140.

Rueden, C. T., Schindelin, J., Hiner, M. C., DeZonia, B. E., Walter, A. E., Arena, E. T., & Eliceiri, K. W. (2017). ImageJ2: ImageJ for the next generation of scientific image data. *BMC Bioinformatics*, *18*(1), 529.

Salmond, G. P., & Fineran, P. C. (2015). A century of the phage: Past, present and future. *Nature Reviews Microbiology*, *13*(12), 777–786.

Salvia-Trujillo, L., Rojas-Graü, M. A., Soliva-Fortuny, R., & Martín-Belloso, O. (2013). Effect of processing parameters on physicochemical characteristics of microfluidized lemongrass essential oil-alginate nanoemulsions. *Food Hydrocolloids*, *30*(1), 401–407.

Sarfraz, M., Ashraf, Y., & Ashraf, S. (2017). A review: Prevalence and antimicrobial susceptibility profile of listeria species in milk products. *Matrix Science Medica*, *1*(1), 03–09.

Sarker, B., Papageorgiou, D. G., Silva, R., Zehnder, T., Gul-E-Noor, F., Bertmer, M., Kaschta, J., Chrissafis, K., Detsch, R., & Boccaccini, A. R. (2014). Fabrication of alginate–gelatin crosslinked hydrogel microcapsules and evaluation of the microstructure and physico-chemical properties. *Journal of Materials Chemistry B*, *2*(11), 1470–1482. https://doi.org/10.1039/C3TB21509A

Sharma, S., Barkauskaite, S., Jaiswal, A. K., & Jaiswal, S. (2020). Essential oils as additives in active food packaging. *Food Chemistry*, 128403.

Sleator, R. D., Gahan, C. G., & Hill, C. (2003). A postgenomic appraisal of osmotolerance in Listeria monocytogenes. *Applied and Environmental Microbiology*, *69*(1), 1–9.

Solanki, H. K., Pawar, D. D., Shah, D. A., Prajapati, V. D., Jani, G. K., Mulla, A. M., & Thakar, P. M. (2013). Development of Microencapsulation Delivery System for Long-Term Preservation of Probiotics

as Biotherapeutics Agent. *BioMed Research International*, *2013*, e620719. https://doi.org/10.1155/2013/620719

Soni, K. A., & Nannapaneni, R. (2010). Removal of Listeria monocytogenes biofilms with bacteriophage P100. *Journal of Food Protection*, *73*(8), 1519–1524.

Soto, M. J., Retamales, J., Palza, H., & Bastías, R. (2018). Encapsulation of specific Salmonella Enteritidis phage f3αSE on alginate-spheres as a method for protection and dosification. *Electronic Journal of Biotechnology*, *31*, 57–60.

Sun, W., & Griffiths, M. W. (2000). Survival of bifidobacteria in yogurt and simulated gastric juice following immobilization in gellan–xanthan beads. *International Journal of Food Microbiology*, *61*(1), 17–25.

Thomas, M. K., Murray, R., Flockhart, L., Pintar, K., Fazil, A., Nesbitt, A., Marshall, B., Tataryn, J., & Pollari, F. (2015). Estimates of foodborne illness–related hospitalizations and deaths in Canada for 30 specified pathogens and unspecified agents. *Foodborne Pathogens and Disease*, *12*(10), 820–827.

Thomas, M. K., Murray, R., Flockhart, L., Pintar, K., Pollari, F., Fazil, A., Nesbitt, A., & Marshall, B. (2013). Estimates of the burden of foodborne illness in Canada for 30 specified pathogens and unspecified agents, circa 2006. *Foodborne Pathogens and Disease*, *10*(7), 639–648.

Thu, B., Bruheim, P., Espevik, T., Smidsrød, O., Soon-Shiong, P., & Skjåk-Bræk, G. (1996). Alginate polycation microcapsules: II. Some functional properties. *Biomaterials*, *17*(11), 1069–1079.

Twede, D. (2005). The origins of paper based packaging. *Proceedings of the Conference on Historical Analysis and Research in Marketing*, *12*, 288–300.

Vartak, N. B., Lin, C. C., Cleary, J. M., Fagan, M. J., & Saier Jr, M. H. (1995). Glucose metabolism in 'Sphingomonas elodea': Pathway engineering via construction of a glucose-6-phosphate dehydrogenase insertion mutant. *Microbiology*, *141*(9), 2339–2350.

Vázquez-Boland, J. A., Kuhn, M., Berche, P., Chakraborty, T., Domínguez-Bernal, G., Goebel, W., González-Zorn, B., Wehland, J., & Kreft, J. (2001). Listeria pathogenesis and molecular virulence determinants. *Clinical Microbiology Reviews*, *14*(3), 584–640.

Walsh, D., Duffy, G., Sheridan, J. J., Blair, I. S., & McDowell, D. A. (2001). Antibiotic resistance among Listeria, including Listeria monocytogenes, in retail foods. *Journal of Applied Microbiology*, *90*(4), 517–522.

Wikswo, M. E., Kambhampati, A., Shioda, K., Walsh, K. A., Bowen, A., & Hall, A. J. (2015). Outbreaks of acute gastroenteritis transmitted by person-to-person contact, environmental contamination, and unknown modes of transmission—United States, 2009–2013. *Morbidity and Mortality Weekly Report: Surveillance Summaries*, *64*(12), 1–16.

Zhang, X., Wang, S., Chen, X., & Qu, C. (2021). Review controlling Listeria monocytogenes in ready-to-eat meat and poultry products: An overview of outbreaks, current legislations, challenges, and future prospects. *Trends in Food Science & Technology*, *116*, 24–35. https://doi.org/10.1016/j.tifs.2021.07.014

Zink, R., & Loessner, M. J. (1992). Classification of virulent and temperate bacteriophages of Listeria spp. On the basis of morphology and protein analysis. *Applied and Environmental Microbiology*, *58*(1), 296–302.

.

Table 1: Properties of capsules formed from different polymers combination.

Capsule	Composition (% w/v)				Phage liberation*	Aspect	Mean diameter (mm) **
	Sodium alginate	Gelatin	Arabic gum	Gellan			
alg	3	0	0	0	+	opaque	3.2 ±0.2
gg	0	0	0	0.8	-	transparent	1.8 ±0.3
ga	0	0	1.2	0	-	translucent	2.1 ±0.2
alg-gg	2.7	0	0	0.3	+	translucent	2.4 ±0.1
alg-gel-gg	2.9	1.1	0	0.3	+	translucent	2.5 ±0.3
alg-gel-ga	3	1.1	1.2	0	+	translucent	2.8 ±0.2

* Phage liberation was determined by following biolytic activity of encapsulated capsules in a liquid culture of *Monocytogenes.*

**50 capsules were counted. Values following ± correspond to the SD.

Table 2: Thermal stability of free and encapsulated P100 phages under extreme temperatures and for different times.

Temperature (°C)	80		90		100		110	
Heating time (min)	1	5	1	5	1	5	1	5
Free P100 phages	x	x	x	x	x	x	x	x
P100 phages encapsulated in alg	√	√	√	√	√	x	x	x
P100 phages encapsulated in alg-gel-ga	√	√	√	√	√	√	√	√
P100 phages encapsulated in alg-gel-gg	√	√	√	√	√	√	√	√

√ : stable phages, x : unstable phages

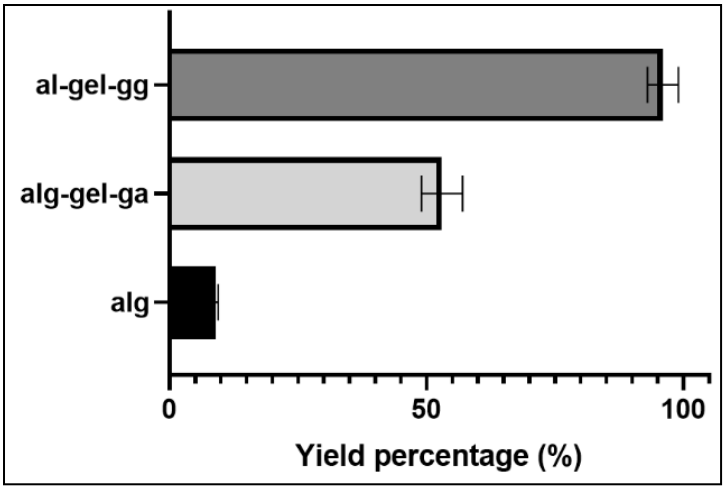


Figure 1: Relative P100 phage content of different capsule formulations in alginate (black), alginate-gelatine and arabic gum (light grey), and alginate-gelatine and gellan gum (dark grey)

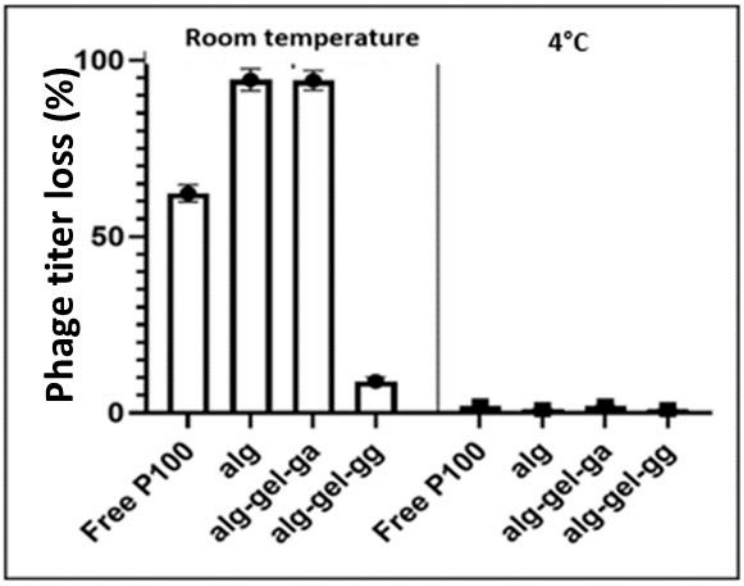


Figure 2: Effect of storage temperatures on phage activity lost of encapsulated and free phages stored at room temperature and 4ºC for 90 days.

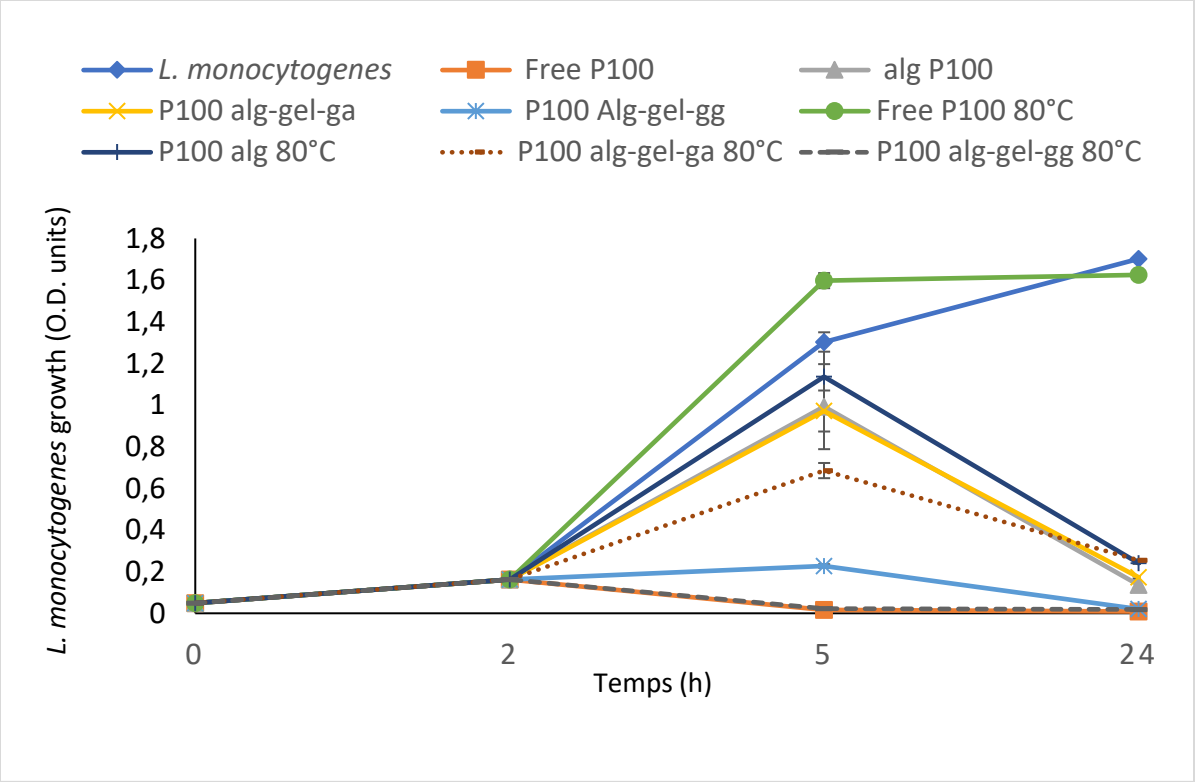


Figure 3: Effect of heat treatment on lytic activity against *L. monocytogenes* of free and encapsulated P100 phage: *L. monocytogenes* optical density without phage (diamond), with free P100 (square), heated free P100 (circle), alginate encapsulated P100 (triangle), heated alginate encapsulated P100 (strike), alginate-gelatine and arabic gum encapsulated P100 (cross), heated alginate-gelatine and arabic gum encapsulated P100 (dotted line), alginate-gelatine and gellan gum encapsulated P100 (asterix) and heated alginate-gelatine and gellan gum encapsulated P100 (dashed line).

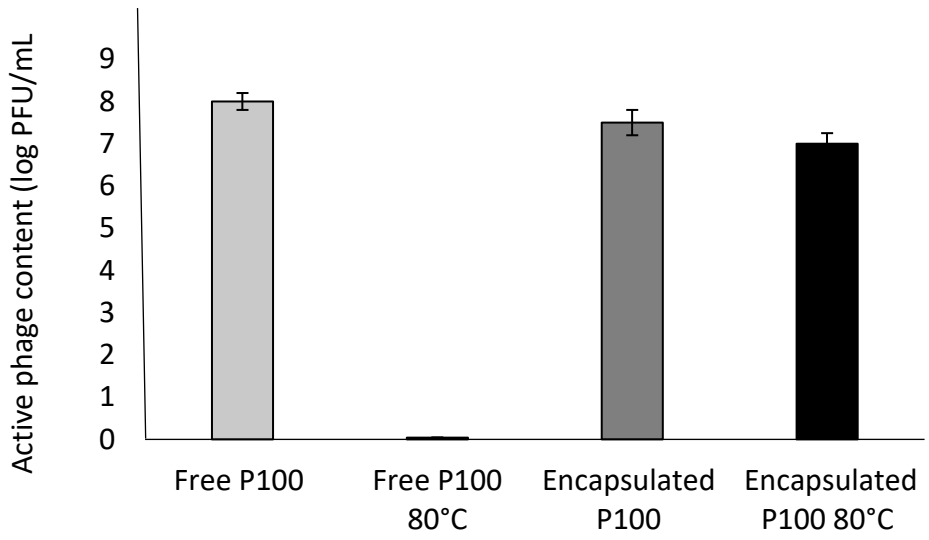


Figure 4: Active phage count (Log PFU/mL) in free and alginate-gelatine-gellan gum encapsulated P100 without and with heat treatment at 80 ºC for 5 min

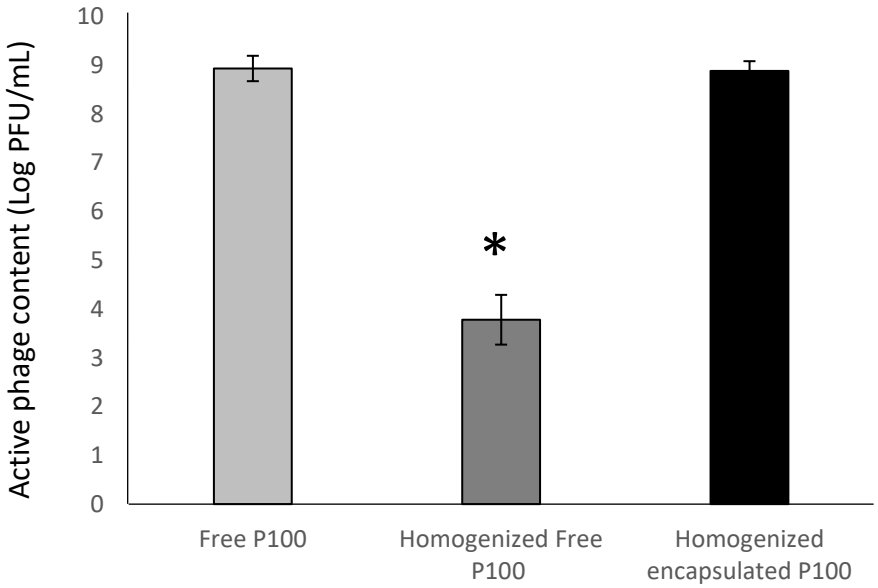


Figure 5: Active phage count (Log PFU/mL) in free P100 phages (light grey) and free P100 phages (dark grey) and encapsulated P100 phages (black) after homogenization in stomacher. Asterix (*) indicates significant difference based on one-way Anova test.

CHAPITRE III

Novel procedure for obtaining bioactive base paper to reduce listeriosis impact in food packaging

Article en attente de soumission à
La revue scientifique *Journal of Food Microbiology.*

Mehdi Harb[1], Lai Thanh Tung[1], Ha Thanh Pham[1], Sylvain Moineau[2], Tarik Jabrane[1,3], Simon Barnabé[1]

[1]Institut d'innovations en Écomatériaux, Écoproduits et Écoénergies, Université du Québec à Trois-Rivières, 3351, boul. des Forges, C.P. 500, Trois-Rivières, QC, Canada G9A 5H7
[2] Université de Laval, Québec, QC, Canada
[3] Innofibre, Cégep de Trois-Rivières, Trois-Rivières, QC, Canada

Corresponding author
simon.barnabe@uqtr.ca (S. Barnabé)

Abstract

Listeria monocytogenes is the causative agent of foodborne disease Listeriosis and is responsible of high hospitalization and fatality rates. Because of its persistence on multiple food packaging material, it is very difficult to reduce *L. monocytogenes* incidence in ready-to-eat food. Here, we propose, for the first time, the conception and characterization of a *Listeria*-phage containing bioactive base paper that allows phages to maintain activity based on coating with a novel biopolymer mixture. The mixture of biopolymers consisting of sodium alginate 2.7 % (w/v), gellan gum 0.3 % (w/v) and gelatin 1.1% with Listex P100 bacteriophages was characterized for phage stability in paper industry environment procedures. This work paves the road toward effective and practical bioactive food packaging systems.

Keywords: Listeriosis, bacteriophages, bioactive food packaging, biopolymers, paper coating

Abbreviations:

AGE acute gastroenteritis

CFU colony forming unit

GRAS generally recognized as safe

PFU phage forming unit

RTE ready-to-eat

1. Introduction

Since late 1800s, papermaking machines and printers have revolutionized the industry in general and specifically food industry. Paper-based packaging initially served the purpose of protecting the product and marketing for the manufacturer (Twede, 2005). Although paper-based machine were initially produced from wood pulp material, specifically a plant cell wall polymer, cellulose, future improvements of the quality of the paper packaging required using other biopolymers such as chitosan, beeswax and alginate; such coating material allowed the obtention of reduced oxygen permeability paper and better oil barrier, essentials proprieties in food packaging. (Deshwal et al., 2019; Khwaldia et al., 2010; Kopacic et al., 2018)

Acute gastroenteritis (AGE) is one of the diseases caused by foodborne bacteria such as *Escherichia coli* O157:H7 and *Listeria monocytogenes,* causing each year around 180 million cases in the United States alone, and 4 millions in Canada (Thomas et al., 2013; Wikswo et al., 2015). Moreover, some researchers established tight relationship between climate changes and increase in hospitalization caused by foodborne diseases related to 13 bacterial strains (Park et al., 2018), which elucidate the importance of finding new tools to limit the propagation and survival of bacteria in ready-to-eat foods such as Deli meats and packaged salads (Churchill et al., 2019). These bacterial species are known to persist for 15 days on common food packaging products such as oriented polyethylene terephthalate (OPET), oriented polypropylene (OPP), and nylon-6 (Kuruwita et al., 2020). *Listeria monocytogenes* specifically, is able to grow in both anaerobic and aerobic conditions, persists at cold and medium high temperatures (−1.5 °C to 45 °C),with a wide range of pH (4.0–9.6) and could survive up to 13% wt of salt content (Zhang et al., 2021). *Listeria* strains are also capable of developing biofilms on multiple surfaces through a mechanism of quorum sensing involving extracellular DNA, lipids and signaling molecules (Osman et al., 2020; Zhang et al., 2021).

One of the most challenging steps in food industry is packaging in a way to preserve food quality and reduce food-based intoxication. Bioactive food packaging is when the packaging offers a protection from outer environment and reduces foodborne bacterial propagation. Multiple approaches in food packaging are relying on incorporating active agents in the packaging like enzymes such as lysozymes that are able to destroy bacterial cell walls (Buruaga-Ramiro et al.,

2020), or essential oils such as terpenes from citrus, lavender or green tea plants as antioxidative agents (Ribeiro-Santos et al., 2017; Sharma et al., 2020). In this paper, we describe the usage of phages as bioactive agents in food packaging, more specifically *Listex* P100 phages that are generally recognized as safe (GRAS) like lysozymes. Beside the FDA approval on Listex P100 as a biocontrol agent, the European European Food Safety Authority (EFSA) conducted a study on the efficacy of this commercial product and a partial positive opinion for its application was issued in 2016 (*Authority: The European Union Summary Report on Trends... - Google Scholar*, n.d.). Nonetheless, the application of phages in food industry was a challenge because of the nature of this bioactive agent and it sensitivity to a variety of conditions such as heat, pH and presence of non-sensitive bacteria, such challenges were encountered in previous attempts of producing bio-inks, or phage-treatment for food surfaces in order to reduce *Listeria* (Miguéis et al., 2017; Prabhu et al., 2018). For instance, (Lone et al.,2016b) and his collaborators established a bioactive cellulose-based film that allows immobilization of phages in order to reduce *E.coli* and *L. monocytogenes* incidence in cantaloupes and other example. In this study, we aim to apply phages on paper in the alginate-gellan gum-gelatin biopolymer solution that was proven to protect Listex phages mainly against high temperatures as shown in previous chapter. The biopolymers are essentially sugars that are accessible and interactive with different humidity and acidity conditions allowing access of phages to the bacteria during the food conservation process.

Commercially available and GRAS Listex phages P100 used in this study, were previously characterized (Miguéis et al., 2017; Prabhu et al., 2018) and proven to be efficient against *L. monocytogenes* in a ratio of 1 CFU to 10 PFU (Miguéis et al., 2017). Listex phages P100 measure between 200 and 400 nm and are able to specifically affect *L. monocytogenes* growth by lytic cycle. The potential use of Listex P100 in RTE-food such as milk, orange juice, cheese and sausages, was previously investigated as a combined approach with hydrostatic pressure to eliminate foodborne listeriosis (Komora et al., 2018). Combination of Listex P100 with antimicrobial chemicals such as potassium lactate have been also investigated showing that these bacteriophages are able to perform an efficient biocontrol function in presence of different chemical and physical conditions (Chibeu et al., 2013). Moreover, multiple studies investigated the effect of pH and temperature on phage activity and recovery through storage (Lone et al., 2016b; Prabhu et al., 2018; Zhang et al., 2021), but all researchers agree on the importance of adapting a suitable application of Listex 100 accordingly with the food matrix. This implies not

only focusing on phage activity and count but also on the accessibility to the bacterial colonies. This paper describes the obtention of a prototype of bioactive paper, Listex P100 is used as a first biocontrol agent but could be combined with different elements in the future.

In this study, bioactive papers were fabricated by coating phages incorporated into biopolymers. The bioactive papers were stored at different temperatures and for different time periods in order to evaluate the kinetics of conserved bioactive phages under different conditions. The procedure of making the bioactive base paper were made in respect with procedure used in North American pulp and paper industry such as instruments recommendation and drying temperatures.

2. Materials and methods

Materials

Coating solution preparation

The alginate-gellan gum-gelatin polymer solution was prepared as follows:.the solution were prepared in sterile deionized water on a percent basis (% w/w) heated in a water bath at 65°C and mixed with a magnetic stirrer plate for 1 h. Sodium alginate in 2.7 % (w/v), gellan gum 0.3 % (w/v) and gelatin 1.1% were combined in 100 ml of water and the final volume was divided into multiple 10 ml in 15 ml falcon tubes. In sterile conditions, 1 mL of phage preparation (10^{11}PFU/mL) or saline magnesium solution (as negative control) was resuspended in 9 ml of each encapsulation solution and mixed gently for 1 min. The polymer solution containing phages was then kept in a water bath at 40^{O}C until coating the paper to avoid early polymerization.

Paper coating

Base paper was manufactured and supplied by Kruger Inc (Ontario, Canada) of basis weight 50 g/m^2. A4 – size base paper sheets were then UV-sterilized for 30 min and stored in a sterile box. The papers were coated with the polymer solution without phage or with the polymer-phage solution using Mayer rod #8 (RD Specialties, Inc, Webster, NY, USA). The coated papers were

then divided into two groups, one that was dried under a sterile hood and one that was undergone a temperature of 75 ^{O} C during drying process under RK print coat instrument (RK Ltd, Littington, UK). A total of ten papers were considered for each condition (n=10).

Paper characterization

Paper thickness was measured using ProGage instrument from Thwing-Albert instrument company (Philadelphia, PA, USA). Paper surface roughness was measured using Parker-Print Surf machine Messmer MS596 as described by the fabricant (Messmer Büchel, Devens, MA, USA). Paper tensile wet and dry strength, breaking length and stretch were measured using Instron 5565 - pull tester (Norwood, MA, USA) with a band width of 15 mm, 100 mm distance between clamps 10 mm/min movement speed. For all measurements, average and standard deviation was calculated on ten samples of paper (n=10), and statistical tests were conducted (two-way ANOVA and Student tests). The full paper coating experiment and all the characterization was performed in three independent experiments separated by two months each. The characterization steps and workflow is described in figure 1.

Phage enumeration

Each category of obtained coated paper was divided into 486 cm^2 cuts (equivalent to 0.5 g of applied phage-containing polymer) and preserved for storage tests and phage titer determination. To determine bacteriophage P100 titers that remained active in paper, each replicate was aseptically transferred into stomacher bags (Stomacher® 80 bags, Seward Laboratory Systems, Inc. Bohemia, NY, United States) containing 9.5 mL of SM buffer and homogenized using a stomacher (Stomacher® 80 microBiomaster lab blender, Seward Laboratory Systems, Inc. Bohemia, NY, United States) set at 100 rpm for 60 s. Samples were used to make serial dilutions in SM buffer and appropriate dilutions plated for enumeration of bacteriophage titers using the agar overlay assay (Kropinski et al., 2009b) using *L. monocytogenes* ATCC 19115 as the host strain, and plating on TSA agar, respectively. Resulting plaques on the agar overlay plates incubated at 30°C for 18 h were counted and presented as PFU/g. For each experiment, ten papers were used per condition (n=10) and 3 triplicates of polymer or biopolymer coted paper were used for each dilution for statistical accuracy. One-way ANOVA test was used to determine significance among independent repetitions and T-test to compare in each repetition ($p \leq 0.05$).

3. Results

Physical properties of coated paper

Phage-polymer-coated paper physical proprieties were tested before and after drying in the RK print coat instrument at a temperature of 75^{O}C. While paper grammage varied between 51,78 g/m^2 before drying and 49.75 g/m^2 after drying, the phage-polymer-coated paper weighed 125.88 g/m^2 before drying and 60.05 g/m^2 after drying the polymer coat using the RT print coat instrument (figure 2). This means that the polymer-phage layer added 10 g/m^2 to the dry paper grammage knowing that in paper and pulp industry, any modification to the base paper should not exceed the initial grammage of the paper without modifications.

The dried paper was also tested for water permeability by using Parker-Print Surf machine Messmer MS596, where the measurement indicates the roughness or the rugosity of the obtained paper. The base paper measurements gave higher than 50 ml/min roughness or rugosity while the coated paper was obviously less rough (1.3 ml/min, figure 3). This indicates that the addition of the polymer sealed the pores of the butcher base paper and made it smoother or less rough.

Another physical propriety that is important in packaging is the thickness of the obtained product. For this, ten samples of base paper were measure against 10 samples of polymer-phage-coated paper (figure 4). Base paper average thickness was 58.52 µm while coated paper was thicker (69.76 µm). This means that the polymer-phage mixture formed a layer of approximatively 10 µm after drying.

Finally, wet and dry textile break or strength, as well as the stretch of the obtained paper, were tested using an Instron pull tester. Difference between base paper and polymer-phage-coated paper were insignificant for the dry strength (around 70 N), while the wet strength of the polymer-phage-coated paper was 6 times less (figure 5a). Regarding stretch percentage, the polymer-phage-coating provided a slightly higher stretch percentage to the butcher base paper, a difference of 0.7% more stretch was observed (Figure 5b).

Bioactivity of coated paper

The polymer-phage-coated paper was then assayed for its phage content since the application and the drying could affect the initial number of active phages. More than one log of active phages

was lost after drying the polymer-phage-coated paper in the RK coat print instrument at 75^{O}C (Table 1). The final titer of active phages after drying is 3.4 x10^{4}PFU/cm^{2}. This value is higher by 3.4 x10^{2} times the maximum *Listeria* counts permitted in food by FDA regulation. Heat dried polymer-phage-coated paper showed a significant loss on phage titer (0.03 x10^{2} PFU/cm^{2}) after storing for 3 weeks at room temperature, while the loss was more than one log lower when the heat dried paper was conserved at 4^{O}C for the same period (3 weeks). This indicates that the storage conditions affect greatly the stability of active phages in the polymer-phage-coated paper.

On the other hand, applying polymer-phage coated paper on a *L. monocytogenes* biofilm lead to the inhibition of the bacterial growth indicating that phages were able to access the bacterial cells on petri dish (supplementary figure 2)

4. Discussion

The obtained paper was characterized for known industrial proprieties such as thickness, roughness, strength and stretch. On the physical aspect, the obtained paper respects the general outlines of paper and pulp industry, especially that the conception was made in respect to pilot-scale paper machinery. The obtained paper grammage is less than double base paper grammage which means the polymer did not add much weigh or thickness to the paper. The permeability of the paper decreased which is a valuable propriety in food packaging guidelines regarding packaging based barrier of oxygen and moisture (Deshwal et al., 2019; Khwaldia et al., 2010). Paper stretch and break properties were improved after coating with the biopolymer layer which is related to chemical and physical properties of alginate and gelatin mainly (Kopacic et al., 2018) and is also considered a positive improvement of the product. On the other side, although the physical proprieties of the paper were improved in general, some storage conditions should be adjusted for a higher bioactivity. It was obvious that heat drying of the phage-biopolymer layer showed an important effect on the bioactivity of the paper. This means a decision of continuing to apply traditional paper-pulp factory procedure might endanger the bioactivity of the product, but in manners that are still effective on target, which is being at least one log higher than the possible *Listeria* count in RTE meat and products(Zhang et al., 2021).

If the procedure is out of discussion, the storage remains a major threat to the bioactivity of the paper. Loss was observed in both room temperature and cold stored paper. In cold, the storage would have affected the polymer-phage mixture (in liquid form) less than what was observed in paper form. This might be related to the storage techniques which was adapted to the need of the experiment such as preserving paper in non-specific plastic bags that might have a retention of polymer-phage mixture causing a loss in the obtained phage count. As this is purely theoretical, the storage conditions need to be addressed as a factor that might affect the bioactive quality of the product.

In the end, this study is a proof of concept that a bioactive paper of biopolymer-phage mixture is a product of interest to be improved to reduce the economical impact on RTE meat market and other *Listeria*-linked food and beverages. This study, although focusing on Listex P100 phages, could be applied to other bacteriophages such as Listshield, T4 and lambda as a mixture to allow the obtention of a multitarget bioactive paper. This sheds the light on the importance of further optimization of the final product storage for a marketable bioactive paper.

5. Conclusion

This paper shows a new approach of incorporating phages as biocontrol agents in food packaging. The application of biopolymer such as sodium alginate, gelatin and gellan gum previously proved effective in protecting Listex P100 phages against heat and physical changes. In this work we showed that applying the biopolymer mix containing phages by coating to base paper also supplies a partial protection against heat and pressure observed in pulp and paper industrial procedures. Although the storage conditions affect the phage activity, and still need to be improved, the obtained prototype is a promising solution for reducing foodborne listeriosis in RTE food.

6. Funding

This project was funded by the Natural Sciences and Engineering Research Council of Canada (NSERC) and Kruger Inc.

References:

Aguirre Calvo, T., & Santagapita, P. (2016). Physicochemical characterization of alginate beads containing sugars and biopolymers. *Journal of Quality and Reliability Engineering*, *2016*.

Ahmadi, H., Wang, Q., Lim, L.-T., & Balamurugan, S. (2018). Encapsulation of Listeria phage A511 by alginate to improve its thermal stability. In *Bacteriophages* (pp. 89–95). Springer.

Akhverdyan, V. Z., Gak, E. R., Tokmakova, I. L., Stoynova, N. V., Yomantas, Y. A., & Mashko, S. V. (2011). Application of the bacteriophage Mu-driven system for the integration/amplification of target genes in the chromosomes of engineered Gram-negative bacteria—Mini review. *Applied Microbiology and Biotechnology*, *91*(4), 857–871.

Authority: The European Union summary report on trends... - Google Scholar. (n.d.). Retrieved 15 February 2022, from https://scholar.google.com/scholar_lookup?title=The%20European%20Union%20summary%20report%20on%20trends%20and%20sources%20of%20zoonoses%2C%20zoonotic%20agents%20and%20food-borne%20outbreaks%20in%202015&publication_year=2016&author=EFSA

Bagheri-Khoulenjani, S., Mirzadeh, H., Etrati-Khosroshahi, M., & Shokrgozar, M. A. (2016). Development of a method for measuring and modeling the NH2 content and crosslinking density of chitosan/gelatin/nanohydroxyapatite based microspheres. *Polymer Testing*, *51*, 20–28.

Buruaga-Ramiro, C., Valenzuela, S. V., Valls, C., Roncero, M. B., Pastor, F. I. J., Díaz, P., & Martinez, J. (2020). Development of an antimicrobial bioactive paper made from bacterial cellulose. *International Journal of Biological Macromolecules*, *158*, 587–594. https://doi.org/10.1016/j.ijbiomac.2020.04.234

Canada, P. H. A. of. (2016, August 10). *Listeriosis (Listeria)* [Education and awareness]. https://www.canada.ca/en/public-health/services/diseases/listeriosis.html

Carlton, R. M., Noordman, W. H., Biswas, B., De Meester, E. D., & Loessner, M. J. (2005). Bacteriophage P100 for control of Listeria monocytogenes in foods: Genome sequence, bioinformatic analyses, oral toxicity study, and application. *Regulatory Toxicology and Pharmacology*, *43*(3), 301–312.

Charpentier, E., Gerbaud, G., Jacquet, C., Rocourt, J., & Courvalin, P. (1995). Incidence of antibiotic resistance in Listeria species. *Journal of Infectious Diseases*, *172*(1), 277–281.

Chen, Y.-L., & Li, Q.-Z. (2007). Prediction of apoptosis protein subcellular location using improved hybrid approach and pseudo-amino acid composition. *Journal of Theoretical Biology*, *248*(2), 377–381.

Chernomordik, A. B. (1989). Bacteriophages and their therapeutic-prophylactic use. *Meditsinskaia Sestra*, *48*(6), 44–47.

Chibeu, A., Agius, L., Gao, A., Sabour, P. M., Kropinski, A. M., & Balamurugan, S. (2013). Efficacy of bacteriophage LISTEX™ P100 combined with chemical antimicrobials in reducing Listeria monocytogenes in cooked turkey and roast beef. *International Journal of Food Microbiology*, *167*(2), 208–214.

Chlebicz, A., & Śliżewska, K. (2018). Campylobacteriosis, salmonellosis, yersiniosis, and listeriosis as zoonotic foodborne diseases: A review. *International Journal of Environmental Research and Public Health*, *15*(5), 863.

CHURCHILL, K. J., SARGEANT, J. M., FARBER, J. M., & O'CONNOR, A. M. (2019). Prevalence of Listeria monocytogenes in Select Ready-to-Eat Foods—Deli Meat, Soft Cheese, and Packaged Salad: A Systematic Review and Meta-Analysis. *Journal of Food Protection*, *82*(2), 344–357. https://doi.org/10.4315/0362-028X.JFP-18-158

Coeffic, T., Thibaut, S., Boutoille, D., & Caillon, J. (2018). Évolution de la résistance aux antibiotiques des souches d'Escherichia coli productrices de bêtalactamase à spectre étendu. *Médecine et Maladies Infectieuses*, *48*(4), S48.

Colom, J., Cano-Sarabia, M., Otero, J., Aríñez-Soriano, J., Cortés, P., Maspoch, D., & Llagostera, M. (2017). Microencapsulation with alginate/CaCO3: A strategy for improved phage therapy. *Scientific Reports*, *7*(1), 1–10.

Conter, M., Paludi, D., Zanardi, E., Ghidini, S., Vergara, A., & Ianieri, A. (2009). Characterization of antimicrobial resistance of foodborne Listeria monocytogenes. *International Journal of Food Microbiology*, *128*(3), 497–500.

Cooper, C. J., Khan Mirzaei, M., & Nilsson, A. S. (2016). Adapting drug approval pathways for bacteriophage-based therapeutics. *Frontiers in Microbiology*, *7*, 1209.

Das, M., & Giri, T. K. (2020). Hydrogels based on gellan gum in cell delivery and drug delivery. *Journal of Drug Delivery Science and Technology*, *56*, 101586. https://doi.org/10.1016/j.jddst.2020.101586

de Noordhout, C. M., Devleesschauwer, B., Angulo, F. J., Verbeke, G., Haagsma, J., Kirk, M., Havelaar, A., & Speybroeck, N. (2014). The global burden of listeriosis: A systematic review and meta-analysis. *The Lancet Infectious Diseases*, *14*(11), 1073–1082.

Deshwal, G. K., Panjagari, N. R., & Alam, T. (2019). An overview of paper and paper based food packaging materials: Health safety and environmental concerns. *Journal of Food Science and Technology*, *56*(10), 4391–4403.

Doss, J., Culbertson, K., Hahn, D., Camacho, J., & Barekzi, N. (2017). A review of phage therapy against bacterial pathogens of aquatic and terrestrial organisms. *Viruses*, *9*(3), 50.

Dufour, N., Delattre, R., Chevallereau, A., Ricard, J.-D., & Debarbieux, L. (2019). Phage therapy of pneumonia is not associated with an overstimulation of the inflammatory response compared to antibiotic treatment in mice. *Antimicrobial Agents and Chemotherapy*, *63*(8), e00379-19.

Ergin, F., Atamer, Z., Göcer, E. M. C., Demir, M., Hinrichs, J., & Kucukcetin, A. (2021). Optimization of Salmonella bacteriophage microencapsulation in alginate-caseinate formulation using vibrational nozzle technique. *Food Hydrocolloids*, *113*, 106456.

Gombotz, W. R., & Wee, S. (1998). Protein release from alginate matrices. *Advanced Drug Delivery Reviews*, *31*(3), 267–285.

Gonzalez-Menendez, E., Fernandez, L., Gutierrez, D., Rodriguez, A., Martinez, B., & Garcia, P. (2018). Comparative analysis of different preservation techniques for the storage of Staphylococcus phages aimed for the industrial development of phage-based antimicrobial products. *PLoS One*, *13*(10), e0205728.

Government of Canada, C. F. I. A. (2019, October 8). *Food safety investigation: Various brands of cooked, diced and shredded chicken products may contain Listeria monocytogenes* [Reference material]. https://inspection.canada.ca/about-cfia/transparency/regulatory-transparency-and-openness/food-safety-investigations/various-brands-of-chicken-products/eng/1570129428140/1570129428596#a5

Government of Canada, H. C. (2021, August 25). *Pinty's brand chicken breast products recalled due to Listeria—Recalls, advisories and safety alerts – Canada.ca*. Government of Canada, Health Canada, CFIA. https://recalls-rappels.canada.ca/en/alert-recall/pinty-s-brand-chicken-breast-products-recalled-due-listeria

Guenther, S., Huwyler, D., Richard, S., & Loessner, M. J. (2009). Virulent bacteriophage for efficient biocontrol of Listeria monocytogenes in ready-to-eat foods. *Applied and Environmental Microbiology*, *75*(1), 93–100.

Hazards (BIOHAZ), E. P. on B. (2016). Evaluation of the safety and efficacy of Listex^{TM} P100 for reduction of pathogens on different ready-to-eat (RTE) food products. *EFSA Journal*, *14*(8), e04565. https://doi.org/10.2903/j.efsa.2016.4565

Heymann, D. L., Chen, L., Takemi, K., Fidler, D. P., Tappero, J. W., Thomas, M. J., Kenyon, T. A., Frieden, T. R., Yach, D., & Nishtar, S. (2015). Global health security: The wider lessons from the west African Ebola virus disease epidemic. *The Lancet*, *385*(9980), 1884–1901.

Jabrane, T., Laloi, M., Dubé, M., & Mangin, P. (2010). Printing and coating of T4 phage based bioactive paper. *Advances in Printing and Media Technology*, *37*, 351–358.

Jamal, M., Bukhari, S. M., Andleeb, S., Ali, M., Raza, S., Nawaz, M. A., Hussain, T., Rahman, S. U., & Shah, S. S. (2019). Bacteriophages: An overview of the control strategies against multiple bacterial infections in different fields. *Journal of Basic Microbiology*, *59*(2), 123–133.

Khwaldia, K., Arab-Tehrany, E., & Desobry, S. (2010). Biopolymer coatings on paper packaging materials. *Comprehensive Reviews in Food Science and Food Safety*, *9*(1), 82–91.

King, V. A. E., & Zall, R. R. (1983). Ethanol fermentation of whey using calcium alginate entrapped yeasts. *Process Biochemistry*, *18*(6), 17–20.

Komora, N., Bruschi, C., Ferreira, V., Maciel, C., Brandão, T. R., Fernandes, R., Saraiva, J. A., Castro, S. M., & Teixeira, P. (2018). The protective effect of food matrices on Listeria lytic bacteriophage P100 application towards high pressure processing. *Food Microbiology*, *76*, 416–425.

Komora, N., Bruschi, C., Magalhães, R., Ferreira, V., & Teixeira, P. (2017). Survival of Listeria monocytogenes with different antibiotic resistance patterns to food-associated stresses. *International Journal of Food Microbiology*, *245*, 79–87.

Kopacic, S., Walzl, A., Zankel, A., Leitner, E., & Bauer, W. (2018). Alginate and chitosan as a functional barrier for paper-based packaging materials. *Coatings*, *8*(7), 235.

Kropinski, A. M., Mazzocco, A., Waddell, T. E., Lingohr, E., & Johnson, R. P. (2009a). Enumeration of bacteriophages by double agar overlay plaque assay. In *Bacteriophages* (pp. 69–76). Springer.

Kropinski, A. M., Mazzocco, A., Waddell, T. E., Lingohr, E., & Johnson, R. P. (2009b). Enumeration of Bacteriophages by Double Agar Overlay Plaque Assay. In M. R. J. Clokie & A. M. Kropinski (Eds.), *Bacteriophages: Methods and Protocols, Volume 1: Isolation, Characterization, and Interactions* (pp. 69–76). Humana Press. https://doi.org/10.1007/978-1-60327-164-6_7

Kuruwita, D. P., Jiang, X., Darby, D., Sharp, J. L., & Fraser, A. M. (2020). Persistence of Escherichia coli O157: H7 and Listeria monocytogenes on the exterior of three common food packaging materials. *Food Control*, *112*, 107153.

Leverentz, B., Conway, W. S., Camp, M. J., Janisiewicz, W. J., Abuladze, T., Yang, M., Saftner, R., & Sulakvelidze, A. (2003). Biocontrol of Listeria monocytogenes on fresh-cut produce by treatment with lytic bacteriophages and a bacteriocin. *Applied and Environmental Microbiology*, *69*(8), 4519–4526.

Liu, D. (2006). Identification, subtyping and virulence determination of Listeria monocytogenes, an important foodborne pathogen. *Journal of Medical Microbiology*, *55*(6), 645–659.

Liu, D., Lawrence, M. L., Ainsworth, A. J., & Austin, F. W. (2005). Comparative assessment of acid, alkali and salt tolerance in Listeria monocytogenes virulent and avirulent strains. *FEMS Microbiology Letters*, *243*(2), 373–378.

Loessner, M. J. (2005). Bacteriophage endolysins—Current state of research and applications. *Current Opinion in Microbiology*, *8*(4), 480–487. https://doi.org/10.1016/j.mib.2005.06.002

Lone, A., Anany, H., Hakeem, M., Aguis, L., Avdjian, A.-C., Bouget, M., Atashi, A., Brovko, L., Rochefort, D., & Griffiths, M. W. (2016a). Development of prototypes of bioactive packaging materials based on immobilized bacteriophages for control of growth of bacterial pathogens in foods. *International Journal of Food Microbiology*, *217*, 49–58.

Lone, A., Anany, H., Hakeem, M., Aguis, L., Avdjian, A.-C., Bouget, M., Atashi, A., Brovko, L., Rochefort, D., & Griffiths, M. W. (2016b). Development of prototypes of bioactive packaging materials based on immobilized bacteriophages for control of growth of bacterial pathogens in foods. *International Journal of Food Microbiology*, *217*, 49–58.

Ma, Y., Pacan, J. C., Wang, Q., Xu, Y., Huang, X., Korenevsky, A., & Sabour, P. M. (2008). Microencapsulation of bacteriophage Felix O1 into chitosan-alginate microspheres for oral delivery. *Applied and Environmental Microbiology*, *74*(15), 4799–4805.

Miguéis, S., Saraiva, C., & Esteves, A. (2017). Efficacy of LISTEX P100 at Different Concentrations for Reduction of Listeria monocytogenes Inoculated in Sashimi. *Journal of Food Protection*, *80*(12), 2094–2098. https://doi.org/10.4315/0362-028X.JFP-17-098

Morton, V. K., Thomas, M. K., Ciampa, N., Cutler, J., Hurst, M., & Currie, A. (2019). Comparison of 3-day and 7-day recall periods for food consumption reference values in foodborne disease outbreak investigations. *Epidemiology & Infection*, *147*.

Moye, Z. D., Woolston, J., & Sulakvelidze, A. (2018). Bacteriophage applications for food production and processing. *Viruses*, *10*(4), 205.

Nayak, A. K., Das, B., & Maji, R. (2012). Calcium alginate/gum Arabic beads containing glibenclamide: Development and in vitro characterization. *International Journal of Biological Macromolecules*, *51*(5), 1070–1078.

Ndoboli, D., Roesel, K., Heilmann, M., Alter, T., Clausen, P.-H., Wampande, E., Grace, D., & Huehn, S. (2018). Sérotypes et profils de résistance aux antibiotiques de Salmonella enterica subsp. Enterica présente dans le porc et les légumes frais servis dans les points de vente de Kampala, Ouganda. *Revue d'élevage et de Médecine Vétérinaire Des Pays Tropicaux*, *71*(1–2), 103–109.

Nemati, S., Rezabakhsh, A., Khoshfetrat, A. B., Nourazarian, A., Biray Avci, Ç., Goker Bagca, B., Alizadeh Sardroud, H., Khaksar, M., Ahmadi, M., Delkhosh, A., Sokullu, E., & Rahbarghazi, R. (2017). Alginate-gelatin encapsulation of human endothelial cells promoted angiogenesis in in vivo and in vitro milieu. *Biotechnology and Bioengineering*, *114*(12), 2920–2930. https://doi.org/10.1002/bit.26395

Olaimat, A. N., Al-Holy, M. A., Shahbaz, H. M., Al-Nabulsi, A. A., Abu Ghoush, M. H., Osaili, T. M., Ayyash, M. M., & Holley, R. A. (2018a). Emergence of antibiotic resistance in Listeria monocytogenes isolated from food products: A comprehensive review. *Comprehensive Reviews in Food Science and Food Safety*, *17*(5), 1277–1292.

Olaimat, A. N., Al-Holy, M. A., Shahbaz, H. M., Al-Nabulsi, A. A., Abu Ghoush, M. H., Osaili, T. M., Ayyash, M. M., & Holley, R. A. (2018b). Emergence of antibiotic resistance in Listeria monocytogenes isolated from food products: A comprehensive review. *Comprehensive Reviews in Food Science and Food Safety*, *17*(5), 1277–1292.

Osman, K. M., Kappell, A. D., Fox, E. M., Orabi, A., & Samir, A. (2020). Prevalence, Pathogenicity, Virulence, Antibiotic Resistance, and Phylogenetic Analysis of Biofilm-Producing Listeria monocytogenes Isolated from Different Ecological Niches in Egypt: Food, Humans, Animals, and Environment. *Pathogens*, *9*(1), 5.

Paredes Juárez, G. A., Spasojevic, M., Faas, M. M., & de Vos, P. (2014). Immunological and technical considerations in application of alginate-based microencapsulation systems. *Frontiers in Bioengineering and Biotechnology*, *2*, 26.

Park, M. S., Park, K. H., & Bahk, G. J. (2018). Combined influence of multiple climatic factors on the incidence of bacterial foodborne diseases. *Science of the Total Environment*, *610*, 10–16.

Prabhu, G., Husak, R., Peterson, R., Hagens, S., Iancu, C., Riemer, Y., & Engle, R. (2018). Validation of the Effect of Listex P100 Bacteriophage on Survival of Listeria Monocytogenes. *Meat and Muscle Biology*, *1*(2).

Radoshevich, L., & Cossart, P. (2018). Listeria monocytogenes: Towards a complete picture of its physiology and pathogenesis. *Nature Reviews Microbiology*, *16*(1), 32–46.

Ribeiro-Santos, R., Andrade, M., de Melo, N. R., & Sanches-Silva, A. (2017). Use of essential oils in active food packaging: Recent advances and future trends. *Trends in Food Science & Technology*, *61*, 132–140.

Rueden, C. T., Schindelin, J., Hiner, M. C., DeZonia, B. E., Walter, A. E., Arena, E. T., & Eliceiri, K. W. (2017). ImageJ2: ImageJ for the next generation of scientific image data. *BMC Bioinformatics*, *18*(1), 529.

Salmond, G. P., & Fineran, P. C. (2015). A century of the phage: Past, present and future. *Nature Reviews Microbiology*, *13*(12), 777–786.

Salvia-Trujillo, L., Rojas-Graü, M. A., Soliva-Fortuny, R., & Martín-Belloso, O. (2013). Effect of processing parameters on physicochemical characteristics of microfluidized lemongrass essential oil-alginate nanoemulsions. *Food Hydrocolloids*, *30*(1), 401–407.

Sarfraz, M., Ashraf, Y., & Ashraf, S. (2017). A review: Prevalence and antimicrobial susceptibility profile of listeria species in milk products. *Matrix Science Medica*, *1*(1), 03–09.

Sarker, B., Papageorgiou, D. G., Silva, R., Zehnder, T., Gul-E-Noor, F., Bertmer, M., Kaschta, J., Chrissafis, K., Detsch, R., & Boccaccini, A. R. (2014). Fabrication of alginate–gelatin crosslinked hydrogel microcapsules and evaluation of the microstructure and physico-chemical properties. *Journal of Materials Chemistry B*, *2*(11), 1470–1482. https://doi.org/10.1039/C3TB21509A

Sharma, S., Barkauskaite, S., Jaiswal, A. K., & Jaiswal, S. (2020). Essential oils as additives in active food packaging. *Food Chemistry*, 128403.

Sleator, R. D., Gahan, C. G., & Hill, C. (2003). A postgenomic appraisal of osmotolerance in Listeria monocytogenes. *Applied and Environmental Microbiology*, *69*(1), 1–9.

Solanki, H. K., Pawar, D. D., Shah, D. A., Prajapati, V. D., Jani, G. K., Mulla, A. M., & Thakar, P. M. (2013). Development of Microencapsulation Delivery System for Long-Term Preservation of Probiotics

as Biotherapeutics Agent. *BioMed Research International*, *2013*, e620719. https://doi.org/10.1155/2013/620719

Soni, K. A., & Nannapaneni, R. (2010). Removal of Listeria monocytogenes biofilms with bacteriophage P100. *Journal of Food Protection*, *73*(8), 1519–1524.

Soto, M. J., Retamales, J., Palza, H., & Bastías, R. (2018). Encapsulation of specific Salmonella Enteritidis phage f3αSE on alginate-spheres as a method for protection and dosification. *Electronic Journal of Biotechnology*, *31*, 57–60.

Sun, W., & Griffiths, M. W. (2000). Survival of bifidobacteria in yogurt and simulated gastric juice following immobilization in gellan–xanthan beads. *International Journal of Food Microbiology*, *61*(1), 17–25.

Thomas, M. K., Murray, R., Flockhart, L., Pintar, K., Fazil, A., Nesbitt, A., Marshall, B., Tataryn, J., & Pollari, F. (2015). Estimates of foodborne illness–related hospitalizations and deaths in Canada for 30 specified pathogens and unspecified agents. *Foodborne Pathogens and Disease*, *12*(10), 820–827.

Thomas, M. K., Murray, R., Flockhart, L., Pintar, K., Pollari, F., Fazil, A., Nesbitt, A., & Marshall, B. (2013). Estimates of the burden of foodborne illness in Canada for 30 specified pathogens and unspecified agents, circa 2006. *Foodborne Pathogens and Disease*, *10*(7), 639–648.

Thu, B., Bruheim, P., Espevik, T., Smidsrød, O., Soon-Shiong, P., & Skjåk-Bræk, G. (1996). Alginate polycation microcapsules: II. Some functional properties. *Biomaterials*, *17*(11), 1069–1079.

Twede, D. (2005). The origins of paper based packaging. *Proceedings of the Conference on Historical Analysis and Research in Marketing*, *12*, 288–300.

Vartak, N. B., Lin, C. C., Cleary, J. M., Fagan, M. J., & Saier Jr, M. H. (1995). Glucose metabolism in 'Sphingomonas elodea': Pathway engineering via construction of a glucose-6-phosphate dehydrogenase insertion mutant. *Microbiology*, *141*(9), 2339–2350.

Vázquez-Boland, J. A., Kuhn, M., Berche, P., Chakraborty, T., Domínguez-Bernal, G., Goebel, W., González-Zorn, B., Wehland, J., & Kreft, J. (2001). Listeria pathogenesis and molecular virulence determinants. *Clinical Microbiology Reviews*, *14*(3), 584–640.

Walsh, D., Duffy, G., Sheridan, J. J., Blair, I. S., & McDowell, D. A. (2001). Antibiotic resistance among Listeria, including Listeria monocytogenes, in retail foods. *Journal of Applied Microbiology*, *90*(4), 517–522.

Wikswo, M. E., Kambhampati, A., Shioda, K., Walsh, K. A., Bowen, A., & Hall, A. J. (2015). Outbreaks of acute gastroenteritis transmitted by person-to-person contact, environmental contamination, and unknown modes of transmission—United States, 2009–2013. *Morbidity and Mortality Weekly Report: Surveillance Summaries*, *64*(12), 1–16.

Zhang, X., Wang, S., Chen, X., & Qu, C. (2021). Review controlling Listeria monocytogenes in ready-to-eat meat and poultry products: An overview of outbreaks, current legislations, challenges, and future prospects. *Trends in Food Science & Technology*, *116*, 24–35. https://doi.org/10.1016/j.tifs.2021.07.014

Zink, R., & Loessner, M. J. (1992). Classification of virulent and temperate bacteriophages of Listeria spp. On the basis of morphology and protein analysis. *Applied and Environmental Microbiology*, *58*(1), 296–302.

Table 1: Results of phage titer **(PFU/cm^2)** in different steps of obtention and storage of phage-polymer-coated paper.

Sample	**Phage titer (PFU/cm^2)**
Non dried polymer-phage coated paper	**$2.98 \pm 0.08 \times 10^5$**
Heat dried polymer-phage coated paper	**$3.4 \pm 0.1 \times 10^4$**
Heat dried coated paper stored for 3 weeks at room temperature	**$0.03 \pm 0.01 \times 10^2$**
Heat dried coated paper stored for 3 weeks at 4° C	**$2.04 \pm 0.02 \times 10^3$**

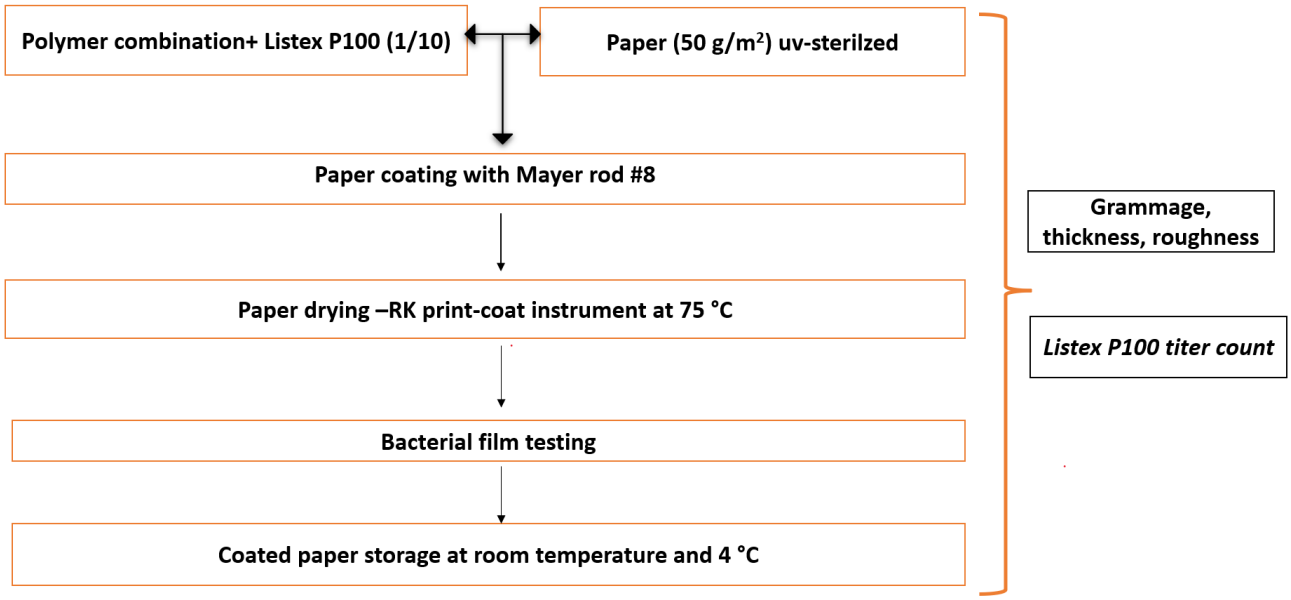


Figure 1: Scheme describing the workflow to obtain bioactive paper and characterize and phage titer count.

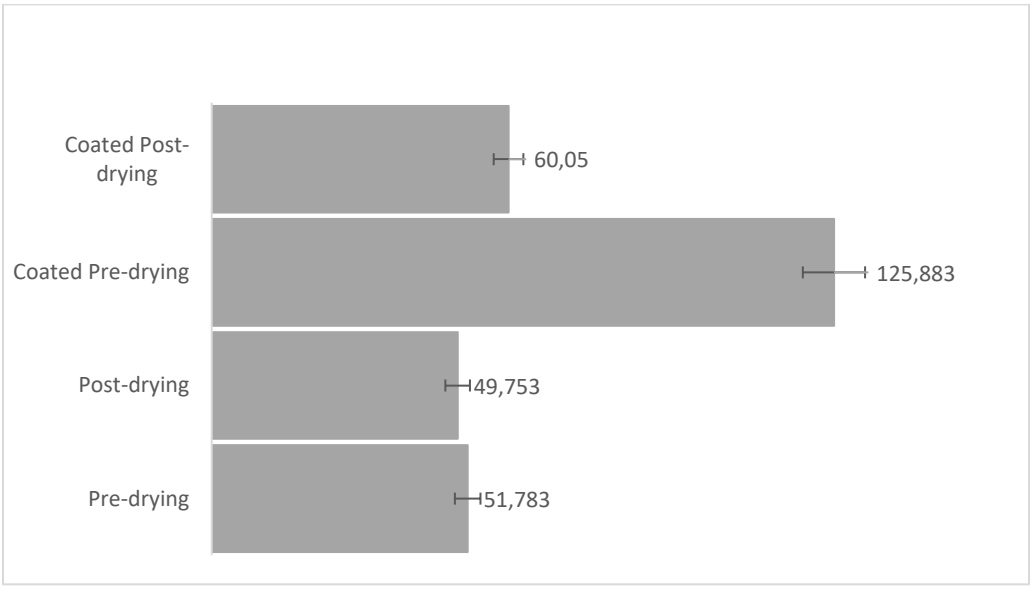


Figure 2: Paper grammage (g/m^2) of base paper, as well as coated base paper before and after drying. Values represent average with standard deviation based on 10 samples per condition.

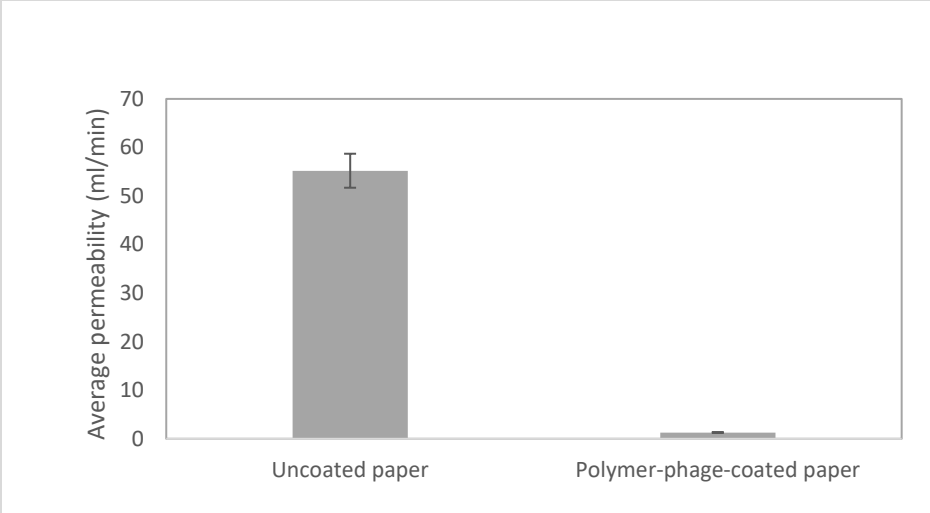


Figure 3: Average permeability to air (ml/min) in butcher base paper and polymer-phage-coated paper. Values represent mean permeability to air (ml/min) and error bars represent standard deviation based on 10 samples per condition.

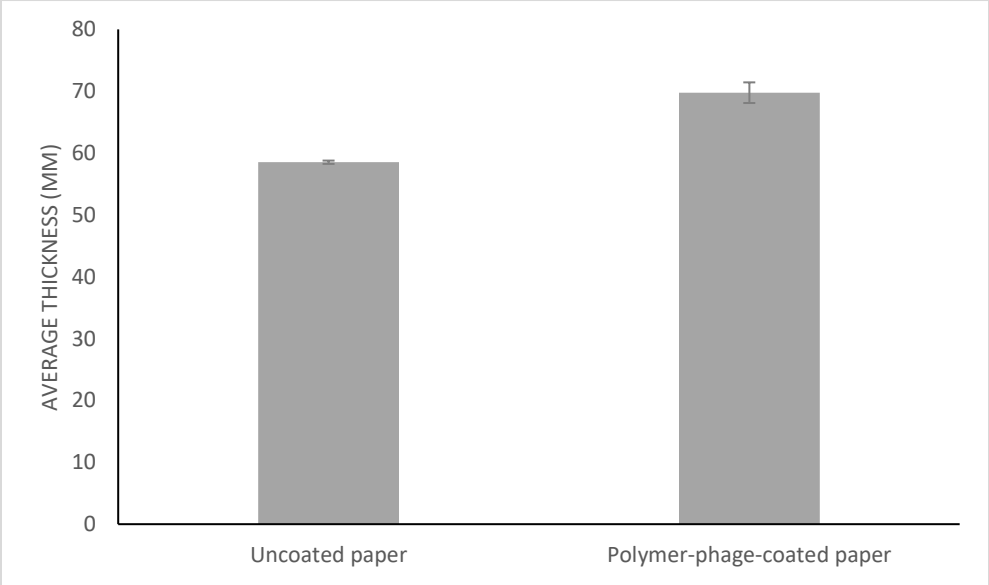


Figure 4: Average thickness (μm) in butcher base paper and polymer-phage-coated paper. Values represent average with standard deviation based on 10 samples per condition.

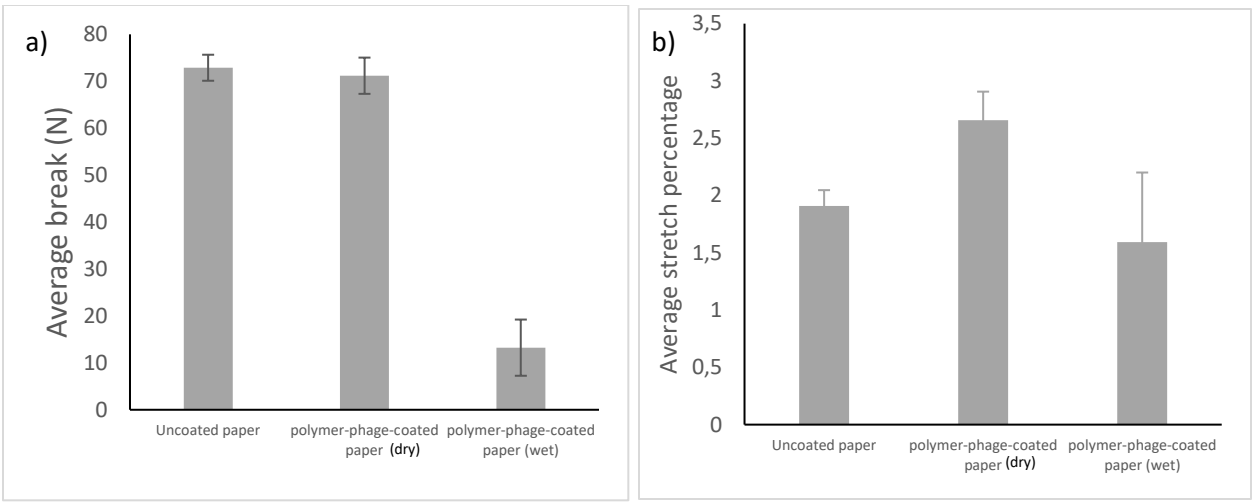
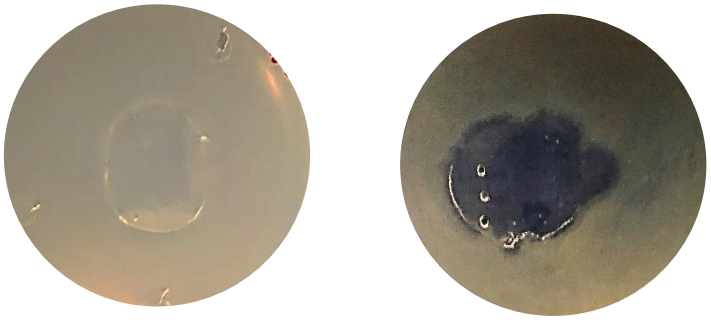


Figure 5: Average textile break in Newton (N) (a) and average stretch percentage (b) in butcher base paper and polymer-phage-coated paper in dry and wet conditions. Values represent average with standard deviation based on 10 samples per condition.

Supplementary figures:

Supplementary Figure 1: Picture of obtained phage-biopolymer coated paper



Supplementary Figure 2: Bacterial film growth inhibition after 24h of application of phage-biopolymer coated paper (right) compared to polymer only coated paper (left).

CHAPITRE IV

CONCLUSION GÉNÉRALE

La listériose est une maladie d'origine alimentaire causant des fatalités importantes partout dans le monde et des dégâts socio-économiques pour l'industrie agroalimentaire. Le but de cette maîtrise était principalement de trouver un mélange de polymère capable de protéger les phages Listex P100 contre des traitements de chaleur, et ce pour répondre à un besoin du partenaire industriel Kruger qui est la création d'emballage alimentaire bioactif capable de freiner les infections alimentaires causé par *Listeria monocytogenes* dans les produits prêts à manger.

4.1 Les capsules

La première partie de l'étude était consacré à tester différentes compositions de biopolymère dans des ratios différents, les mélanger avec les bactériophages et voir si la matrice formée est capable de maintenir l'activité lytique après différentes applications de chaleur. De plus la maintenance de la stabilité, la durée d'entreposage et ses conditions ont été aussi testées. Le produit final est sous forme de capsule, appelé parfois dans la littérature bille, donc une matrice sphérique composée d'un hydrogel contenant des alginates de sodium, de la gomme gellane et de la gélatine. Un trio qui a pu préserver non seulement la forme sphérique et le visuel, mais aussi un dynamisme de libération de bactériophages lors de la mise en contact avec une solution contenant de *Listeria monocytogenes* de façon efficace. La cinétique du relâchement a été étudiée pour les différents polymères et la combinaison alginate-gélatine-gellane a prouvé un relâchement efficace et pas excessivement rapide ou lent pour se différencier des autres combinaisons de polymères pour cet aspect. Toutes les combinaisons ont démontré une protection de l'activité lytique contre des degrés accrus de chaleurs arrivant à 110^{O}C.

Pour conclure, la combinaison alginate-gélatine-gellane a été étudiée pour la première fois dans le cadre de cette maîtrise. Cette combinaison a été testée pour la protection thermique des phages, la

vitesse de relâchement et la durée d'entreposage. Une application est suggérée pour les capsules bioactives obtenues dans différentes matrices liquides pour évaluer le pouvoir bioactif dans des produits comme du lait, du jus d'oranges, des vinaigrettes et sauces, pour essayer de comprendre les dynamiques de relâchement des phages et la préservation de leur bioactivité dans des pH divers pour tenter à optimiser ces capsules par l'ajout d'une couche extérieure ou pas.

4.2 Le papier bioactif

La deuxième partie de cette étude impliquait l'application de la combinaison optimale de biopolymères sur papier de base fourni par le partenaire Kruger. L'application s'est faite par couchage et étalement puis séchage du papier à 75^{O} C de façon à respecter les normes des industries nord-américaines de pâte et papier. Plusieurs tentatives d'impression de phages sur papier ou d'implémentation d'élément bioactif dans le papier ont eu lieu dans le passé par divers chercheurs (Jabrane et al., 2010; Khwaldia et al., 2010). Mais dans ces études, les phages étaient immobilisés. Tel que décrit dans le chapitre 3, la présence des phages dans la couche de biopolymère, cette couche étant plastique, permet une interaction dynamique pouvant permettre aux phages d'accéder à la profondeur des aliments emballés.

Bien que ce soit un prototype, avec les améliorations de l'entretien et le changement de la charge phagique initiale, ce papier pourrait répondre aux besoins du marché. Mais avant ceci, en perspectives à court terme, une application sur viande prête-à-manger sera nécessaire pour évaluer l'interaction du papier et de la matrice protectrice avec l'humidité relative de la viande et son pH.

À long terme, tester différentes procédures d'application du mélange polymère-phage sera nécessaire pour évaluer la capacité d'optimisation du papier pour obtenir un papier bioactif amélioré.

4.3 Discussion générale

Les résultats de cette maîtrise suggèrent l'utilisation d'une même combinaison alginate-gélatine-gellane avec des phages dans des capsules ou du papier. La combinaison gagnante a démontré plusieurs niveaux de protection ainsi que plusieurs caractéristiques physico-chimiques

recherchées. Ces propriétés sont principalement dues aux propriétés de base de chaque composant du polymère mais aussi à l'effet matrice combinant les interactions entre les 3 polymères. Pour être plus précis, l'alginate de sodium est formé par des résidues de (1, 4)- β-D-mannuronate et d' α-L-guluronate. Le sodium est remplacé par le calcium en solution d'encapsulation permettant un rapprochement entre les différents monomères et une compaction supérieure (Colom et al., 2017). La gélatine de son côté offre une fois refroidie une structure de triple hélice augmentant la stabilité et l'organisation de la combinaison de polymère (Nemati et al., 2017; Sarker et al., 2014). Finalement, la gomme gellane, utilisée dans des capsules bioactives pour la première fois dans cette étude, confère une meilleure thermostabilité et est connu pour garder sa structure sous différents pH (Das & Giri, 2020). La combinaison des trois biopolymères résulte d'un hydrogel capable de donner une forme sphérique semi transparente, des capsules bioactives qui conservent l'activité lytique sous différentes températures et durant de longue durée d'entreposage. Cette même combinaison était étalée sur papier, et malgré les pertes observées après séchage du papier, ce dernier était capable de garder une charge phagique active non négligeable.

4.4 Applicabilité des résultats

Que ce soient des capsules ou du papier, chacun a son application probable dans l'industrie de l'agroalimentaire, comme illustré dans la figure 1.5., Les capsules bioactives seront principalement appliquées pour la conservation des produits de breuvages comme les jus de fruits ou de légumes, du lait, de la crème, des yaourts et des sauces. D'autre part, le papier bioactif vise des applications d'emballage de produits solides tel que les viandes et les produits connexes, les fromages, les pâtes…

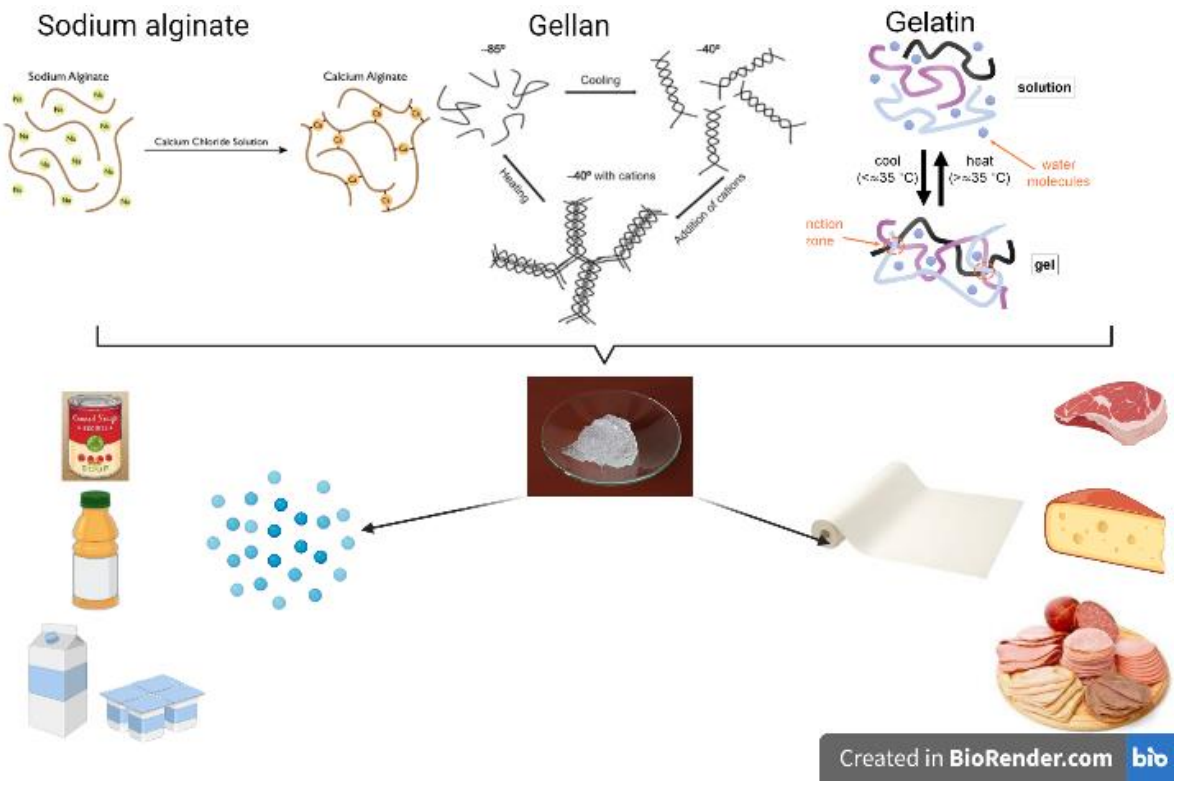


Figure 1.5 : Schématisation de la composition de la combinaison des polymères et des applications prévues en capsules et en papiers bioactifs. (Générée dans Biorender)

RÉFÉRENCES BIBLIOGRAPHIQUES

Aguirre Calvo, T., & Santagapita, P. (2016). Physicochemical characterization of alginate beads containing sugars and biopolymers. *Journal of Quality and Reliability Engineering*, *2016*.

Ahmadi, H., Wang, Q., Lim, L.-T., & Balamurugan, S. (2018). Encapsulation of Listeria phage A511 by alginate to improve its thermal stability. In *Bacteriophages* (pp. 89–95). Springer.

Akhverdyan, V. Z., Gak, E. R., Tokmakova, I. L., Stoynova, N. V., Yomantas, Y. A., & Mashko, S. V. (2011). Application of the bacteriophage Mu-driven system for the integration/amplification of target genes in the chromosomes of engineered Gram-negative bacteria—Mini review. *Applied Microbiology and Biotechnology*, *91*(4), 857–871.

Authority: The European Union summary report on trends... - Google Scholar. (n.d.). Retrieved 15 February 2022, from https://scholar.google.com/scholar_lookup?title=The%20European%20Union%20summary%20report%20on%20trends%20and%20sources%20of%20zoonoses%2C%20zoonotic%20agents%20and%20food-borne%20outbreaks%20in%202015&publication_year=2016&author=EFSA

Bagheri-Khoulenjani, S., Mirzadeh, H., Etrati-Khosroshahi, M., & Shokrgozar, M. A. (2016). Development of a method for measuring and modeling the NH2 content and crosslinking density of chitosan/gelatin/nanohydroxyapatite based microspheres. *Polymer Testing*, *51*, 20–28.

Buruaga-Ramiro, C., Valenzuela, S. V., Valls, C., Roncero, M. B., Pastor, F. I. J., Díaz, P., & Martinez, J. (2020). Development of an antimicrobial bioactive paper made from bacterial cellulose. *International Journal of Biological Macromolecules*, *158*, 587–594. https://doi.org/10.1016/j.ijbiomac.2020.04.234

Canada, P. H. A. of. (2016, August 10). *Listeriosis (Listeria)* [Education and awareness]. https://www.canada.ca/en/public-health/services/diseases/listeriosis.html

Carlton, R. M., Noordman, W. H., Biswas, B., De Meester, E. D., & Loessner, M. J. (2005). Bacteriophage P100 for control of Listeria monocytogenes in foods: Genome sequence, bioinformatic analyses, oral toxicity study, and application. *Regulatory Toxicology and Pharmacology*, *43*(3), 301–312.

Charpentier, E., Gerbaud, G., Jacquet, C., Rocourt, J., & Courvalin, P. (1995). Incidence of antibiotic resistance in Listeria species. *Journal of Infectious Diseases*, *172*(1), 277–281.

Chen, Y.-L., & Li, Q.-Z. (2007). Prediction of apoptosis protein subcellular location using improved hybrid approach and pseudo-amino acid composition. *Journal of Theoretical Biology*, *248*(2), 377–381.

Chernomordik, A. B. (1989). Bacteriophages and their therapeutic-prophylactic use. *Meditsinskaia Sestra*, *48*(6), 44–47.

Chibeu, A., Agius, L., Gao, A., Sabour, P. M., Kropinski, A. M., & Balamurugan, S. (2013). Efficacy of bacteriophage LISTEX™ P100 combined with chemical antimicrobials in reducing Listeria monocytogenes in cooked turkey and roast beef. *International Journal of Food Microbiology*, *167*(2), 208–214.

Chlebicz, A., & Śliżewska, K. (2018). Campylobacteriosis, salmonellosis, yersiniosis, and listeriosis as zoonotic foodborne diseases: A review. *International Journal of Environmental Research and Public Health*, *15*(5), 863.

CHURCHILL, K. J., SARGEANT, J. M., FARBER, J. M., & O'CONNOR, A. M. (2019). Prevalence of Listeria monocytogenes in Select Ready-to-Eat Foods—Deli Meat, Soft Cheese, and Packaged Salad: A Systematic Review and Meta-Analysis. *Journal of Food Protection*, *82*(2), 344–357. https://doi.org/10.4315/0362-028X.JFP-18-158

Coeffic, T., Thibaut, S., Boutoille, D., & Caillon, J. (2018). Évolution de la résistance aux antibiotiques des souches d'Escherichia coli productrices de bêtalactamase à spectre étendu. *Médecine et Maladies Infectieuses*, *48*(4), S48.

Colom, J., Cano-Sarabia, M., Otero, J., Aríñez-Soriano, J., Cortés, P., Maspoch, D., & Llagostera, M. (2017). Microencapsulation with alginate/CaCO3: A strategy for improved phage therapy. *Scientific Reports*, *7*(1), 1–10.

Conter, M., Paludi, D., Zanardi, E., Ghidini, S., Vergara, A., & Ianieri, A. (2009). Characterization of antimicrobial resistance of foodborne Listeria monocytogenes. *International Journal of Food Microbiology*, *128*(3), 497–500.

Cooper, C. J., Khan Mirzaei, M., & Nilsson, A. S. (2016). Adapting drug approval pathways for bacteriophage-based therapeutics. *Frontiers in Microbiology*, *7*, 1209.

Das, M., & Giri, T. K. (2020). Hydrogels based on gellan gum in cell delivery and drug delivery. *Journal of Drug Delivery Science and Technology*, *56*, 101586. https://doi.org/10.1016/j.jddst.2020.101586

de Noordhout, C. M., Devleesschauwer, B., Angulo, F. J., Verbeke, G., Haagsma, J., Kirk, M., Havelaar, A., & Speybroeck, N. (2014). The global burden of listeriosis: A systematic review and meta-analysis. *The Lancet Infectious Diseases*, *14*(11), 1073–1082.

Deshwal, G. K., Panjagari, N. R., & Alam, T. (2019). An overview of paper and paper based food packaging materials: Health safety and environmental concerns. *Journal of Food Science and Technology*, *56*(10), 4391–4403.

Doss, J., Culbertson, K., Hahn, D., Camacho, J., & Barekzi, N. (2017). A review of phage therapy against bacterial pathogens of aquatic and terrestrial organisms. *Viruses*, *9*(3), 50.

Dufour, N., Delattre, R., Chevallereau, A., Ricard, J.-D., & Debarbieux, L. (2019). Phage therapy of pneumonia is not associated with an overstimulation of the inflammatory response compared to antibiotic treatment in mice. *Antimicrobial Agents and Chemotherapy*, *63*(8), e00379-19.

Ergin, F., Atamer, Z., Göcer, E. M. C., Demir, M., Hinrichs, J., & Kucukcetin, A. (2021). Optimization of Salmonella bacteriophage microencapsulation in alginate-caseinate formulation using vibrational nozzle technique. *Food Hydrocolloids*, *113*, 106456.

Gombotz, W. R., & Wee, S. (1998). Protein release from alginate matrices. *Advanced Drug Delivery Reviews*, *31*(3), 267–285.

Gonzalez-Menendez, E., Fernandez, L., Gutierrez, D., Rodriguez, A., Martinez, B., & Garcia, P. (2018). Comparative analysis of different preservation techniques for the storage of Staphylococcus phages aimed for the industrial development of phage-based antimicrobial products. *PLoS One*, *13*(10), e0205728.

Government of Canada, C. F. I. A. (2019, October 8). *Food safety investigation: Various brands of cooked, diced and shredded chicken products may contain Listeria monocytogenes* [Reference material]. https://inspection.canada.ca/about-cfia/transparency/regulatory-transparency-and-openness/food-safety-investigations/various-brands-of-chicken-products/eng/1570129428140/1570129428596#a5

Government of Canada, H. C. (2021, August 25). *Pinty's brand chicken breast products recalled due to Listeria—Recalls, advisories and safety alerts – Canada.ca*. Government of Canada, Health Canada, CFIA. https://recalls-rappels.canada.ca/en/alert-recall/pinty-s-brand-chicken-breast-products-recalled-due-listeria

Guenther, S., Huwyler, D., Richard, S., & Loessner, M. J. (2009). Virulent bacteriophage for efficient biocontrol of Listeria monocytogenes in ready-to-eat foods. *Applied and Environmental Microbiology*, *75*(1), 93–100.

Hazards (BIOHAZ), E. P. on B. (2016). Evaluation of the safety and efficacy of Listex^{TM} P100 for reduction of pathogens on different ready-to-eat (RTE) food products. *EFSA Journal*, *14*(8), e04565. https://doi.org/10.2903/j.efsa.2016.4565

Heymann, D. L., Chen, L., Takemi, K., Fidler, D. P., Tappero, J. W., Thomas, M. J., Kenyon, T. A., Frieden, T. R., Yach, D., & Nishtar, S. (2015). Global health security: The wider lessons from the west African Ebola virus disease epidemic. *The Lancet*, *385*(9980), 1884–1901.

Jabrane, T., Laloi, M., Dubé, M., & Mangin, P. (2010). Printing and coating of T4 phage based bioactive paper. *Advances in Printing and Media Technology*, *37*, 351–358.

Jamal, M., Bukhari, S. M., Andleeb, S., Ali, M., Raza, S., Nawaz, M. A., Hussain, T., Rahman, S. U., & Shah, S. S. (2019). Bacteriophages: An overview of the control strategies against multiple bacterial infections in different fields. *Journal of Basic Microbiology*, *59*(2), 123–133.

Khwaldia, K., Arab-Tehrany, E., & Desobry, S. (2010). Biopolymer coatings on paper packaging materials. *Comprehensive Reviews in Food Science and Food Safety*, *9*(1), 82–91.

King, V. A. E., & Zall, R. R. (1983). Ethanol fermentation of whey using calcium alginate entrapped yeasts. *Process Biochemistry*, *18*(6), 17–20.

Komora, N., Bruschi, C., Ferreira, V., Maciel, C., Brandão, T. R., Fernandes, R., Saraiva, J. A., Castro, S. M., & Teixeira, P. (2018). The protective effect of food matrices on Listeria lytic bacteriophage P100 application towards high pressure processing. *Food Microbiology*, *76*, 416–425.

Komora, N., Bruschi, C., Magalhães, R., Ferreira, V., & Teixeira, P. (2017). Survival of Listeria monocytogenes with different antibiotic resistance patterns to food-associated stresses. *International Journal of Food Microbiology*, *245*, 79–87.

Kopacic, S., Walzl, A., Zankel, A., Leitner, E., & Bauer, W. (2018). Alginate and chitosan as a functional barrier for paper-based packaging materials. *Coatings*, *8*(7), 235.

Kropinski, A. M., Mazzocco, A., Waddell, T. E., Lingohr, E., & Johnson, R. P. (2009a). Enumeration of bacteriophages by double agar overlay plaque assay. In *Bacteriophages* (pp. 69–76). Springer.

Kropinski, A. M., Mazzocco, A., Waddell, T. E., Lingohr, E., & Johnson, R. P. (2009b). Enumeration of Bacteriophages by Double Agar Overlay Plaque Assay. In M. R. J. Clokie & A. M. Kropinski (Eds.), *Bacteriophages: Methods and Protocols, Volume 1: Isolation, Characterization, and Interactions* (pp. 69–76). Humana Press. https://doi.org/10.1007/978-1-60327-164-6_7

Kuruwita, D. P., Jiang, X., Darby, D., Sharp, J. L., & Fraser, A. M. (2020). Persistence of Escherichia coli O157: H7 and Listeria monocytogenes on the exterior of three common food packaging materials. *Food Control*, *112*, 107153.

Leverentz, B., Conway, W. S., Camp, M. J., Janisiewicz, W. J., Abuladze, T., Yang, M., Saftner, R., & Sulakvelidze, A. (2003). Biocontrol of Listeria monocytogenes on fresh-cut produce by treatment with lytic bacteriophages and a bacteriocin. *Applied and Environmental Microbiology*, *69*(8), 4519–4526.

Liu, D. (2006). Identification, subtyping and virulence determination of Listeria monocytogenes, an important foodborne pathogen. *Journal of Medical Microbiology*, *55*(6), 645–659.

Liu, D., Lawrence, M. L., Ainsworth, A. J., & Austin, F. W. (2005). Comparative assessment of acid, alkali and salt tolerance in Listeria monocytogenes virulent and avirulent strains. *FEMS Microbiology Letters*, *243*(2), 373–378.

Loessner, M. J. (2005). Bacteriophage endolysins—Current state of research and applications. *Current Opinion in Microbiology*, *8*(4), 480–487. https://doi.org/10.1016/j.mib.2005.06.002

Lone, A., Anany, H., Hakeem, M., Aguis, L., Avdjian, A.-C., Bouget, M., Atashi, A., Brovko, L., Rochefort, D., & Griffiths, M. W. (2016a). Development of prototypes of bioactive packaging materials based on immobilized bacteriophages for control of growth of bacterial pathogens in foods. *International Journal of Food Microbiology*, *217*, 49–58.

Lone, A., Anany, H., Hakeem, M., Aguis, L., Avdjian, A.-C., Bouget, M., Atashi, A., Brovko, L., Rochefort, D., & Griffiths, M. W. (2016b). Development of prototypes of bioactive packaging materials based on immobilized bacteriophages for control of growth of bacterial pathogens in foods. *International Journal of Food Microbiology*, *217*, 49–58.

Ma, Y., Pacan, J. C., Wang, Q., Xu, Y., Huang, X., Korenevsky, A., & Sabour, P. M. (2008). Microencapsulation of bacteriophage Felix O1 into chitosan-alginate microspheres for oral delivery. *Applied and Environmental Microbiology*, *74*(15), 4799–4805.

Miguéis, S., Saraiva, C., & Esteves, A. (2017). Efficacy of LISTEX P100 at Different Concentrations for Reduction of Listeria monocytogenes Inoculated in Sashimi. *Journal of Food Protection*, *80*(12), 2094–2098. https://doi.org/10.4315/0362-028X.JFP-17-098

Morton, V. K., Thomas, M. K., Ciampa, N., Cutler, J., Hurst, M., & Currie, A. (2019). Comparison of 3-day and 7-day recall periods for food consumption reference values in foodborne disease outbreak investigations. *Epidemiology & Infection*, *147*.

Moye, Z. D., Woolston, J., & Sulakvelidze, A. (2018). Bacteriophage applications for food production and processing. *Viruses*, *10*(4), 205.

Nayak, A. K., Das, B., & Maji, R. (2012). Calcium alginate/gum Arabic beads containing glibenclamide: Development and in vitro characterization. *International Journal of Biological Macromolecules*, *51*(5), 1070–1078.

Ndoboli, D., Roesel, K., Heilmann, M., Alter, T., Clausen, P.-H., Wampande, E., Grace, D., & Huehn, S. (2018). Sérotypes et profils de résistance aux antibiotiques de Salmonella enterica subsp. Enterica présente dans le porc et les légumes frais servis dans les points de vente de Kampala, Ouganda. *Revue d'élevage et de Médecine Vétérinaire Des Pays Tropicaux*, *71*(1–2), 103–109.

Nemati, S., Rezabakhsh, A., Khoshfetrat, A. B., Nourazarian, A., Biray Avci, Ç., Goker Bagca, B., Alizadeh Sardroud, H., Khaksar, M., Ahmadi, M., Delkhosh, A., Sokullu, E., & Rahbarghazi, R. (2017). Alginate-gelatin encapsulation of human endothelial cells promoted angiogenesis in in vivo and in vitro milieu. *Biotechnology and Bioengineering*, *114*(12), 2920–2930. https://doi.org/10.1002/bit.26395

Olaimat, A. N., Al-Holy, M. A., Shahbaz, H. M., Al-Nabulsi, A. A., Abu Ghoush, M. H., Osaili, T. M., Ayyash, M. M., & Holley, R. A. (2018a). Emergence of antibiotic resistance in Listeria monocytogenes isolated from food products: A comprehensive review. *Comprehensive Reviews in Food Science and Food Safety*, *17*(5), 1277–1292.

Olaimat, A. N., Al-Holy, M. A., Shahbaz, H. M., Al-Nabulsi, A. A., Abu Ghoush, M. H., Osaili, T. M., Ayyash, M. M., & Holley, R. A. (2018b). Emergence of antibiotic resistance in Listeria monocytogenes isolated from food products: A comprehensive review. *Comprehensive Reviews in Food Science and Food Safety*, *17*(5), 1277–1292.

Osman, K. M., Kappell, A. D., Fox, E. M., Orabi, A., & Samir, A. (2020). Prevalence, Pathogenicity, Virulence, Antibiotic Resistance, and Phylogenetic Analysis of Biofilm-Producing Listeria monocytogenes Isolated from Different Ecological Niches in Egypt: Food, Humans, Animals, and Environment. *Pathogens*, *9*(1), 5.

Paredes Juárez, G. A., Spasojevic, M., Faas, M. M., & de Vos, P. (2014). Immunological and technical considerations in application of alginate-based microencapsulation systems. *Frontiers in Bioengineering and Biotechnology*, *2*, 26.

Park, M. S., Park, K. H., & Bahk, G. J. (2018). Combined influence of multiple climatic factors on the incidence of bacterial foodborne diseases. *Science of the Total Environment*, *610*, 10–16.

Prabhu, G., Husak, R., Peterson, R., Hagens, S., Iancu, C., Riemer, Y., & Engle, R. (2018). Validation of the Effect of Listex P100 Bacteriophage on Survival of Listeria Monocytogenes. *Meat and Muscle Biology*, *1*(2).

Radoshevich, L., & Cossart, P. (2018). Listeria monocytogenes: Towards a complete picture of its physiology and pathogenesis. *Nature Reviews Microbiology*, *16*(1), 32–46.

Ribeiro-Santos, R., Andrade, M., de Melo, N. R., & Sanches-Silva, A. (2017). Use of essential oils in active food packaging: Recent advances and future trends. *Trends in Food Science & Technology*, *61*, 132–140.

Rueden, C. T., Schindelin, J., Hiner, M. C., DeZonia, B. E., Walter, A. E., Arena, E. T., & Eliceiri, K. W. (2017). ImageJ2: ImageJ for the next generation of scientific image data. *BMC Bioinformatics*, *18*(1), 529.

Salmond, G. P., & Fineran, P. C. (2015). A century of the phage: Past, present and future. *Nature Reviews Microbiology*, *13*(12), 777–786.

Salvia-Trujillo, L., Rojas-Graü, M. A., Soliva-Fortuny, R., & Martín-Belloso, O. (2013). Effect of processing parameters on physicochemical characteristics of microfluidized lemongrass essential oil-alginate nanoemulsions. *Food Hydrocolloids*, *30*(1), 401–407.

Sarfraz, M., Ashraf, Y., & Ashraf, S. (2017). A review: Prevalence and antimicrobial susceptibility profile of listeria species in milk products. *Matrix Science Medica*, *1*(1), 03–09.

Sarker, B., Papageorgiou, D. G., Silva, R., Zehnder, T., Gul-E-Noor, F., Bertmer, M., Kaschta, J., Chrissafis, K., Detsch, R., & Boccaccini, A. R. (2014). Fabrication of alginate–gelatin crosslinked hydrogel microcapsules and evaluation of the microstructure and physico-chemical properties. *Journal of Materials Chemistry B*, *2*(11), 1470–1482. https://doi.org/10.1039/C3TB21509A

Sharma, S., Barkauskaite, S., Jaiswal, A. K., & Jaiswal, S. (2020). Essential oils as additives in active food packaging. *Food Chemistry*, 128403.

Sleator, R. D., Gahan, C. G., & Hill, C. (2003). A postgenomic appraisal of osmotolerance in Listeria monocytogenes. *Applied and Environmental Microbiology*, *69*(1), 1–9.

Solanki, H. K., Pawar, D. D., Shah, D. A., Prajapati, V. D., Jani, G. K., Mulla, A. M., & Thakar, P. M. (2013). Development of Microencapsulation Delivery System for Long-Term Preservation of Probiotics

as Biotherapeutics Agent. *BioMed Research International*, *2013*, e620719. https://doi.org/10.1155/2013/620719

Soni, K. A., & Nannapaneni, R. (2010). Removal of Listeria monocytogenes biofilms with bacteriophage P100. *Journal of Food Protection*, *73*(8), 1519–1524.

Soto, M. J., Retamales, J., Palza, H., & Bastías, R. (2018). Encapsulation of specific Salmonella Enteritidis phage f3αSE on alginate-spheres as a method for protection and dosification. *Electronic Journal of Biotechnology*, *31*, 57–60.

Sun, W., & Griffiths, M. W. (2000). Survival of bifidobacteria in yogurt and simulated gastric juice following immobilization in gellan–xanthan beads. *International Journal of Food Microbiology*, *61*(1), 17–25.

Thomas, M. K., Murray, R., Flockhart, L., Pintar, K., Fazil, A., Nesbitt, A., Marshall, B., Tataryn, J., & Pollari, F. (2015). Estimates of foodborne illness–related hospitalizations and deaths in Canada for 30 specified pathogens and unspecified agents. *Foodborne Pathogens and Disease*, *12*(10), 820–827.

Thomas, M. K., Murray, R., Flockhart, L., Pintar, K., Pollari, F., Fazil, A., Nesbitt, A., & Marshall, B. (2013). Estimates of the burden of foodborne illness in Canada for 30 specified pathogens and unspecified agents, circa 2006. *Foodborne Pathogens and Disease*, *10*(7), 639–648.

Thu, B., Bruheim, P., Espevik, T., Smidsrød, O., Soon-Shiong, P., & Skjåk-Bræk, G. (1996). Alginate polycation microcapsules: II. Some functional properties. *Biomaterials*, *17*(11), 1069–1079.

Twede, D. (2005). The origins of paper based packaging. *Proceedings of the Conference on Historical Analysis and Research in Marketing*, *12*, 288–300.

Vartak, N. B., Lin, C. C., Cleary, J. M., Fagan, M. J., & Saier Jr, M. H. (1995). Glucose metabolism in 'Sphingomonas elodea': Pathway engineering via construction of a glucose-6-phosphate dehydrogenase insertion mutant. *Microbiology*, *141*(9), 2339–2350.

Vázquez-Boland, J. A., Kuhn, M., Berche, P., Chakraborty, T., Domínguez-Bernal, G., Goebel, W., González-Zorn, B., Wehland, J., & Kreft, J. (2001). Listeria pathogenesis and molecular virulence determinants. *Clinical Microbiology Reviews*, *14*(3), 584–640.

Walsh, D., Duffy, G., Sheridan, J. J., Blair, I. S., & McDowell, D. A. (2001). Antibiotic resistance among Listeria, including Listeria monocytogenes, in retail foods. *Journal of Applied Microbiology*, *90*(4), 517–522.

Wikswo, M. E., Kambhampati, A., Shioda, K., Walsh, K. A., Bowen, A., & Hall, A. J. (2015). Outbreaks of acute gastroenteritis transmitted by person-to-person contact, environmental contamination, and unknown modes of transmission—United States, 2009–2013. *Morbidity and Mortality Weekly Report: Surveillance Summaries*, *64*(12), 1–16.

Zhang, X., Wang, S., Chen, X., & Qu, C. (2021). Review controlling Listeria monocytogenes in ready-to-eat meat and poultry products: An overview of outbreaks, current legislations, challenges, and future prospects. *Trends in Food Science & Technology*, *116*, 24–35. https://doi.org/10.1016/j.tifs.2021.07.014

Zink, R., & Loessner, M. J. (1992). Classification of virulent and temperate bacteriophages of Listeria spp. On the basis of morphology and protein analysis. *Applied and Environmental Microbiology*, *58*(1), 296–302.

ANNEXE

Bacteriophage as a biocontrol agent: large-scale production overview and advances

Bacteriophage discovery by, separately, Fredrick twirt in 1915 and Felix d'Hérelle in 1917 revolutionized modern microbiology. Whether in medicine, biotechnology or agronomy, these viruses started the turning point of of biological control as we know it [1].

Phage generalities

Bacteriophages measures between 24 and 400 nm and are typically formed by a capsid containing a single genome and a tail essential for adhesion on bacterial interface, it also contains enzymes such as endolysins, capable of degrading bacterial cell wall components [2,3]. It was in 1921 that the action of phages against bacterial infection was first proven. Since then, phages have been used to treat human diseases, *Escherichia* infections of cattle and even bacterial diseases of plants [4]. Phages offer a treatment with a minimal risk of secondary infections found during treatment with antibiotics that compromise the beneficial bacterial flora, due to their specificities and the limitation of bacterial resistance [5].

Studies showed in the last hundred years that, unlike antibiotics, phage are very specific and effective against host bacteria. These criteria are the main reason behind the scientific community supporting of the usage of phages in food and feed as a biological control. But many barriers are contributing to retarding the application of this biological control procedure. In order to benefit from the antibacterial actions of phages, they must be produced on a large scale and be as pure as possible. In this paper, we discuss the different models of large-scale phage production and describe the main steps as well as challenges encountered during the process.

Phage large-scale production challenges

Phage production was for long done in purpose of phage-bacterial interaction studies, but since the proof of phage therapy in 1920's, the focus on possible and proper usage of phages led scientist

to start optimizing phage production in the 1940's. First assays were conducted at small level in flasks before transiting to bioreactors to achieve more important volumes [1,5]. Production volume varies between 8 ml and more than 1000 L for concentration around 10^{11}pfu/ml [1,3], but many obstacles influence this production quality and quantity. Among the barriers comes first the logistics of phage large-scale production in terms of concentration and purity, as well as application strategies of phages in food safety, not to forget ethical judgment (Lewis and Hill, 2020). Another factor in phage production is the production type, batches could be produced in continuous mode or in batches.

Batch production consists of a single addition of phage inoculum once the bacterial density has reached a peak in the bioreactor. On the other hand, continuous mode allows addition on the flow of bacteria and phages as well as continuous harvest of the media with contained phages, this is usually achieved in a single bioreactor. Continuous mode could be achieved as well in two stages, the first stage is dedicated for bacterial growth in one bioreactor, then once optimal bacterial density is reached, a flow of the culture is allowed to reach the second bioreactor where phage meet the host and the phage production takes place in the second bioreactor: this is known as two-stage continuous mode, and the transfer between bioreactors always happen between the bacterial growth bioreactor toward the phage-bacterial interface [6, 18]. One last known model of phage production consists of two-stage cycle, comparable to the two-stage continuous model, to the difference that culture flow is reversible; depending on the phage-bacterial equilibrium, bacterial culture could be carried into the phage containing bioreactor and vice-versa, in a perpetual cycle. In this kind of montage, the harvest does not impose the arrest of the cycle, and this engenders many profits at the industrial level.

After optimizing host bacteria concentration through bioreactor's build-in detectors, phage production is triggered by initial inoculum of concentrated phage strain or cocktail, then the bacterial cell density decrease is monitored in order to optimize the endpoint timing. Endpoint timing is essential since studies have proven that waiting longer than 3 hours to collect phages could lead to an important loss in phage concentration due to DNAse and RNAse released following bacterial lysis [8,12,14]. Large-scale phage production varies in volume between 8 ml and 1500L depending on bioreactors' functional volume. Following the arrest of bacteriophage cycle production, the process of phage purification and concentration debuts, this step is essential

to eliminate bacterial debris and remaining media and increases the phage concentration at the same time. Many types of concentration techniques are applied at large scale, such as membrane filtration, centrifugation or sedimentation using polyethylene glycol gradient [17]. Many aspects of phage purification and concentration still need to be improved in order to achieve minimal loss of phages during the recovery stage, and this is among the main barriers of phage large-scale production nowadays.

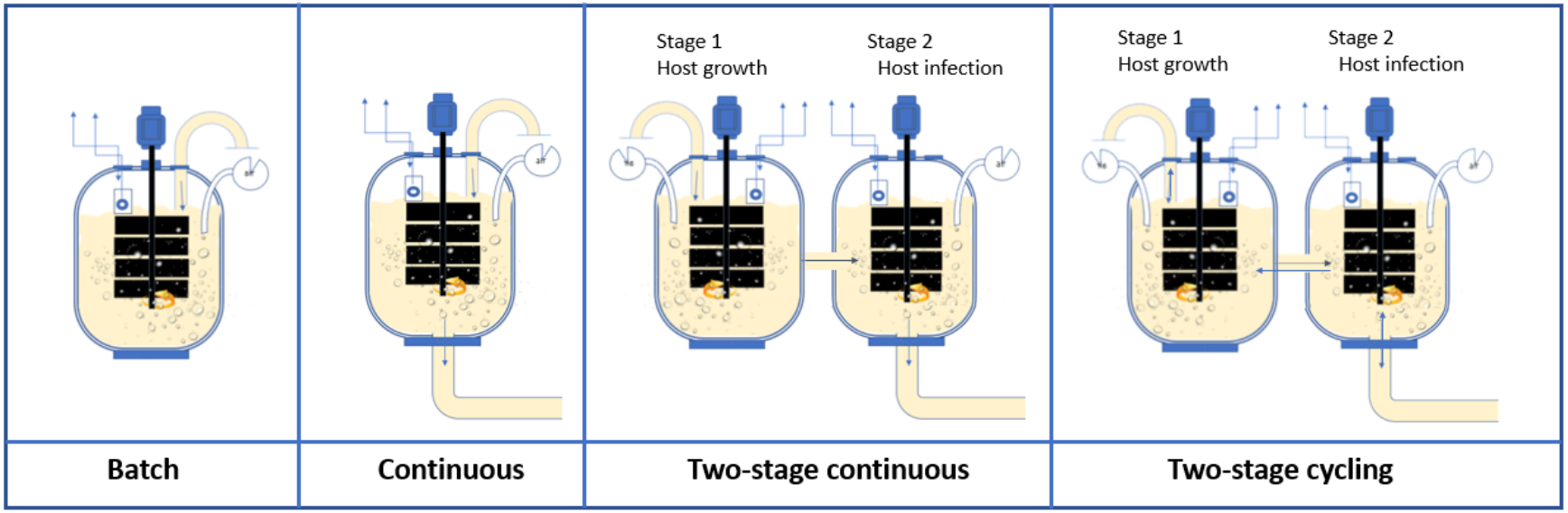


Figure 1: Different large-scale production models (adapted from 6 & 18)

Phage production models

The production of phage at a laboratory scale is limited by the volume in case of absence of large bioreactors. To be able to produce phages on a large scale and in high concentration, dedicated bioreactors and monitoring of bacterial density and phage titer over time are required. The bioreactor most found in the literature for the production of phages is the BioStat, this assembly allows to produce a large concentration of phage due to the monitoring of the parameters of the culture and to choose the exact moment of addition phages. [6,8]

Established phage production models tend to describe mathematically parameters that are most critical for optimal phage production. Depending on the model, these parameters could involve temperature, aeration, bacterial density, initial phage inoculum and time of incubation. [6,8]

Other models describe more detailed parameters such as the number of phages per infected cells [10], bacterial division rate [11] and burst size or free phages in media [11].

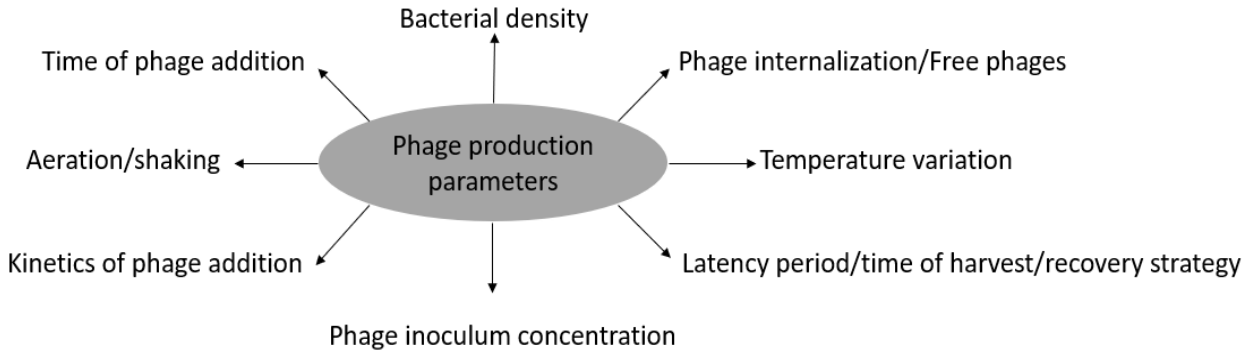


Figure 2: Parameters of phage production.

Some studies show that beyond the final phage production time, the waiting times before purifying phages can affect their stability. In fact, leaving phages in the culture medium with lysed bacteria leads to degradation of phages following DNAse attack on their genome. This was confirmed by the viral DNA assay which did indeed drop after a certain time of incubation in the culture medium. To confront this problem, monitoring of phage titer is essential, followed by an appropriate

concentration of EDTA in the medium, this allows the recovery of approximately 90% of the phage produced. Although this study is performed on T7 phages and the BL21 strain of E. coli, but the phage degradation process can be observed with other specific phages in the presence of the DNAse of the corresponding host bacterium [7]. To optimize the conservation of the phages without degradation, it is also essential not to delay the purification of the phages and their conservation in a stable buffer.

Examples of industrial large-scale production

The Swiss company Nestlé produces 1010 PFU / ml of T4 phages and collects them by filtration on a 0.22-µm membrane without loss. Then the phages are concentrated into a smaller volume by differential centrifugation, this step also allows the separation of phages from bacterial debris. The concentrated phages are cold stable in a solution containing 10 mM Magnesium ions. The purity of the phage solution is analyzed by electron microscopy and mass spectrometry. Nestlé chooses to purify phages by differential centrifugation although this is possible by simple medium speed centrifugation or exchange by anion exchange chromatography column or by ultrafiltration [8].

A review of the literature shows that phage production is not complicated if phage quantity is the only desired parameter, the procedure is comparable to large-scale fermentation. However, when phage purity and concentration are in question, it becomes important to think about the kinetics of bacteria / phage co-culture and post-production purification techniques. Among the purification techniques, we find centrifugation at different speeds, differential centrifugation with PEG or with a sucrose gradient, filtration on membranes and others [8,9]. It remains for each industry to specify which phage production and purification procedure is appropriate for its facility and which meets the appropriate Standard Operating Procedure (SOP).

Across the world, several companies have attempted to produce phages on a large scale. These phages will be for medical applications (phage therapy), agricultural or for surface treatment purposes in the food industries.

Table 1: List of top phages producing companies.

Company name	Country	Production volume	Company's website
Phage consultants	Poland	25-200L	phageconsultants.com
Phagelux	Canada + China	variable	phagelux.com
PhagoMed Biopharma GmbH	Germany	variable	phagomed.com
Technophage	Portugal	variable	technophage.pt
Omnilytics	United States	variable	phageguard.com/
Intralytix	United States	1,500 L	ntralytix.com
Cytophage	Canada	variable	cytophage.com
Armata pharmaceuticals	United States	variable	armatapharma.com
Eligo Biosience	France	variable	eligo.bio

Conclusion

As shown in figure 2, to be able to predict the amount of newly formed phages, study of certain factors effects such a adsorption constant, initial phage concentration, phage tail formation and dilution in the bioreactor is needed. Some models develop and describe the effect of bioreactor programming and the online. Compared to conventional bioreactors, BioStat bioreactors ate most adjustable according to the needs of production. In the following table a list of currently producing phage companies is established.

Whether in small, pilot or large-scale, phage production is a complex process that needs both advanced parameter setting and online follow-ups. But regardless of the complexity of this process, a lot is still to be done regarding phage recovery and optimal phage production parameters. Further studies will allow phage production in high quality and quantity to be possible and would allow further application of phage therapy and phages as a biocontrol agent.

Références

1. Vandamme, E. J., & Mortelmans, K. (2019). A century of bacteriophage research and applications: impacts on biotechnology, health, ecology and the economy. *Journal of Chemical Technology & Biotechnology*, 94(2), 323-342.

2. Loessner MJ. Bacteriophage endolysins − current state ofresearch and applications. *Curr Opin Microbiol* 2005;8:480–7.

3. Akhverdyan VZ, Gak ER, Tokmakova IL, Stoynova NV. Application of the bacteriophage Mu-driven system for the integration/amplification of target genes in the chromosomes of engineered Gram-negative bacteria − mini review. *Appl Microbiol Biotechnol* 2011;91:857.

4. Jamal, M., Bukhari, S. M., Andleeb, S., Ali, M., Raza, S., Nawaz, M. A., ... & Shah, S. S. (2019). Bacteriophages: an overview of the control strategies against multiple bacterial infections in different fields. Journal of Basic Microbiology, 59(2), 123-133.

5. Chernomordik AB. Bacteriophages and their therapeutic prophylacticuse. *Med Sestra* 1989;6:44–7.

6. García R, Latz S, Romero J, Higuera G, García K, Bastías R. Bacteriophage Production Models: An Overview. *Front Microbiol*. 2019 Jun 4;10:1187. doi: 10.3389/fmicb.2019.01187. PMID: 31214139; PMCID: PMC6558064.

7. Smrekar, F., Ciringer, M., Jančar, J., Raspor, P., Štrancar, A. and Podgornik, A. (2011), Optimization of lytic phage manufacturing in bioreactor using monolithic supports. *J. Sep. Science*, 34: 2152-2158. doi:10.1002/jssc.201100182

8. Bourdin G, Schmitt B, Marvin Guy L, et al. Amplification and purification of T4-like escherichia coli phages for phage therapy: from laboratory to pilot scale. *Appl Environ Microbiol*. 2014;80(4):1469-1476. doi:10.1128/AEM.03357-13

9. Jurač, K., Nabergoj, D., & Podgornik, A. (2019). Bacteriophage production processes. *Applied Microbiology and Biotechnology*, 103(2), 685-694.

10. Campbell, A. (1961). Conditions for the existence of bacteriophage. *Evolution* 15, 153–165. doi: 10.2307/240607

11. Santos, S. B., Carvalho, C., Azeredo, J., and Ferreira, E. C. (2014). Population dynamics of a Salmonella lytic phage and its host: implications of the host bacterial growth rate in modelling. *PLoS* One 9:e102507. doi: 10.1371/journal.pone.0102507

12. Nabergoj, D., Modic, P., and Podgornik, A. (2018b). Effect of bacterial growth rate on bacteriophage population growth rate. *Microbiologyopen* 7:e00558. doi:10.1002/mbo3.558

13. Grieco, S. H. H., Wong, A. Y., Dunbar, W. S., MacGillivray, R. T., & Curtis, S. B. (2012). Optimization of fermentation parameters in phage production using response surface methodology. *Journal of Industrial Microbiology & Biotechnology*, 39(10), 1515-1522.

14. Lemaître, J. P., Duroux, A., Pimpie, R., Duez, J. M., & Milat, M. L. (2015). Listeria phage and phage tail induction triggered by components of bacterial growth media (phosphate, LiCl, nalidixic acid, and acriflavine). *Applied and Environmental Microbiology*, 81(6), 2117-2124.

15. Ward, J. M., Branston, S., Stanley, E., & Keshavarz-Moore, E. (2019). Scale-Up and Bioprocessing of Phages. In *Bacteriophages-Biology and Applications*. IntechOpen.

16. Mancuso, F., Shi, J., & Malik, D. J. (2018). High throughput manufacturing of bacteriophages using continuous stirred tank bioreactors connected in series to ensure optimum host bacteria physiology for phage production. *Viruses*, 10(10), 537.

17. Yamamoto, K. R., Alberts, B. M., Benzinger, R., Lawhorne, L., & Treiber, G. (1970). Rapid bacteriophage sedimentation in the presence of polyethylene glycol and its application to large-scale virus purification. *Virology*, *40*(3), 734-744.

18. Agboluaje M, Sauvageau D. Bacteriophage Production in Bioreactors. *Methods Mol Biol.* 2018;1693:173-193. doi: 10.1007/978-1-4939-7395-8_15. PMID: 29119441.