Enriching the Unculturable Microbial Majority of the Human Gastrointestinal Tract through Microfluidic Encapsulation

by

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To Mark and my family,

for their endless support and encouragement.

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Réanimer et enrichir la majorité microbienne non cultivable du tractus gastro-intestinal humain par encapsulation microfluidique

Sydney WHEATLEY

RÉSUMÉ

Le microbiote intestinal humain est un consortium de microorganismes composé principalement de bactéries, de mycètes et d'archées. La communauté complexe des bactéries, en particulier, fait l'objet de nombreuses recherches en raison de son lien avec la santé et les maladies humaines. Malheureusement, les études visant à comprendre cette relation alambiquée manquent d'informations essentielles, car une grande partie de cette population commensale n'a pas été cultivée *in vitro*. L'une des principales théories expliquant l'absence de souches cultivées est l'impossibilité d'imiter les conditions naturelles *in vitro* nécessaires à la croissance des bactéries. En raison de la prise de conscience croissante de l'importance du microbiote intestinal pour la santé humaine, il est nécessaire d'améliorer les techniques de culture. En particulier, l'encapsulation des cellules est une approche prometteuse pour améliorer les rendements de culture en raison des effets mécanostimulants et de l'isolement spatial des cellules. La génération de gouttelettes microfluidiques est une méthode d'encapsulation cellulaire préférable en raison du contrôle élevé de la formation des microbilles et de la compatibilité des biomatériaux.

Dans cette thèse, l'encapsulation microfluidique de bactéries intestinales anaérobies est étudiée pour améliorer la culture *in vitro*. La génération de gouttelettes microfluidiques a été caractérisée et optimisée pour la compatibilité avec un biomatériau synthétique, à savoir le poly(éthylène glycol maleimide) (PEG4MAL) à quatre bras. L'aptitude du PEG4MAL à la culture de bactéries anaérobies a été caractérisée par l'évaluation des propriétés mécaniques, de la dégradation, de la microstructure et de la biocompatibilité. Enfin, l'effet de l'encapsulation a été évalué en examinant la viabilité cellulaire et la formation de colonies.

L'application de la microencapsulation PEG4MAL pour l'enrichissement des bactéries intestinales anaérobies difficiles à cultiver a conduit à une forte densité de croissance à l'intérieur des microbilles. Ce travail jette les bases d'études futures explorant la possibilité d'une culture, d'un échantillonnage et d'une distribution *in situ* dans le tractus gastro-intestinal humain.

Mots-clés: microfluidique, microencapsulation, microbiote intestinal humain, bactéries

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ABSTRACT

The human gut microbiota is a consortium of microorganisms made up primarily of bacteria, fungi, and archaea. The complex community of bacteria, in particular, is highly investigated due to its link to human health and disease. Unfortunately, studies aimed at understanding this convoluted relationship are missing key information as a large portion of this commensal population has not been cultured *in vitro*. One of the dominating theories for the lack of cultured strains is due to the inadequacies of imitating native conditions *in vitro* that are required for bacterial growth. Due to the growing awareness of gut microbiota's importance for human health, there is an established need for improved culture techniques. Notably, cell encapsulation is a promising approach to improve culture outputs due to the mechanostimulatory effects and spatial isolation of cells. Microfluidic droplet generation is a preferable method of cell encapsulation due to the high control of microbead formation and biomaterial compatibility.

In this thesis, the microfluidic encapsulation of anaerobic gut bacteria is explored to improve *in vitro* culture. The microfluidic droplet generation was characterized and optimized for compatibility with a synthetic biomaterial, namely four-arm poly(ethylene glycol maleimide)(PEG4MAL). The suitability of PEG4MAL for anaerobic bacteria culture was characterized by assessing mechanical properties, microstructure, and biocompatibility. Lastly, the effect of encapsulation was evaluated by examining cell viability and colony formation.

The application of PEG4MAL microencapsulation for the enrichment of difficult-to-grow anaerobic gut bacteria has led to high-density growth within the microbeads. This work provides the foundation for future studies exploring the possibility of *in situ* culture, sampling, and delivery within the human gastrointestinal tract.

Keywords: microfluidics, microencapsulation, human gut microbiota, bacteria

PREFACE

The experimental work presented in this thesis was conducted in the Biomaterial and BioFabrication Lab at the University of Montreal Hospital Research Center and École de technologie supérieure. All experimental work involving the use of cells was conducted in the Maurice Lab, a part of the McGill Center for Microbiome Research at McGill University. The content of this thesis is under preparation for publication in a peer-reviewed journal.

Melanie Rodger performed the rheological test presented in this thesis. Lisa Dupeyroux and Hanna Hamoud-Michel performed the mechanical test presented in this thesis. The experiments presented in sections 2.4 and 3.4 were performed with assistance from Lisa Dupeyroux.

Peer-Reviewed Journal Articles

1. **Wheatley, S. K.**, Dupeyroux, L., Rodger, M., Lerouge, S., Maurice, C. F., & Ahmadi, A. (2025) Microfluidic Encapsulation of the Human Microbiota: A Tool for Research and Beyond, *Microsystems and Nanoengineering* (In Preparation).
2. **Wheatley, S. K.**, Lerouge, S., Maurice, C. F., & Ahmadi, A. (2025) Culturing a Cure: Bacteria in Cancer Therapy, (In preparation)
3. Safeghianmaryan, A., Ahmadian, N., **Wheatley, S. K.**, Sardroud, H. A., Nasrollah, S. A. S., Naseri, E., & Ahmadi, A. (2024) Advancements in 3D-Printable Polysaccharides, Proteins and Synthetic Polymers for Wound Dressing and Skin Scaffolding A Review, *International Journal of Biological Macromolecules*.
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Conference Proceedings

1. Hamoud-Michel, H., **Wheatley, S. K.**, King, I., Maurice, C. F., Ahmadi, A. Smart Capsules for the Targeted Sampling of Microbiota within the Gastrointestinal Tract. Proceedings of the Canadian Society for Mechanical Engineering International Congress. CSME 2025, Montreal, Canada.
2. Dupeyroux, L., **Wheatley, S. K.**, Lerouge, S., Maurice, C. F., & Ahmadi, A. Intestinal Bacteria Microencapsulation: Optimization of Microbead Composition to Improve Cell

Adhesion and Enrichment. The Canadian Biomaterials Society Congress. CBS 2025, Kingston, Canada.

3. **Wheatley, S. K.**, Hamoud-Michel, H., Phan, C., Lerouge, S., Maurice, C. F., & Ahmadi, A. A Gut Feeling: Understanding the Gut Microbiota through Microencapsulation: The 28th International Conference on Miniaturized Systems for Chemistry and Life Sciences. MicroTAS 2024, Montreal, Canada.
4. Hamoud-Michel, H., **Wheatley, S. K.**, Phan, C., King, I., Maurice, C. F., & Ahmadi, A. An Electronic-Free Device for the Targeted Samplign of Microbiota within the Gastrointestinal Tract. The 28th International Conference on Miniaturized Systems for Chemistry and Life Sciences. MicroTAS 2024, Montreal, Canada.
5. **Wheatley, S. K.**, Phan, C., Lerouge, S., Maurice, C. F., & Ahmadi, A. *In Situ* Culture of the Unculturable Human Gastointestinal Microbiota Bacteria. The 12th World Biomaterials Congress. WBC 2024, Daegu, South Korea.
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7. **Wheatley, S. K.**, Navaei, T., Pope, E., Alkayyali, T., Cartmell, C., Haltli, B. A., Kerr, R. G., & Ahmadi, A. Growing the Unknown: Investigation into a New Approach for *In Situ* Microbial Growth. The 27th International Conference on Miniaturized Systems fro Chemistry and Life Sciences. MicroTAS 2023, Katowice, Poland.
8. **Wheatley, S. K.**, Navaei, T., Cartmell, C., Haltli, B. A., Kerr, R. G., & Ahmadi, A. Microfabrication of Domestication Pods for *In Situ* Cultivation of Marine Bacteria and Natural Product Discovery. The Canadian Biomaterials Society Congress. CBS 2023, Halifax, Canada.

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LIST OF ABBREVIATIONS

ETS	École de Technologie Supérieure
CRCHUM	University of Montreal Hospital Research Center
PEG4MAL	Four-arm poly(ethylene glycol) maleimide
GI	Gastrointestinal
3D	Three-Dimensional
SCFA	Short-Chain Fatty Acid
FMT	Fecal Microbiota Transplant
VBNC	Viable but Nonculturable
A. muciniphila	*Akkermansia muciniphila*
HEPES	4- (2-hydroxyethyl) -1-piperazine ethanesulfonic acid
DTT	Dithiothreitol
PBS	Phosphate-Buffered Saline
E. coli	*Escherichia coli*
BHI	Brain Heart Infusion
PI	Propidium Iodide
RGD	Arginylglycylaspartic acid
ANOVA	Analysis of Variance
2D	Two-Dimensional
SEM	Scanning Electron Microscope

LIST OF SYMBOLS AND UNITS OF MEASUREMENTS

Q_m	mass swelling ratio (%)
m	mass (g)
Ca	capillary number
μ	viscosity (Pa·s)
U	velocity (m/s)
σ	interfacial tension (mN/m)
E	molar extinction coefficient (L/mol·cm)
A	absorbance
C	absorbance of control
ϕ	void fraction (%)

INTRODUCTION

The human gut microbiota is an assemblage of microorganisms that colonize the gastrointestinal (GI) tract. The consolidation of its genes, otherwise known as the microbiome, accounts for many biological functions that can be mutually beneficial for the microorganisms and human host (Almeida *et al.*, 2021). However, it has also been shown that changes in the abundance of certain species can be linked to adverse health effects and disease (Keshet & Segal, 2024; Zheng *et al.*, 2024; Cheng, Wu & Yu, 2020). Although establishing clear causality between health states and microbiota composition is daunting due to the inter-individuality and ever-evolving community compositions of bacteria within the GI tract.

Technical limitations have also impeded gut microbiota research as many species of bacteria resist *in vitro* cultivation (Lloyd, Steen, Ladau, Yin & Crosby, 2018; Almeida *et al.*, 2021; Pribyl, Hugenholtz & Cooper, 2025). In particular, the human GI tract is dominated by strictly obligate anaerobes with specific environmental requirements (Bodor *et al.*, 2020). These bacteria are also heavily adapted to thrive under co-culture conditions with access to unique nutritional and environmental factors (Culp & Goodman, 2023). As a result, recapitulating these complex requirements *in vitro* is laborious. Alternative approaches to culture have been investigated to surmount bacterial sensitivity within other ecological contexts with promising results (Kaeberlein, Lewis & Epstein, 2002; Bollmann, Lewis & Epstein, 2007; Alkayyali *et al.*, 2021). However, translation of this technology to the human gut microbiota has yet to be explored.

Of these techniques, microencapsulation of bacteria is of particular interest due to its ability to physically isolate cells during culture. The physical confinement of bacteria to microbeads can decrease nutrient competition between species while providing a more realistic three-dimensional (3D) environment for culture. Historically, bacteria encapsulation has been dominated by the

use of naturally derived materials; however, this can be considered a major limitation for studies exploring gut microbiota.

Herein, this thesis presents the microfluidic encapsulation of difficult-to-culture strictly anaerobic bacteria as the first step of a multi-pronged pursuit to explore the human gut microbiota. This thesis explores the novel application of synthetic PEG4MAL to achieve isolation and 3D culture of gut-derived bacteria to enrich historically unculturable bacteria, which will be discussed over the following three chapters (Figure 0.1).

Chapter 1 of this thesis provides a detailed account of the motivation for this work, highlighting the importance of the human gut microbiota and the current technological challenges that limit discovery. Chapter 1 also includes a list of the specific objectives of this work.

The methodologies for experimental work are presented in Chapter 2. The experiments broadly consist of biomaterial physical and mechanical property assessment, microfluidic setup and optimization, biomaterial biocompatibility, and measures of cell enrichment.

Chapter 3 presents the results of the microfluidic optimization for microbead generation in anaerobic environments. Additionally, the suitability of PEG4MAL for bacteria encapsulation is supported by evidence of biocompatibility and desirable physical properties. Lastly, the impact of encapsulation on bacteria enrichment is presented by measures of cell viability, and colony formation.

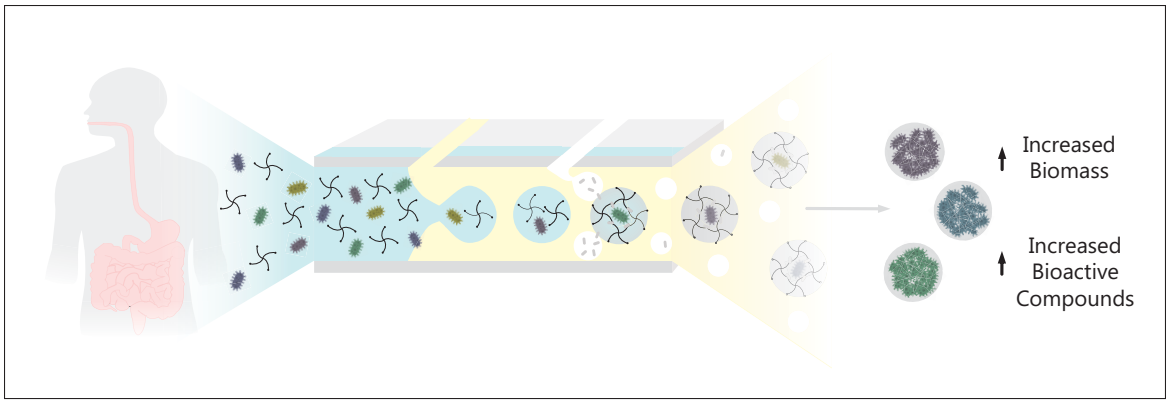

Figure 0.1 A microfluidic approach to explore the human gut microbiota through encapsulation to increase diversity, biomass, and metabolic secretions of cultured species

CHAPTER 1

LITERATURE REVIEW

1.1 The Human Gut Microbiota

Recently, studies focusing on the gut microbiota have increased exponentially, and in concert, so has the awareness of its important role in human health and disease. Contestably crowned one of the newest organ systems, the gut microbiota is made up of a diverse community of bacteria, fungi, archaea, and phages capable of multidirectional interactions with the body, though not yet fully understood (Collado, Devkota & Ghosh, 2024; Cani, 2017; Afzaal *et al.*, 2022). Of the microorganisms within the gastrointestinal (GI) tract, bacteria are considered the most significant players with prominent phyla consisting of Bacillota (formerly Firmicutes), Bacteroidota (formerly Bacteroidetes), Actinomycetota (formerly Actinobacteria), Pseudomonadota (formerly Proteobacteria), and Verrucomicrobiota (formerly Verrucomicrobia) (Tremaroli & Bäckhed, 2012; Oren, Göker & Sutcliffe, 2022).

One of the most dominant host-microbe interactions consists of bacteria's role in host metabolism by breaking down complex carbohydrates and polysaccharides that are nondigestible by host enzymes (Tremaroli & Bäckhed, 2012; Ramakrishna, 2013). During this fermentation process, several metabolic by-products are produced, such as short-chain fatty acids (SCFAs) or secondary bile salts, which contribute to healthy host and resident microbial function (Anwer *et al.*, 2025; Vogt, Peña-Díaz & Finlay, 2015). Despite the perceived benefit for the host, the overgrowth of bacteria within the mucosal layer of the GI tract is actively prevented by numerous host cell response pathways (Geuking, Köller, Rupp & McCoy, 2014; Walter & Ley, 2011). Additional support in maintaining GI community homeostasis comes from commensal microbes as they use targeted approaches to prevent the outbreak of pathogenic bacteria (Kamada, Chen, Inohara & Núñez, 2013; Bäumler & Sperandio, 2016).

Not only is the presence of microbes important, but the relative abundance of the microbial community also influences host health (Pepke, Hansen & Limborg, 2024). It is hypothesized

that significant shifts in the relative abundance of the resident community are closely linked to adverse health outcomes. For example, recent evidence suggests that a lower diversity of bacteria within the GI tract has been associated with a higher prevalence of metabolic risk factors, bowel disorders, cancers, and more (Keshet & Segal, 2024; Zheng *et al.*, 2024; Cheng *et al.*, 2020). Additionally, the gut microbiota can affect gut epithelial permeability, which is closely related to host inflammation and insulin sensitivity (Tremaroli & Bäckhed, 2012). As such, the age-old question of whether the chicken or the egg came first finds a parallel in microbiome research as scientists question whether the microbiota's composition is the catalyst of disease or merely a response to environmental changes (Cani, 2017; Hammer, 2024). However, the transient nature and inter-individuality of the gut microbiota make establishing a causal relationship between the microbiota and human health a daunting task, and therefore, many unknowns remain (Procházková *et al.*, 2024; Odamaki *et al.*, 2016)(Figure 1.1).

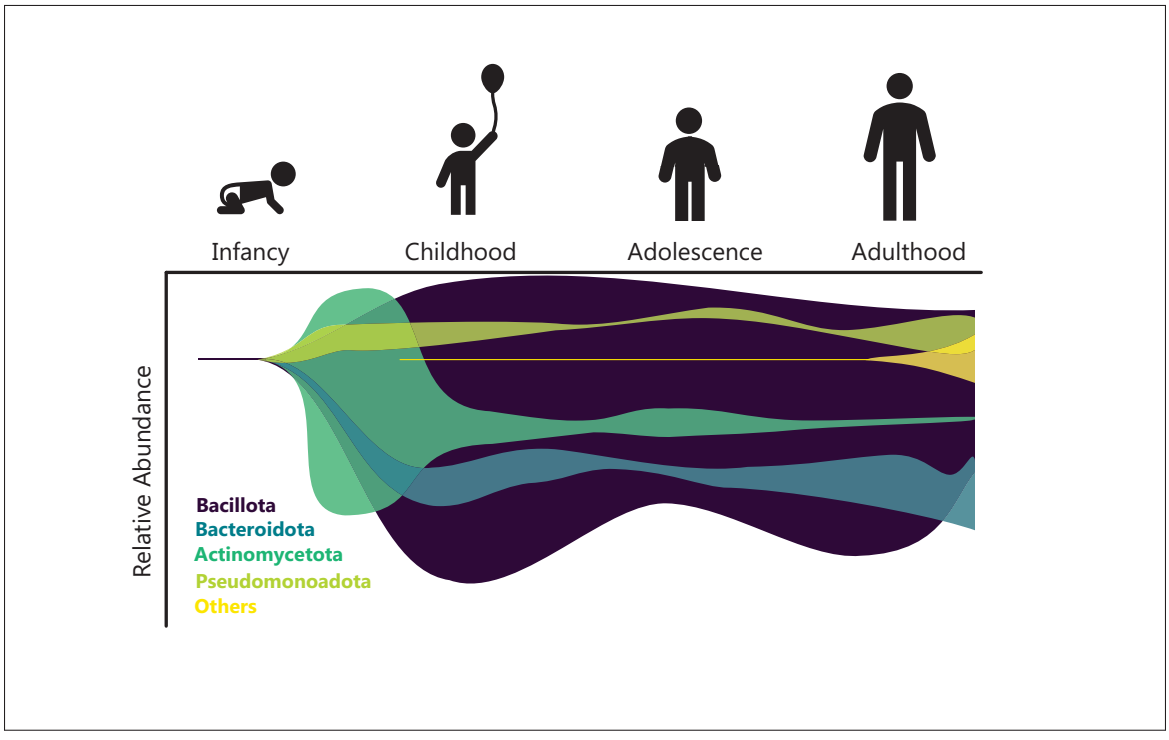

Figure 1.1 A graphical depiction of the variation of dominant gut microbiota phyla from infancy to adulthood (Odamaki *et al.*, 2016)

1.2 Unculturable Microbial Majority

Of the vast number of species that can colonize the human gut, it is estimated that up to 71% remain “uncultured” (Lloyd *et al.*, 2018; Almeida *et al.*, 2021; Pribyl *et al.*, 2025). Within the context of the human microbiota, these uncultured species have been labelled the “Most Wanted Taxa.” These species are highly sought after for key information such as whole genome data and morphological characterization (Fodor *et al.*, 2012). This "yet-to-be-cultured" phenomenon is unsurprising in the field of microbiology as it was first brought into the spotlight in 1985 (Staley & Konopka, 1985). Originally crowned the "Great Plate Count Anomaly," it was estimated that only 1% of environmental microorganisms can be cultured *in vitro* Staley & Konopka (1985).

Overcoming the "unculturable" phenomenon *in vitro* is important in the context of gut microbiota research as many findings on its role in human health remain speculative in nature (Relman, 2020; Allen-Vercoe, 2013). Notably, *in vitro* culture allows researchers to limit confounding variables by isolating individual parameters of interest. For example, the gut microbiota can be studied *in vitro* at the individual species level or via the interaction of a controlled consortia of several species. The co-culture of mammalian and microbial species can also further elucidate specific host-microbe interactions that otherwise cannot be causally ascertained (Kim, Wang, Sims & Allbritton, 2022; Donkers *et al.*, 2024; Wang *et al.*, 2024). Optimization of biologics, such as fecal microbiota transplants (FMTs), may be achieved through *in vitro* culture and standardization of beneficial species (Singh *et al.*, 2024). Lastly, understanding the metabolite profile of the gut microbiota is also important, and *in vitro* culture can enable direct measurement of these critical molecules. Merely scraping the surface, *in vitro* culture can empower microbiome researchers with a means to critically assess and validate their findings in a controlled environment; although, current challenges with *in vitro* culture restrict its applicability.

The leading theory for the resistance to culture is the lack of mimicry of environmental conditions *in vitro* such as the nutrient profile, pH, degree of oxygenation, and more. As the site of host nutrient digestion, the GI tract has a complex nutritional landscape available to the microbiota

that is spatially variable (Kiela & Ghishan, 2016; Pereira & Berry, 2017). The nutrient niches of the GI tract can drive changes in microbial composition, but imitation of these conditions *in vitro* is complex and requires extensive experimental optimization (Dupont *et al.*, 2019). Both pH and oxygen levels can help dictate the local microbial composition, but are also spatially variable along the GI tract (Ng *et al.*, 2023; Duncan, Louis, Thomson & Flint, 2009; Espey, 2013). In addition to difficulties in recapitulating conditions of the GI tract, physical conditions subjected to bacteria during *in vitro* culture, such as white light exposure, have been shown to negatively impact the success of culture (Bodor *et al.*, 2020). Lastly, recent studies have also shown evidence that bacteria can sense and respond to external forces which can act as a modulator of cell fitness, also referred to as mechanostimulation (Dufrêne & Persat, 2020). The cell's ability to tolerate mechanical stressors can be linked to membrane composition and can promote distinct phenotypes such as substrate adhesion (Dufrêne & Persat, 2020). Therefore, in order to successfully culture bacteria, various chemical and physical parameters must be taken into consideration.

During culture, when these favourable environmental conditions have not been satisfied, bacteria are capable of entering dormant states. The most characterized state of dormancy is the sporulation of bacteria, where cells can undergo a physical transformation and cease metabolic activity (Koopman, Remijas, Seppen, Setlow & Brul, 2022). Less understood is the idea of viable but nonculturable (VBNC) cells, which can be detected through standard microbiology imaging and screening as viable and metabolically active, but do not reproduce or form colonies (Bodor *et al.*, 2020). VBNCs are of particular interest as mounting evidence indicates that these cells are capable of resuscitation back to their viable and culturable state; however, the exact mechanisms of resuscitation are not yet well-understood (Pan & Ren, 2022; Bodor *et al.*, 2020). The combination of spore and VBNC presence is hypothesized to contribute to the lack of representative cultured isolates of the gut microbiota (Figure 1.2).

In addition to the ecological specificities of the GI tract, the community composition of microorganisms can also impact individual species' ability to thrive *in vitro*. Inter-species relationships have been well characterized, such as synergism, commensalism, and competition,

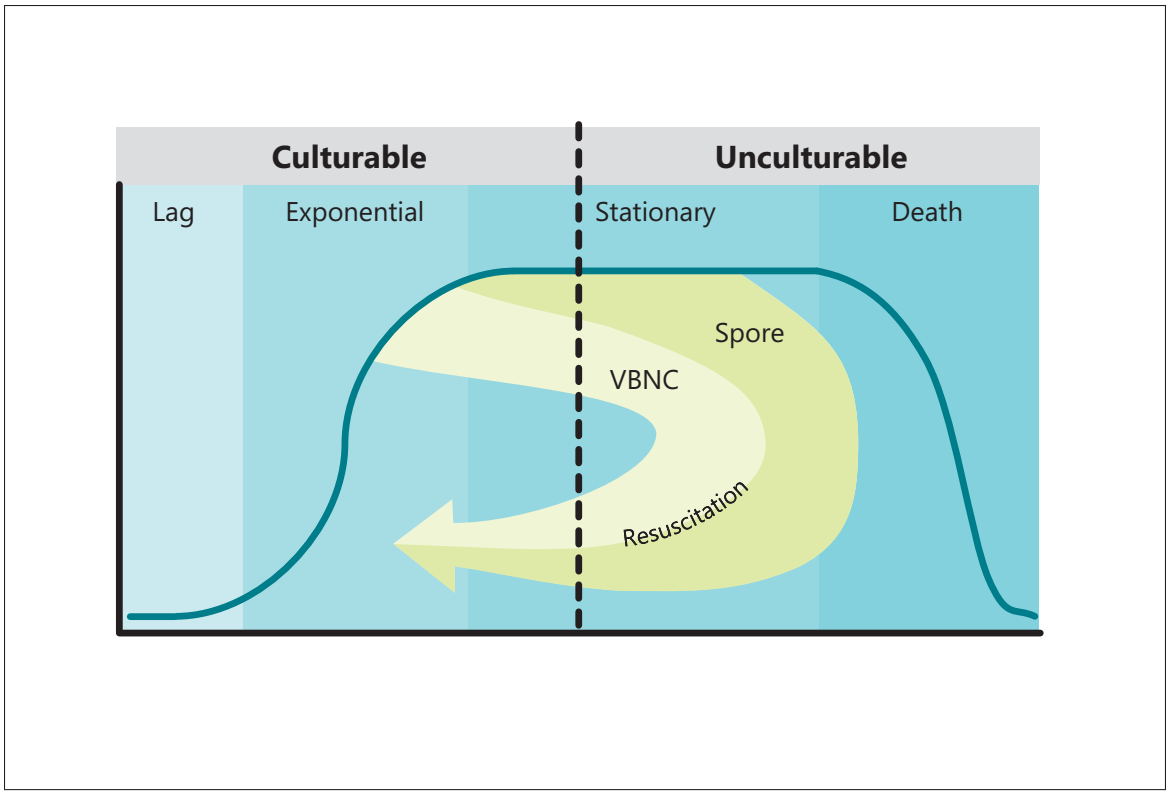

Figure 1.2 A schematic of a microbial growth curve including the VBNC and spoulative states

and are present in every ecological community of microbial growth (Weiland-Bräuer, 2021). Within the GI tract, many types of interaction between species have been identified and can occur simultaneously to drive these inter-species relationships (Coyte & Rakoff-Nahoum, 2019). A dominating theory of microbe-microbe interactions is cross-feeding within the GI tract, where the metabolic products of one species act as an energy source for another (Culp & Goodman, 2023). Conversely, researchers argue that nutrient competition is a core influence on community composition (Pereira & Berry, 2017; Horrocks, King, Yip, Marques & McDonald, 2023). Other mechanisms of bacterial cross-talk, such as signaling molecules and gene transfer, have also been shown to influence community composition (Sultan, Mottawea, Yeo & Hammami, 2021; Martín, Bermúdez-Humarán & Langella, 2016).

The complex cellular interactions of the gut microbiota are theorized to contribute to axenic culture resistance, but likewise can contribute to the underrepresentation of some species during

community culture. This dynamic, evolutionary relationship between the resident microbiota and host environment is the primary driver perpetuating the existence of the unculturable microbial majority (Lewis, Tahon, Geesink, Sousa & Ettema, 2021). Despite microbial sensitivities to culture, culture-based methods are the foundation of current microbiology knowledge and continue to be an important approach to new discovery.

1.3 Bacteria Culture and Sequencing

The plight of traditional culture-based gut microbiota research stems from bacterial sensitivity to many environmental conditions (Pribyl *et al.*, 2025; Browne *et al.*, 2016; Kaeberlein *et al.*, 2002). These issues are often exacerbated for sensitive and low-abundant strains prevalent within the GI tract, resulting in a significant barrier to human health research and discovery. However, culture-based methods enable investigation into microbiota inheritance, cell morphology, function, metabolism, and immune system development and yield whole-genome samples (Fodor *et al.*, 2012; Almeida *et al.*, 2021; Lewis *et al.*, 2021; Giovannoni & Stingl, 2007).

In recent years, there has been less emphasis on culture-based GI studies due to the complexities in accounting for the diverse microbial requirements. In turn, sequencing-based approaches have dominated microbiome research and have led to significant data-driven advancements, including the identification of new species, improved diagnostics, and complex analyses (Almeida *et al.*, 2021; Nayfach, Shi, Seshadri, Pollard & Kyrpides, 2019; Almeida *et al.*, 2019). Genomic analysis enables rapid and detailed detection of bacteria up to the strain level using many different techniques; however, these methods fall short with low biomass samples and can be biased by amplification requirements (Almeida *et al.*, 2021; Hiergeist, Gläsner, Reischl & Gessner, 2015). As a result, researchers are faced with a catch-22 from sequencing- and culture-based approaches; therefore, important information continues to fall between the cracks.

The need for improved culture techniques is no secret and has forced researchers to deviate from traditional broth- or solid medium-based culture. New innovations have appeared to better recapitulate or reincorporate bacteria into native ecological contexts such as soil and seawater

with promising results (Kaeberlein *et al.*, 2002; Bollmann *et al.*, 2007; Nichols *et al.*, 2010; Wheatley *et al.*, 2022; Pope, Cartmell, Haltli, Ahmadi & Kerr, 2022). Such technology has helped overcome the optimization bottlenecks inherent to culturomic-based studies, which depend on precise *in vitro* manipulation of nutrients, selective inhibitors, and environmental conditions. The human GI tract presents a unique challenge for mimicking culture conditions due to its spatially variable microbial community, pH, enzymatic presence, and anoxic conditions (Sheth *et al.*, 2019). While *in situ* cultivation may offer a promising experimental model for future gut microbiota research, there remains an urgent need to improve *in vitro* culture strategies.

1.4 Microfluidics in Microbiota Research

To overcome obstacles in culture, the adaptation of microfluidic technologies to the field of microbiome research has increased rapidly, enabling the creation and manipulation of micro-environments in a high-throughput fashion (Yin *et al.*, 2022; Pajoumshariati *et al.*, 2018; Tauzin *et al.*, 2020; Penarete-Acosta *et al.*, 2024). More specifically, microfluidics is the study of fluid manipulation within micron- or submicron-sized channels (Hajam & Khan, 2024). The devices used to perform such studies, referred to as microfluidic chips, take on many different forms and have been customized for widespread use. However, the commercialization and real-world translation of this technology remain in its infancy (Nelson *et al.*, 2021; Jalili-Firoozinezhad *et al.*, 2019; Min *et al.*, 2022).

Nonetheless, the application of microfluidics to various fields of research has allowed for the miniaturization and biomimicry of complex experimental and biological pathways (Wong Hawkes, Chapela & Montembault, 2005; Özyurt, Uludağ, İnce & Sezgintürk, 2023; Trujillo-de Santiago, Lobo-Zegers, Montes-Fonseca, Zhang & Alvarez, 2018). For example, gut-on-a-chip technology has enabled researchers to recreate human physiology *in vitro* as a means to shift away from the reliance on animal models (Ashammakhi *et al.*, 2020; Huh *et al.*, 2013). In general, the primary goal of gut-on-a-chip devices is to create a more representative and functional system to assess drug absorption, toxicity, GI physiology, and disease pathology. Recently, rudimentary models have also begun to explore the incorporation of fecal-derived microorganisms into

gut-on-a-chip models (Donkers *et al.*, 2024; Wang *et al.*, 2024). Although, the scope of this work has primarily focused on the assessment of host response, and little has been explored through a community-based microbiological lens (Lee *et al.*, 2023; Shah *et al.*, 2016).

Other microfluidic approaches have explicitly focused on the human gut microbiota through the isolation and compartmentalization of bacteria exclusively (Yu *et al.*, 2022). The primary method for microfluidic microbial segregation consists of microarrays or water-in-oil droplets for on-chip culture (Sharma *et al.*, 2014; Yin *et al.*, 2022; Tauzin *et al.*, 2020; Villa *et al.*, 2020). Mounting evidence has shown that on-chip cultivation is a high-throughput approach that can better characterize microbial functions and metabolism of isolates from complex communities (Villa *et al.*, 2019; Ingham *et al.*, 2007). It has also streamlined and accelerated screening for antibiotic resistance, bacteriophages, and uncovered other complex cell-cell interactions (Ge, Girguis & Buie, 2016; Watterson *et al.*, 2020; Mirzaei & Deng, 2022). On-chip culture can be integrated jointly with different technologies for the detection of new species and mining of bioactive products (Pryszlak *et al.*, 2022; Ingham *et al.*, 2007; Uehling *et al.*, 2019; Andreasen *et al.*, 2018). Unfortunately, this technology presents many drawbacks, primarily from insufficient imitation of *in situ* conditions. On-chip culture often experiences gas diffusion, insufficient access to nutrients, limited waste diffusion, and a finite experimental time due to the coalescence of droplets (Villa *et al.*, 2019; Liu *et al.*, 2021; Watterson *et al.*, 2020). Therefore, a more robust approach is needed to capitalize on the benefits of microfluidic technologies while better imitating native environmental conditions.

1.5 Bacteria Encapsulation

As previously mentioned, droplet microfluidics is particularly advantageous as it can physically isolate cells in a high-throughput fashion. Another avenue of droplet microfluidic research involves using polymeric materials to create stable 3D microenvironments, which have been investigated as an adjunct approach to culture (Alkayyali *et al.*, 2021). This strategy, dubbed cell encapsulation, can overcome the obstacles apparent in on-chip culture by implementing more physical, chemical, and mechanical cues for microbial culture. Herein, the application of cell

encapsulation in the context of the human gut microbiota is explored. Additionally, common approaches and polymeric materials used for cell encapsulation are described.

1.5.1 Existing Applications

Cell encapsulation is a widespread approach for studying cell behaviour, delivery, and culture. For example, cell encapsulation has been extensively explored for mammalian cell culture in natural and synthetic biomaterials for multifaceted applications such as immune system modulation and tissue regeneration with promising results (Cunningham *et al.*, 2023; Desai & Shea, 2017; Weir & Xu, 2010). The gel-based 3D environment can alter mammalian cell survival and differentiation by modifying parameters such as material stiffness, porosity, and adhesion sites (Podhorská *et al.*, 2024). However, polymeric cell encapsulation has yet to be explored for human gut microbiota culture.

Within different ecological contexts, the encapsulation of bacteria has indicated preferential growth compared to standard culture methods and has been implemented for diverse applications (Alkayyali *et al.*, 2021; Pope *et al.*, 2022; Wang, Ishii & Novak, 2021; Gutierrez, Reed, McElroy & Hansen, 2024). The prevailing use of bacteria encapsulation is for environmental bioremediation with a particular focus on soil contamination, crude oil contamination, and wastewater treatment. Mounting evidence suggests that the encapsulation and immobilization of bacteria leads to a more rapid metabolic removal of toxic compounds from the environment with reduced fouling (Armanu *et al.*, 2025; Bayat, Hassanshahian & Cappello, 2015; Juntawang, Rongsayamanont & Khan, 2017). Encapsulation has also been used to explore one of the least understood ecological environments of bacteria growth—the vast ocean depths (Pope *et al.*, 2022). Previous work has shown that the encapsulation of bacteria isolated from marine sources can be employed as a means of "unculturable" species enrichment and drug discovery (Alkayyali *et al.*, 2021; Pope *et al.*, 2022; Navaei *et al.*, 2023). Overall, the encapsulation of environmentally derived bacteria has shown promising results for a wide range of uses.

To date, the encapsulation of gut-associated bacteria has been limited to probiotic delivery. Probiotics, a current buzzword, refers to a bacterial species or community of species that are administered orally to correct microbiota imbalances, but very few commercially available probiotics in Canada have approved clinical indications. Some of the primary issues observed with probiotics are low cell survival and engraftment due to the harsh encapsulation methods and influence from the resident microbiota (Huq, Khan, Khan, Riedl & Lacroix, 2013; Walter, Maldonado-Gómez & Martínez, 2018). Although probiotics are a promising avenue for personalized and preventative medicine, further work is required to improve live cell retention time and ensure the ultimate engraftment of bacteria in the gastrointestinal tract.

1.5.2 Droplet-Based Encapsulation Techniques

The appeal for the entrapment of cells within hydrogels stems from the need for cell protection. A rudimentary technique for achieving cell encapsulation is through the emulsification of two immiscible phases (Figure 1.3 A). Emulsification is a rapid approach to achieve polymeric encapsulation of cells and can be easily scaled to industrial-sized applications, but it leads to substantial size and morphological variances of droplets (Neufeld & Poncelet, 2004; Alinejad *et al.*, 2019). Similarly, an oscillatory mechanical motion can be used to break up fluid flow and create droplets (Figure 1.3 B). This technique, referred to as vibrating nozzle extrusion, involves a high-velocity laminar fluid flow extruded through a vibrating nozzle (Whelehan & Marison, 2011). The droplets are typically generated in air and collected in an aqueous or oleaginous solution for crosslinking. However, a shortcoming of this technology is the limited compatibility with biomaterials due to a restrictive viscosity range (Whelehan & Marison, 2011; Dorati, Genta, Modena & Conti, 2013).

Alternatively, electrospraying permits higher control of droplet formation down to the nano-range (Jaworek, 2008; Boda, Li & Xie, 2018). As opposed to the utilization of mechanical force, the mechanism of droplet generation stems from an applied electric field (Watanabe, Matsuyama & Yamamoto, 2003) (Figure 1.3 B). However, the voltage applied to the nozzle must be sufficiently high to overcome the surface tension of the fluid to generate droplets

(Jaworek, 2008). Despite the high required voltages, the current remains low and is generally tolerated by living cells, leading to success in cell encapsulation (Wang, Jansen & Yang, 2019; Young, Poole-Warren & Martens, 2012; Wu *et al.*, 2019). However, issues regarding viability begin to arise at voltages in excess of 20 kV, and unfortunately, this technique is considered low-throughput (Ye *et al.*, 2015; Jaworek, 2008). Another encapsulation technique relies on the principle of charge, albeit in a very different manner. This technique, referred to as complex coacervation, involves the interaction between both an anionic and cationic polymer, resulting in phase separation (Timilsena, Akanbi, Khalid, Adhikari & Barrow, 2019) (Figure 1.3 B). Although coacervation is predominantly applied to the food and pharmaceutical industries, it can also be used for cell encapsulation (Timilsena *et al.*, 2019). Coacervation faces drawbacks akin to vibrating nozzle extrusion due to the limited material compatibility, but also exposes cells to harsh pH and temperature conditions during a time-consuming process (de Vos, Lazarjani, Poncelet & Faas, 2014; Baruch & Machluf, 2006; Eratte, Dowling, Barrow & Adhikari, 2018).

Microfluidic encapsulation provides a unique alternative to these brute force approaches, offering high cell viability, easily tailored microbead sizes, compatibility with many biomaterials, and the ability to achieve high-throughput single-cell encapsulation. There are many different approaches to microfluidic encapsulation, such as flow focusing, T-junctions, and co-flow of two immiscible phases (Alkayyali, Cameron, Haltli, Kerr & Ahmadi, 2019) (Figure 1.3 B). Each of these approaches can be conveniently adapted to different applications with the use of syringe, peristaltic, or pressure-driven pumps. Of the aforementioned encapsulation methods, microfluidics is highly coveted due to its ease of manipulation and optimization with various biomaterials, but faces pushback due to the dependency on oil and its historically tedious removal (Figure 1.3).

1.5.3 Biomaterial Selection

Regardless of the technique for droplet formation, there are various approaches to crosslink the resulting droplets into gelled microbeads. The crosslinking mechanism is highly dependent on the biomaterial of choice and can significantly impact the biocompatibility and stability

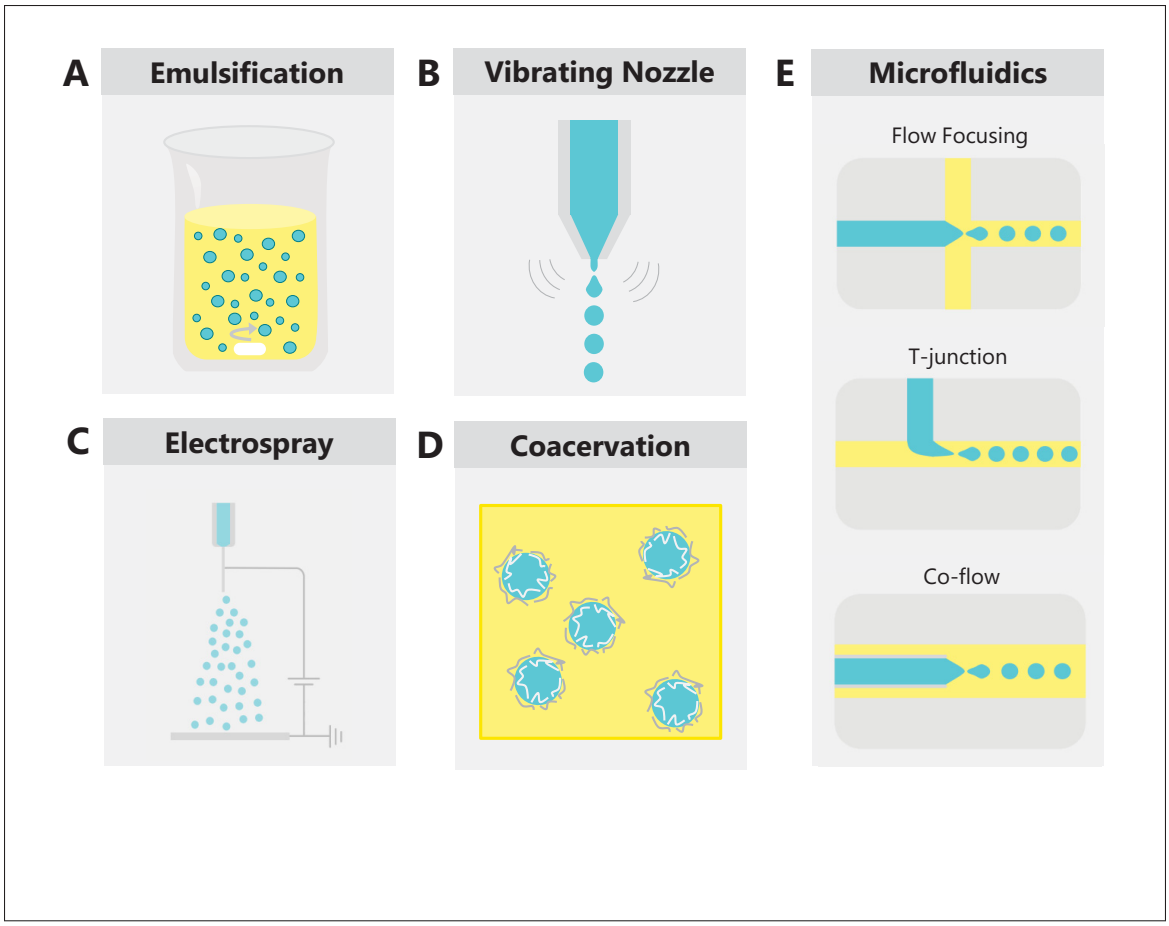

Figure 1.3 A schematic of common cell encapsulation modalities including A) emulsification; B) vibrating nozzle extrusion; C) electrospray; D) complex coacervation; E) microfluidics including flow focusing, T-junctions, and co-flow

of the microbeads. Historically, cell encapsulation has been dominated by naturally derived biomaterials as a testament to their intrinsic biocompatible properties; however, countless synthetic options are also available.

Natural biomaterials for microbial cell encapsulation are predominantly derived from animal and plant sources, with the primary distinction as proteinaceous or polysaccharidic (Brovold *et al.*, 2018). Protein-based biomaterials, such as gelatin and fibrin, offer enhanced biocompatibility due to their inherent bioactivity. However, several disadvantages restrict their application to *in situ* gut microbiota studies, such as undesirable degradation (Sikorski, Gzyra-Jagieła & Draczyński, 2021). Alternatively, polysaccharide-based biomaterials, such as alginate and agarose, offer

excellent biocompatibility and affordability for ease of widespread application (López-Marcial *et al.*, 2018). Polysaccharidic biomaterials are resistant to human enzyme digestion; however, the resident gut microbiota has the ability to metabolize and degrade these materials, again limiting their applicability to microbiota research (Flint, Bayer, Rincon, Lamed & White, 2008).

In contrast, the use of synthetic biomaterials has been met with backlash in health applications due to fears of low biocompatibility (Brovold *et al.*, 2018). Many of these issues arise from the combination of biomaterial and crosslinking agents, as they can require harsh pH, temperature, or UV exposure for crosslinking, which is not amenable to cell survival, and can also lead to free radical formation (Olabisi, 2015). However, synthetic biomaterials offer enhanced customization compared to naturally-derived materials, which can be leveraged to optimize crosslinking and biocompatibility (Gao, Peng & Mitragotri, 2021). Four-arm poly(ethylene glycol) maleimide (PEG4MAL), for example, presents as a unique solution for gut bacteria encapsulation due to its mechanical stability, rapid and oxygen-independent crosslinking, and cell-friendly reaction conditions (Phelps *et al.*, 2011; Cruz-Acuña *et al.*, 2018; Gonçalves, 2019; Gutierrez *et al.*, 2024). To date, PEG4MAL has been extensively utilized for *in vitro* mammalian cell growth and organoid fabrication, arthritis treatment, and *in vivo* cell, bacteriophage, and drug delivery (Cruz-Acuña *et al.*, 2018; Burnham *et al.*, 2025; Quizon *et al.*, 2024; Mora-Boza, Ahmedin & García, 2024; Medina *et al.*, 2022; Holyoak, Wheeler, van der Meulen & Singh, 2019; Wroe, Johnson & García, 2020). However, bacteria encapsulation in PEG4MAL remains largely understudied.

There is a current need to explore the encapsulation of GI-derived bacteria in synthetic polymers to ensure stability and avoid degradation during incubation. With tunable properties, synthetic biomaterials can act as a 3D microenvironment for cell growth, enabling nutrient and waste diffusion. Additionally, stable microencapsulated bacteria offer the ability to be integrated with an *in situ* cultivation approach to alleviate culture-based biases.

1.6 Knowledge Gap

PEG4MAL is a promising synthetic biomaterial with highly tunable properties and innate biocompatibility. Recent work has utilized PEG4MAL to support the growth of organoids *in vivo*, enable insulin release from encapsulated pancreatic islets, prevent knee cartilage degradation, and more (Cruz-Acuña *et al.*, 2018; Phelps *et al.*, 2011; Headen, Aubry, Lu & García, 2014; Holyoak *et al.*, 2019). To date, however, research on PEG4MAL's interaction with bacteria remains limited. A recent study has focused on evaluating the suitability of poly(ethylene glycol)-based materials for bacteria culture (Gutierrez *et al.*, 2024). The evidence suggests that maleimide-terminated and thiol-crosslinked poly(ethylene glycol) can support the growth of microbial cell aggregates (Gutierrez *et al.*, 2024). In spite of this promising evidence, no studies have explored the encapsulation of anaerobic gut bacteria using PEG4MAL for improved *in vitro* culture. Additionally, the porosity, low stiffness, and ease of functionalization of PEG4MAL may provide a desirable microstructure to support the enrichment of gut-derived anaerobic bacteria.

Additionally, *Akkermansia muciniphila* (*A. muciniphila*) presents as a noteworthy model species for highly sensitive strict anaerobes due to its preferred localization to a polymeric material, the mucosal layer, within the GI tract (Ioannou, Berkhout, Geerlings & Belzer, 2025). Thus far, no studies have investigated the *in vitro* enrichment of *A. muciniphila* in a non-mucin containing polymeric network. Therefore, the importance of the surrounding physical environment for *A. muciniphila* enrichment remains unknown despite its preferential inhabitation of the host mucus layer. Moreover, *A. muciniphila* is of recent interest for human health due to its promising associations with positive immunotherapy response and, therefore, novel approaches are required to improve GI engraftment of such beneficial microbes (Routy *et al.*, 2018; Walter *et al.*, 2018)

1.7 Hypothesis

It is hypothesized that the encapsulation of an anaerobic gut-derived bacteria, *A. muciniphila*, will lead to improved enrichment during *in vitro* culture due to the mechanical and physical properties of the PEG4MAL network.

1.8 Research Objectives

The aim of this work is to investigate the use of PEG4MAL for the microfluidic encapsulation of human gut microbiota bacteria. The objectives of this thesis are outlined as follows:

Objective 1: Optimize a microfluidic setup for droplet generation of bacteria-laden PEG4MAL microbeads by quantifying fluid properties and droplet behaviour.

Objective 2: Quantify the physical and mechanical properties of PEG4MAL to mimic the gut mucosal environment.

Objective 3: Quantify the effect of PEG4MAL-based encapsulation on bacterial enrichment *in vitro*.

CHAPTER 2

MATERIALS AND METHODS

2.1 Biomaterial Characterization

2.1.1 Biomaterial Preparation

The PEG4MAL precursor solution was prepared, as needed, by dissolving 2, 5, 10, and 20% w/v 20 kDa PEG4MAL (Advanced Biochemicals, US) in HEPES buffer (Thermofisher Scientific, CA) and phenol red (Millipore Sigma, CA) at a 1:1 ratio for microfluidic experiments or exclusively HEPES buffer alternatively (Headen *et al.*, 2014; Holyoak *et al.*, 2019). The PEG4MAL concentrations were selected based on previously reported studies investigating PEG4MAL for cell encapsulation (Holyoak *et al.*, 2019). A 20 mM solution of Dithiothreitol (DTT) (Millipore Sigma, CA) was prepared using phosphate buffered saline (PBS) to act as the crosslinking agent for PEG4MAL (Figure 2.1). Lastly, mineral oil (Millipore Sigma, CA) was mixed with 3% v/v SPAN 80 surfactant (Millipore Sigma, CA), and all solutions were 0.22 μm filter sterilized (Millipore Sigma, CA) (Alkayyali *et al.*, 2021).

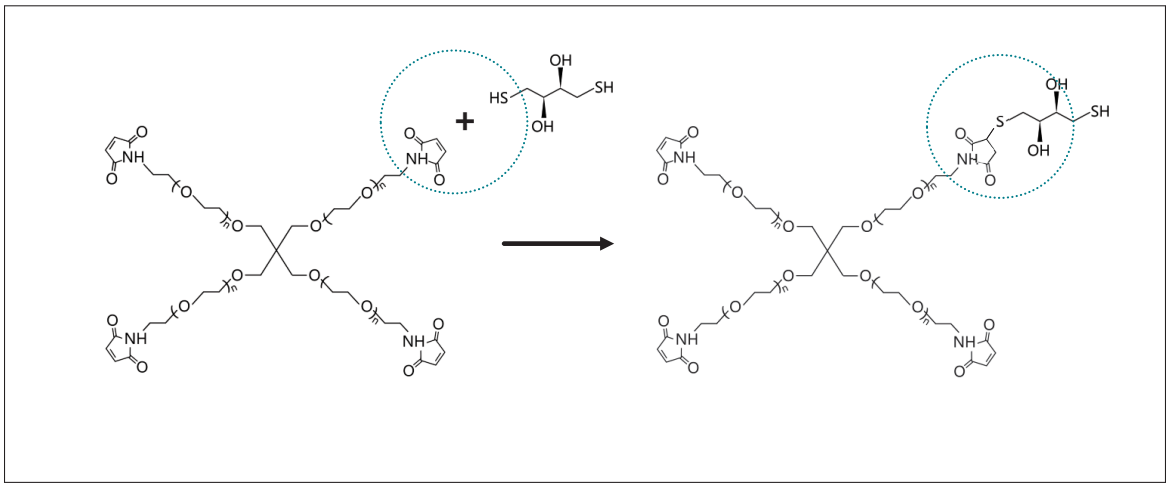

Figure 2.1 A schematic of the chemical structure of PEG4MAL with the corresponding DTT crosslinking molecule including an illustrative depiction of the crosslinking reaction

2.1.2 Mechanical Property Characterization

The mechanical properties of PEG4MAL microbeads were assessed using a MicroTester (CellScale, CA). Washed microbeads were resuspended in PBS and pipetted onto the MicroTester sample stage for isolation and compression. A 76.2 μm diameter beam and 2 mm by 2 mm plate were used to compress the 2% and 5% PEG4MAL microbeads, and a 203.2 μm diameter beam was used for 10% and 20% PEG4MAL microbeads. The beam and plate were controlled by a vertical actuator to induce microbead deformation. Visual detection of plate deformation was used to calculate the force during a three-phase compression cycle. The Young's modulus of the microbeads was calculated at 20% strain to remain within the linear region of the stress-strain relationship.

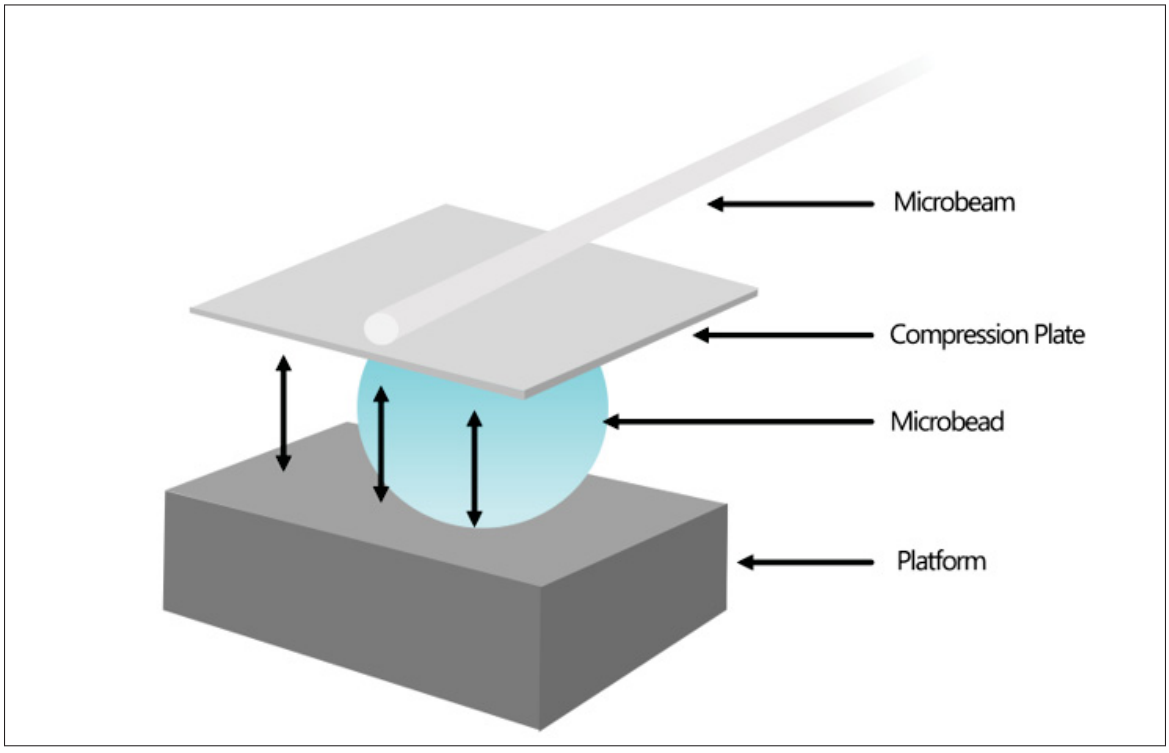

Figure 2.2 A schematic of the MicroTester setup used for mechanical property assessment

2.1.3 Microstructure and Swelling

The microstructure of the various concentrations of PEG4MAL was assessed visually using scanning electron microscopy. To prepare the samples for imaging, 20 μL droplets were formed by manually adding PEG4MAL to the crosslinker at a ratio of 4:1, based on previous studies, which underwent immediate crosslinking (Cruz-Acuña *et al.*, 2018). The droplets were freeze-dried for 8 hours (HarvestRight, US) prior to imaging and sputter-coated with a 20 nm layer of gold nanoparticles using a K550X Sputter Coater (Quorum, UK).

To assess swelling, crosslinked droplets of PEG4MAL were prepared in a petri dish, as previously described, and completely submerged in de-ionized water. After 24 hours, the water was removed and the samples were freeze-dried as previously described. The mass swelling ratio,

$$Q_m = \frac{m_{\text{swollen}}}{m_{\text{dry}}}, \tag{2.1}$$

was determined as a function of the swollen mass (m_{swollen}) to the freeze-dried mass (m_{dry}).

2.2 Microfluidic Optimization

2.2.1 Microfluidic Setup

A 140 μm channel double-emulsion microfluidic chip (Microfluidic ChipShop, DE) was used to generate water-in-oil microbeads, with flow controlled by syringe pumps (Chemyx, US) (Figure 2.3 A). Flow rates were optimized experimentally by ranging the oil-based continuous phase from 0.001 μL/min to 5 μL/min to confirm droplet formation within the dripping regime visually. The PEG4MAL precursor and crosslinker channel flow rates were constant at 0.5 μL/min and 2.85 μL/min, respectively. The range of flow rates was determined based on previously published work (Headen *et al.*, 2014). All tubing and connections were sterilized via autoclaving, and the microfluidic chip was sterilized using cyclic passing of 70% ethanol and sterile deionized water. Following microbead generation, the collected microbeads were washed to remove the oil-based

continuous phase. For washing, the microbead suspension was sequentially filtered through a 200 μm cell strainer, followed by a 70 μm cell strainer (PluriSelect, US). The microbeads retained on the 70 μm cell strainer were repeatedly washed and filtered to remove residual oil (Alkayyali *et al.*, 2021). The microfluidic process was tested under both aerobic and anaerobic conditions (Figure 2.3 B).

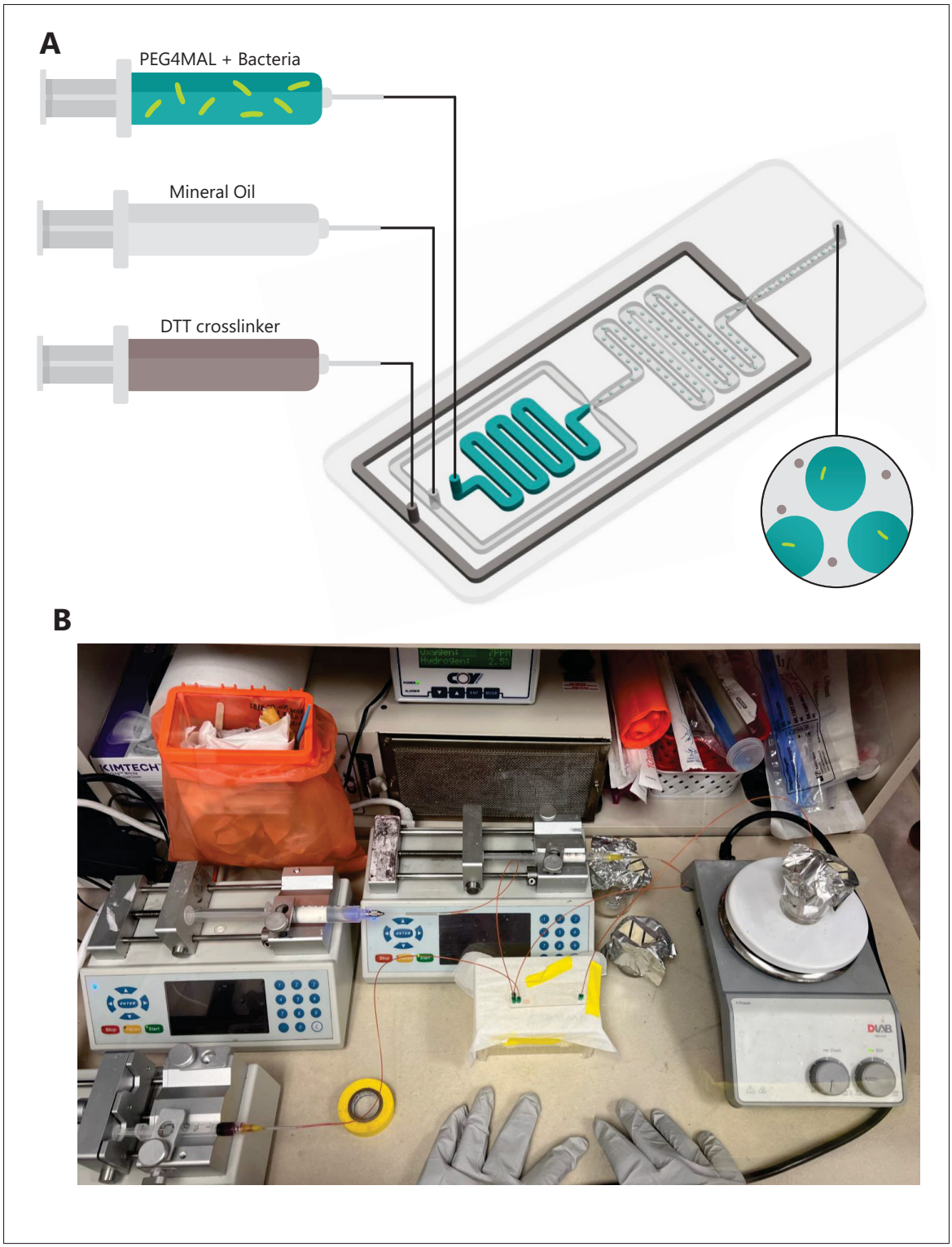

Figure 2.3 A) A schematic of the dual-junction microfluidic chip including the corresponding system inputs and outputs; B) the microfluidic setup in an anaerobic chamber

2.2.2 Fluid Properties

Interfacial tension was characterized using a DSA30R tensiometer (Kruss Scientific, DE). The pendant drop method and manual depositor were used to form a suspended droplet of the PEG4MAL precursor in mineral oil containing surfactant to assess the interfacial tension between the two immiscible fluids.

The viscosity of the PEG4MAL precursor solutions and mineral oil with surfactant was assessed using a Physica MCR 301 Rheometer (Anton Paar, DE). Experimental conditions included a parallel plate geometry with a 1 mm gap sealed with oil at 22 °C, and a frequency shear sweep was conducted from 100 to 1000 s^{-1}.

Lastly, the osmolality of the PEG4MAL precursor solutions was measured for 2, 5, 10, and 20% w/v. Fluid osmolality was assessed using a Model 3320 Osmometer (Advanced Instruments Inc; US) calibrated with a 290 mOsm/L solution.

2.2.3 Bead Size Distribution and Capillary Number

The size distribution of microbeads was evaluated for three different conditions by varying the flow rate of the continuous phase. The collected microbeads were separated from the oil phase by the addition of PBS, but were not subjected to filtering. The microbeads were imaged using an inverted brightfield Revolve microscope (Echo, US).

Following the assessment of fluid properties, the capillary number,

$$Ca = \frac{\mu U}{\sigma}, \tag{2.3}$$

was calculated assuming a shear rate-independent viscosity to determine the different flow regimes of droplet generation where U represents fluid velocity (m/s), μ represents viscosity (Pa·s), and σ represents interfacial tension (mN/m) (Samanipour, Wang, Ahmadi & Kim, 2016).

2.3 Assessment of Cell Behaviour and Tolerance

2.3.1 Biocompatibility

To verify the biocompatibility of PEG4MAL, a resuspended culture of bacteria was exposed to crosslinked droplets of PEG4MAL, created as previously described. *Escherichia coli* (*E. coli*) was used as a model species to ensure rapid cell proliferation, enabling rapid assessment of the corresponding response to PEG4MAL exposure. *E. coli* was grown overnight from frozen stock in Brain Heart Infusion (BHI) media under aerobic conditions at 37 °C. The optical density was recorded using a spectrophotometer (Thermofisher Scientific, CA) and diluted to reach a final concentration of approximately 9×10^7 cells/mL. The diluted suspension was added to a culture tube containing a 40 μL droplet of crosslinked PEG4MAL. On days 1, 2, and 3, aliquots were incubated with the alamarBlue Assay (Thermofisher Scientific, CA) for 4 hours while protected from light. Post-incubation, an aliquot was pelleted at 9500 x g for 5 minutes, and the supernatant was removed for optical density measurements of the sample (A) and the control (C). The percent reduction of alamarBlue,

$$\%\text{Reduction} = \frac{(E_{600,\text{oxi}}A_{570}) - (E_{570,\text{oxi}}A_{600})}{(E_{570,\text{red}}C_{600}) - (E_{600,\text{red}}C_{570})} \times 100, \tag{2.4}$$

was calculated based on absorbance readings at 570 nm and 600 nm using manufacturer measurements for molar extinction coefficients (E).

2.3.2 Culturability

During the aforementioned experiment, the culturability of cells post-exposure to crosslinked PEG4MAL droplets was measured. Aliquots of each condition were serially diluted from 10×10^{-4} to 10×10^{-11} and plated on BHI agar using a spot titer method. The culture plates were incubated aerobically at 37 °C overnight and imaged for colony counting.

2.3.3 Effect of Biomaterial Concentration

The optimal concentration of PEG4MAL was determined by observing cellular behaviour following encapsulation. To achieve this, *E. coli* was grown overnight under aerobic conditions in BHI media at 37 °C. The diluted culture was resuspended in 1 mL of each concentration of the PEG4MAL precursor solutions. Microbeads were formed by a double emulsion generated via stirred emulsification and washed, as previously described. The encapsulated *E. coli* was resuspended in BHI media and grown under aerobic conditions at 37 °C. After a 24-hour incubation, the microbeads were stained using SYTO 9 and imaged with an Axiovert 3 epifluorescence microscope (Zeiss, DE). Z-stacking was performed for a 70 μm region with 0.2 μm slice thickness to visualize 3D colony structure within the microbead using an Axiovert 3 epifluorescence microscope (Zeiss, DE).

2.3.4 Viability and Colony Formation

Metrics for cell viability were obtained by staining the encapsulated cells with a Live Dead Assay of Propidium Iodide (PI) and SYTO 9 fluorescent stains (Thermofisher Scientific, CA), per manufacturer's instructions. Samples were imaged using an Axiovert 3 microscope (Zeiss, Germany), and images were assessed by fluorescent area coverage.

Subsequent experiments involved the encapsulation of bacteria in 5% PEG4MAL via microfluidics, as previously described, using a 2 μL/min, 0.5 μL/min, and 2.85 μL/min flow rate for the oil, PEG4MAL, and crosslinker phase, respectively. The following experiments involved the use of *A. muciniphila* which is a strictly anaerobic gut-derived bacteria. Both *E. coli* and *A. muciniphila* were independently encapsulated under aerobic and anaerobic conditions, respectively. The encapsulated cells were stained and imaged as previously described. A 2D analysis of the colony formation was assessed by comparing the size of cell aggregation within the microbead to that of resuspended cell culture.

2.4 Modification of PEG4MAL with RGD

The addition of plasticizers was explored as a means to improve the cellular response of 5% PEG4MAL by approaching the fluid and mechanical properties of 2% PEG4MAL. Arginylglycylaspartic acid (RGD) was selected to act as a plasticizing agent as it is a small organic molecule that does not chemically interact with the crosslinking agent or the PEG4MAL backbone. RGD is a peptide sequence composed primarily of arginine, glycine, and aspartic acid. In mammalian cell culture, RGD binds to the cell membrane; however, the same binding motifs are not present on microbial membranes. The inclusion of 9 μM RGD in the PEG4MAL precursor solution was hypothesized to reduce the crosslinking efficiency via steric hindrance. The fluid and mechanical properties and cellular response, as previously described, were characterized for the RGD-containing PEG4MAL biomaterial.

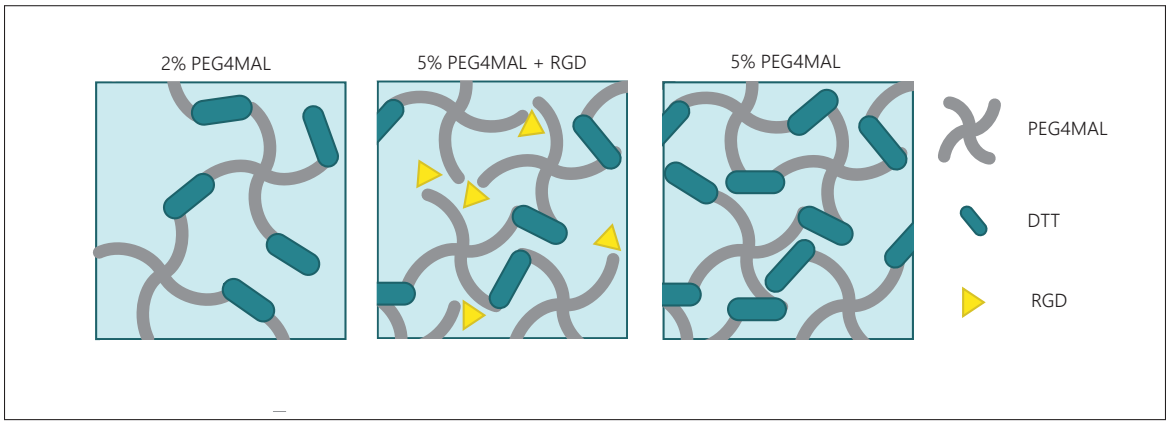

Figure 2.4 An illustration of 2%, 5%, and 5% PEG4MAL precursor solutions with RGD peptide

2.5 Statistical Analysis

Results are presented as mean ± standard deviation, as median and inter-quartile range, or as a percentage of the population. Statistical analysis was performed in RStudio, and a p-value of <0.05 was considered significant. The Shapiro-Wilk test was used to test for normality and skewness of the data. Levene's test was used to test for equal variance. The statistical tests used for analysis are listed in Table 2.1.

Table 2.1 The statistical tests and post hoc analyses performed

Experimental Data	**Statistical Test**	**Post Hoc Test**
Viscosity	One-Way ANOVA	Tukey's HSD
Interfacial Tension	Kruskall Wallis	Dunn's with Bonferroni Correction
Metabolic Activity	Two-Way ANOVA	Tukey's HSD
Culturability	Two-Way ANOVA with Welch's Correction	Games-Howell
Cell Aggregation	Kruskall Wallis	Dunn's
VIability	Two-Way ANOVA with Welch's Correction	Games-Howell
Swelling	Kruskall Wallis	Dunn's with Bonferroni Correction
Osmolality	One-Way ANOVA	Tukey's HSD
Mechanical Properties	One-Way ANOVA and One-Way ANOVA with Welch's Correction	Tukey's HSD and Games-Howell

CHAPTER 3

RESULTS AND DISCUSSION

3.1 Microfluidic Optimization

The dual-junction microfluidic chip enabled the controlled formation of microdroplets (Figure 3.1 A). The coefficient of diffusion for DTT in PBS was reported as $2.83 \times 10^{-6} cm^2/s^{-1}$, thus enabling rapid downstream crosslinking prior to collection (Santarino, Oliveira & Oliveira-Brett, 2012) (Figure 3.1 B). The setup enabled portability and was successfully integrated into an anaerobic chamber, ensuring encapsulation under conditions conducive to gut microbiota survival (Figure 3.1 C). Herein, this section discusses the assessment of fluid properties and droplet generation behaviour within the microfluidic chip.

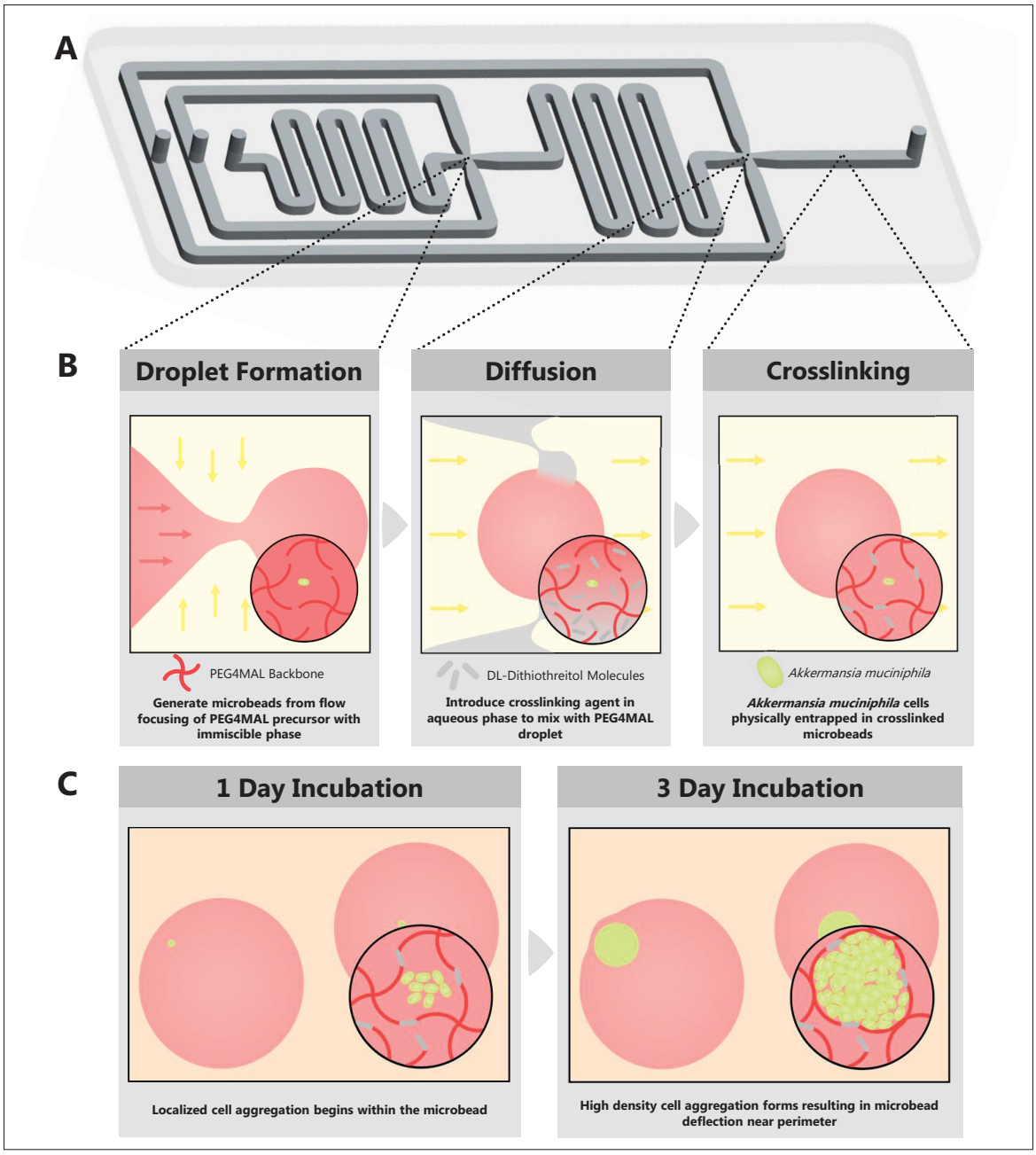

Figure 3.1 A) A schematic of the dual-junction microfluidic chip; B) a schematic of droplet formation and crosslinking, C) a schematic of cell growth within microbeads

3.1.1 Fluid Properties

To better characterize PEG4MAL for use in microfluidic droplet generation, the fluid properties were measured to assess droplet behaviour. The viscosity of PEG4MAL at concentrations of 2, 5, 10, and 20% w/v were constant within the observed shear range and measured as 4.4×10^{-3}, 1.2×10^{-2}, 1.7×10^{-2}, and 3.6×10^{-2} Pa·s, respectively and performed in triplicate at 22 °C (Figure 3.2 A). Using the same rheological conditions, the viscosity of the mineral oil containing surfactant was also constant and determined to be 5.8×10^{-2} Pa·s (Figure 3.2 A). All fluids displayed Newtonian characteristics as there were no shear-rate dependencies observed over the same shear range previously reported in literature (Utama *et al.*, 2021). The minimal apparent variations in the 2% PEG4MAL viscosity were assumed to be a result of evaporation and plate slipping. At higher concentrations of PEG4MAL, the increased number of molecules contributes to a resistance to fluid movement, thus corresponding to the rise in observed viscosity.

Another important fluid property influencing droplet formation is the interfacial tension between the continuous and dispersed phases. The interfacial tension of PEG4MAL precursor droplets suspended in oil with surfactant was measured as 2.95, 3.75, 3.93, and 4.21 mN/m for 2, 5, 10, and 20% w/v PEG4MAL, respectively, in triplicate over 60 seconds (Figure 3.2 B). The role of the surfactant, an amphiphilic molecule, is to reduce the interfacial tension between the oleaginous and aqueous phases. Acting at the interface between the two immiscible phases, the surfactant also helps to stabilize the water-in-oil droplets. The increase in interfacial tension between PEG4MAL concentrations can be attributed to the corresponding increase in density of the precursor solutions.

The osmolality of PEG4MAL was assessed in triplicate and determined to follow the same increasing trend with concentration (Figure 3.2 C). The rise in osmolality can be attributed to the increased number of free-floating PEG4MAL molecules in solution. Osmolarity plays a crucial role in bacteria's survival, morphology, and function (Wood, 2015). Some species exhibit osmotolerance or osmoadaptation in which they express internal mechanisms to regulate the response to changes in osmolarity (Gil *et al.*, 2023; Sleator & Hill, 2002). Due to this inherent

adaptability, bacteria are capable of surviving in a wide range of environmental osmolarities (Gil *et al.*, 2023; Wood, 2015). Within the human body, for example, the microbiota have adapted to exist within a physiological range similar to that of mammalian cell culture, which is typically between 260 and 320 mOsm/kg (Watson & Austin, 2021; Ozturk & Palsson, 1991). As a result, bacteria sampled from the gastrointestinal tract would experience a significant osmotic shock when suspended in 10% and 20% PEG4MAL precursor solutions.

In addition to the cells, it has been hypothesized that biofilms also react to extreme osmotic conditions by expanding under hyperosmotic stress and shrinking during hypoosmotic conditions (Dufrêne & Persat, 2020). As a result, the behaviour of biofilms can promote microbial survival by controlling the diffusion of solutes. Studies also suggest that osmotic pressure differences between the biofilm and the surrounding environment can act as a driver for colony growth (Yan, Nadell, Stone, Wingreen & Bassler, 2017). Therefore, osmotic pressure differences in the crosslinked PEG4MAL microbead and the surrounding medium may assist in the proliferation of cells within the microbead.

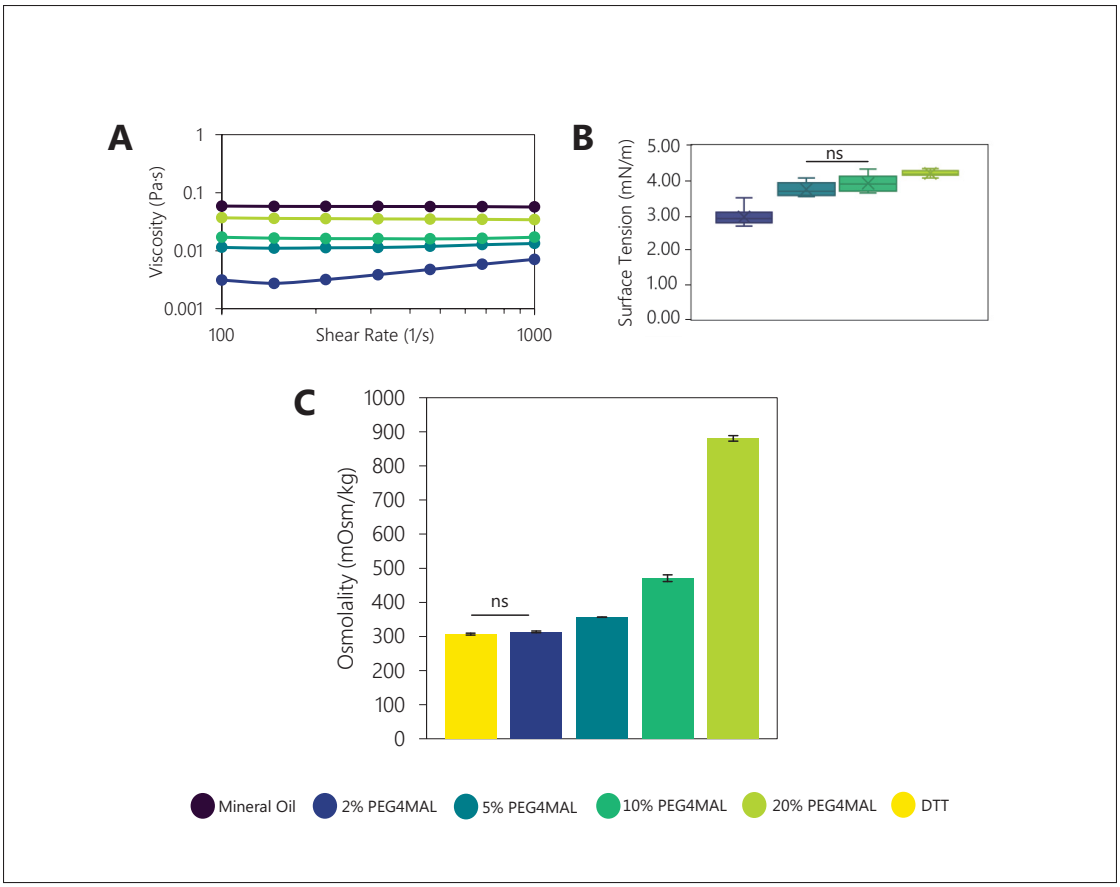

Figure 3.2 Fluid properties of PEG4MAL including A) the viscosity of various PEG4MAL concentrations and mineral oil with surfactant, $n = 3$ and $p < 0.0001$ between all conditions; B) the interfacial tension between various PEG4MAL concentrations and mineral oil with surfactant, $n = 39$ and $p < 0.0001$; C) the osmolality of various PEG4MAL solutions prior to crosslinking, $n = 3$, $p < 0.0001$

3.1.2 Capillary Number and Bead Size Distribution

Collectively, fluid viscosity and interfacial tension data were used to calculate the capillary number over a range of fluid velocities. The capillary number is a key dimensionless parameter that determines the dominant force driving droplet generation, with lower values indicating a greater influence from surface tension. Combined with visual observation, fluid regimes—squeezing, dripping, and jetting—were classified (Figure 3.3 A). Experimentally, the flow rate of the continuous phase was varied to visually confirm the transitions between these regimes, which can be associated with capillary number thresholds, while maintaining constant flow rates for both discrete phases (Figure 3.3 B). Critical capillary numbers for transition states are dependent on channel geometry, the ratio of fluid viscosities, and are influenced by user interpretation; however, the observed transitions were within a similar range to reported values of squeezing to dripping and dripping to jetting occurring at continuous phase capillary numbers approaching 10^{-2} and 10^{-1}, respectively (Samanipour *et al.*, 2016; Phelps *et al.*, 2011). By manipulating the continuous phase flow rate, microbead size could be modulated within a narrow range, and this precise control is particularly advantageous for achieving single-cell encapsulation (Figure 3.4 A, B). Maintaining operation within the dripping regime was prioritized to achieve controlled and uniform microbead production, leading to the selection of an optimal continuous phase flow rate of 2 μL/min. However, optimization of the crosslinker flow rate is required to further reduce microbead size discrepancies.

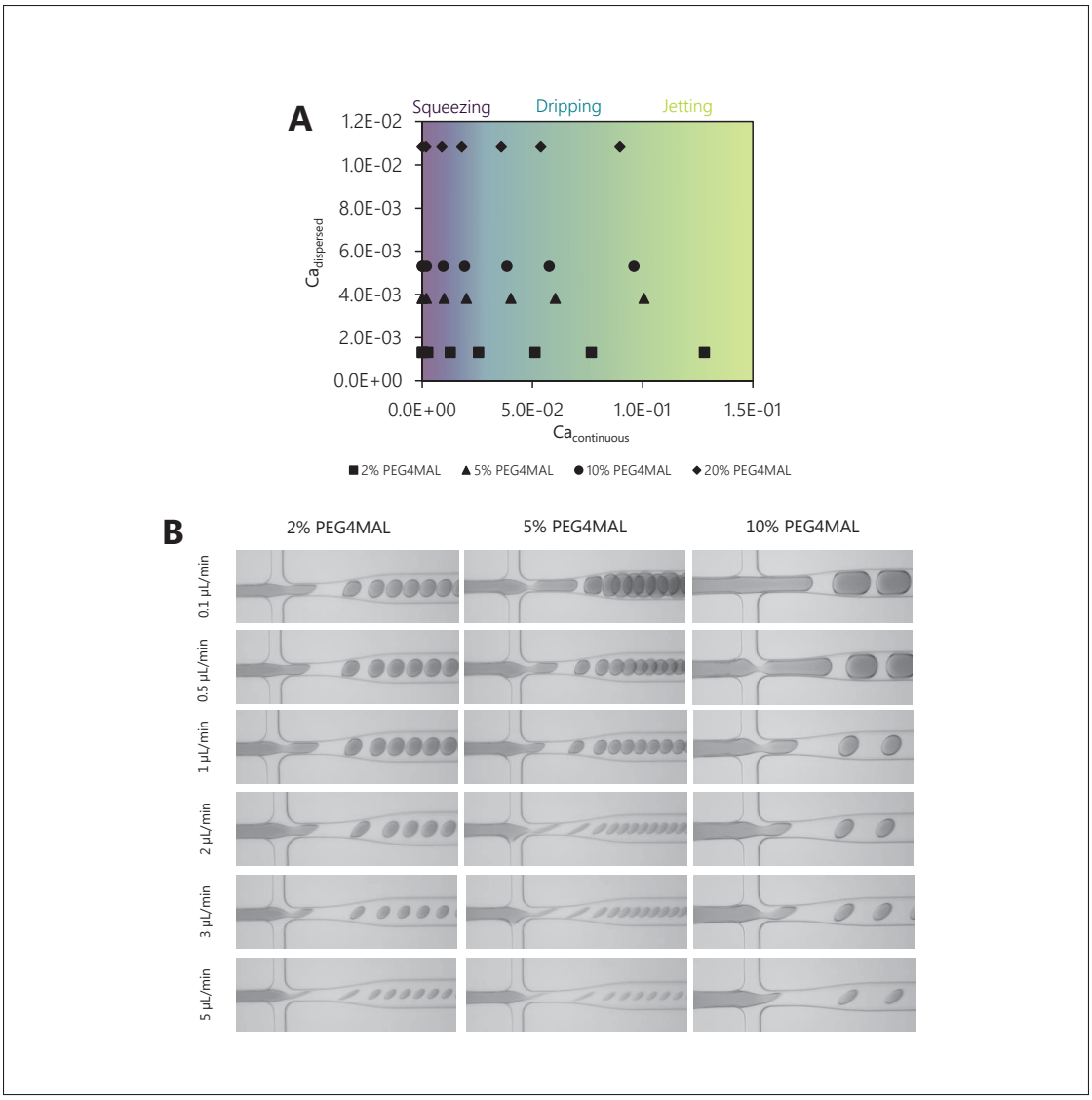

Figure 3.3 Characterization of the microfluidic setup including A) the capillary number range for the continous and dispered phases at various PEG4MAL concentrations

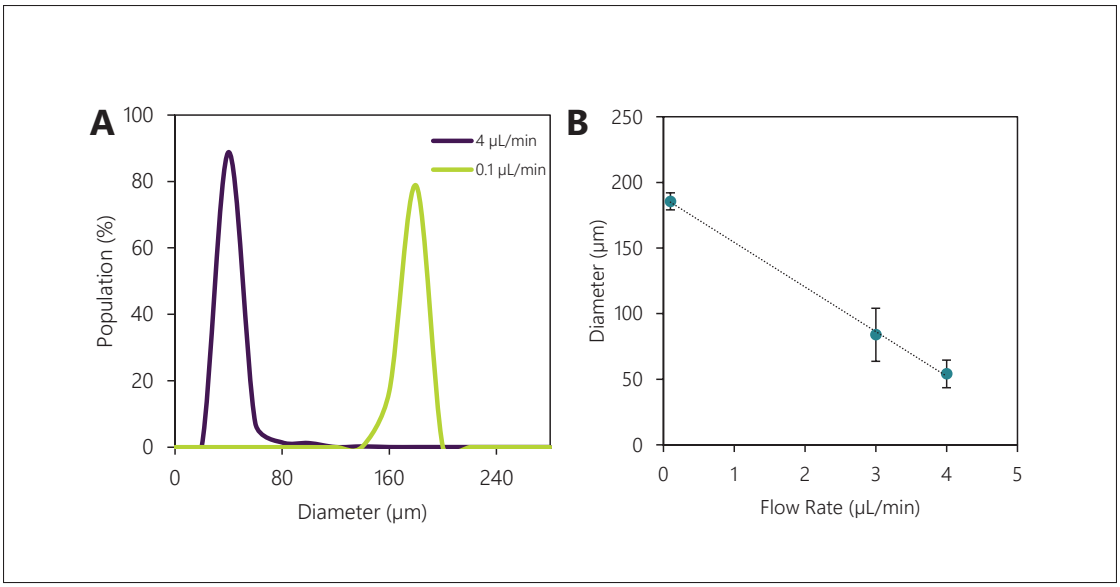

Figure 3.4 The characterization of microbead size including A) the 5% PEG4MAL microbead size distribution with varying oil phase flow rates ranging from 0.1 μL/min to 4 μL/min; B) the trend of microbead size by varying continous phase flow rate

3.2 Biomaterial Characterization

3.2.1 Mechanical Properties

It was observed that by optimizing PEG4MAL concentration, the mechanical properties can be precisely tuned. The Young's modulus was measured to range from 1.5-12 kPa for 2% to 20% w/v PEG4MAL as determined via individual microbead compression for a minimum of 6 microbeads per sample (Figure 3.5 A). The increasing elastic modulus can be attributed to the increase in crosslinks from the additional macromer chains present, thus reducing network mobility. Previous work has reported a lower elastic modulus of 1.5 kPa for 10% PEG4MAL as a result of using a larger crosslinking molecule, which gives rise to a looser network (Phelps *et al.*, 2011). There was a larger recorded range for 20% PEG4MAL microbeads, which may be a result of a heterogeneous microstructure due to rapid, highly crosslinked pockets. Additionally, a larger microbeam was required for higher concentrations of PEG4MAL, which could account for the increase in measurement variability. At lower concentrations of PEG4MAL, the magnitude of the Young's modulus was more comparable to that of the ileum mucosal layer, a primary region for gut microbiota growth (Sotres, Jankovskaja, Wannerberger & Arnebrant, 2017).

Recently, there has been an interest in exploring the mechanostimulatory effects on bacteria growth, and the modulation of hydrogel stiffness for cell encapsulation can offer important insights (Dufrêne & Persat, 2020). It has been shown that external mechanical forces can dictate cell orientation during division and correspondingly influence cell interaction (Wittmann, Nguyen, Löwen, Schwarzendahl & Sengupta, 2023). Biofilms, considered a natural state of bacterial growth, can provide a source of external mechanostimulation, although this is not well understood (Dufrêne & Persat, 2020). The incorporation of mucins into the hydrogel network may enhance cell adhesion and enable microbial-driven control of hydrogel stiffness, similar to biofilm behaviour (Dufrêne & Persat, 2020).

3.2.2 Microstructure and Swelling

Variations in PEG4MAL microstructure were apparent across the different concentrations observed, with a denser network observed at higher concentrations (Figure 3.5 B). These differences in porosity may be a driving influence of encapsulated cell behaviour, which will be further discussed in sections 3.3.2 and 3.3.4.

Swelling is an important parameter that can also dictate changes in microstructure. Some slight differences in swelling between the PEG4MAL concentrations were observed after 24 hours of immersion in water with 5 samples measured per condition (Figure 3.5 C). The significant decrease in swelling ratio observed at higher concentrations of PEG4MAL is assumed to be a result of the denser network structure. Additionally, evidence of a greater magnitude of the swelling ratio has been shown for PEG4MAL crosslinked with larger molecules (Phelps *et al.*, 2011). Lastly, the ratio of PEG4MAL to crosslinking agent has also been shown to directly impact network structure and resulting swelling capabilities of the hydrogel (Holyoak *et al.*, 2019). Swelling of the biomaterial is important to help adjust for the internal volume increase of the microbial cells over time.

Due to the rapid thiol-based reaction between PEG4MAL and DTT, heterogeneous crosslinking can occur at high PEG4MAL volumes; therefore, the maximum droplet volume investigated was 20 μL for both microstructure analysis and swelling and otherwise tests were conducted with microbeads (Gonçalves, 2019) (Figure 3.5 D).

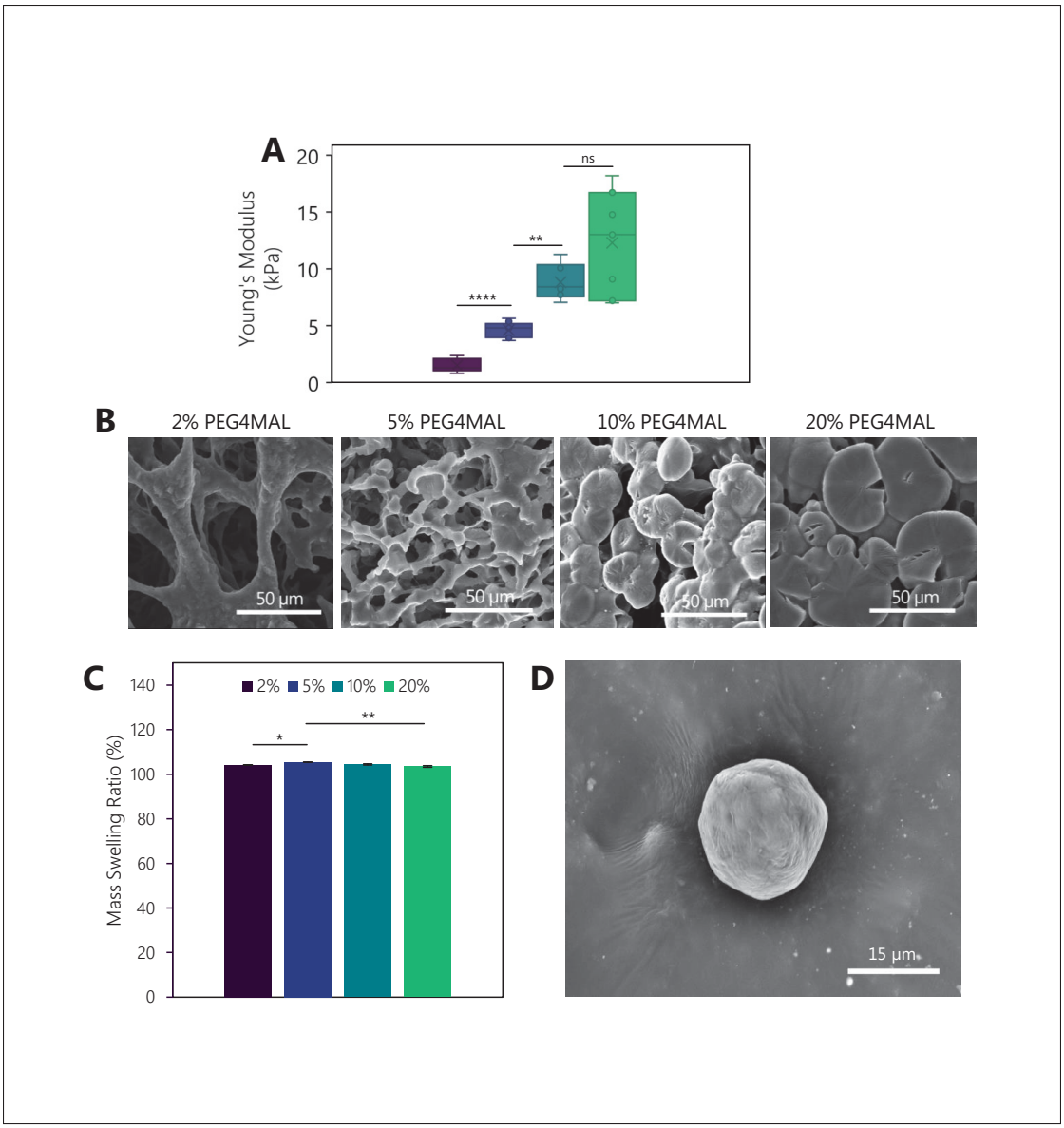

Figure 3.5 The mechanical proprieties and struction of PEG4MAL including A) the Young's modulus calculated at 20% strain, $n \geq 6$ and $p < 0.00001$; B) the microstructure of various PEG4MAL concentrations; C) the mass swelling ratio of 20 μL PEG4MAL droplets, $n = 5$ and $p < 0.05$ and $p < 0.001$; D) an SEM image of a 5% PEG4MAL microbead;

3.3 Assessment of Cell Behaviour and Tolerance

3.3.1 Biocompatiblity

PEG4MAL has previously demonstrated a high degree of biocompatibility for mammalian cell culture; however, evidence supporting its biocompatibility for bacterial culture remains limited (Gutierrez *et al.*, 2024). *E. coli* exposed to crosslinked PEG4MAL in triplicate demonstrated a high degree of metabolic activity even after three days in culture, with no significant differences compared to standard culture (Figure 3.6 A). The high metabolic activity confirms that the presence of PEG4MAL does not negatively affect cell function; however, it is suggested that future work investigates biocompatibility in the context of anaerobic bacteria with assays that are not oxygen-dependent (Rampersad, 2012). Additionally, the culture was plated 4 times per condition post-exposure to crosslinked PEG4MAL, and no significant differences in culturability were observed between samples. However, there was a significant decrease in culturability observed over time for all conditions, including the resuspended control (Figure 3.6 B). As such, it can be concluded that microbial exposure to PEG4MAL does not ellicit a shift to a sporulative or VBNC state non-conducive to cell culture.

3.3.2 Effect of Biomaterial Concentration

Although the various PEG4MAL concentrations showed no differences regarding biocompatibility, differences were observed in microstructure and mechanical properties. The biological impact of cells encapsulated within these crosslinked networks was investigated. Fluorescently stained *E. coli* enabled visualization of cell distribution across different PEG4MAL concentrations. Notably, at 2% PEG4MAL, cells were evenly distributed through the microbead in a two-dimensional (2D) projection (Figure 3.6 C). In contrast, at 20% PEG4MAL, a greater degree of cell escape and preferential adhesion to the microbead surface was observed (Figure 3.6 C). This behaviour aligns with established knowledge of bacteria biofilm formation, wherein bacteria preferentially adhere to stiffer surfaces. Additionally, it has been shown that bacteria can undergo up to 10% strain when experiencing a 10 kPa stress (Chu, Kilic, Cho, Groisman & Levchenko, 2018). Based on

these observations, lower PEG4MAL concentrations were favoured to enhance cellular retention and fitness within the microbead. However, using 2% PEG4MAL presented practical challenges. Particularly, a significant degree of 2% PEG4MAL bead loss occurred during cleaning as microbeads were retained on the filters used to remove residual oil, decreasing overall throughput. Additionally, microbial enrichment was primarily quantified using image-based approaches and it was more challenging to observe 2% PEG4MAL microbeads under the microscope. Therefore, subsequent experiments used 5% PEG4MAL as a balance between mechanical integrity and bacterial retention; however, future investigation into additional PEG4MAL concentrations is recommended.

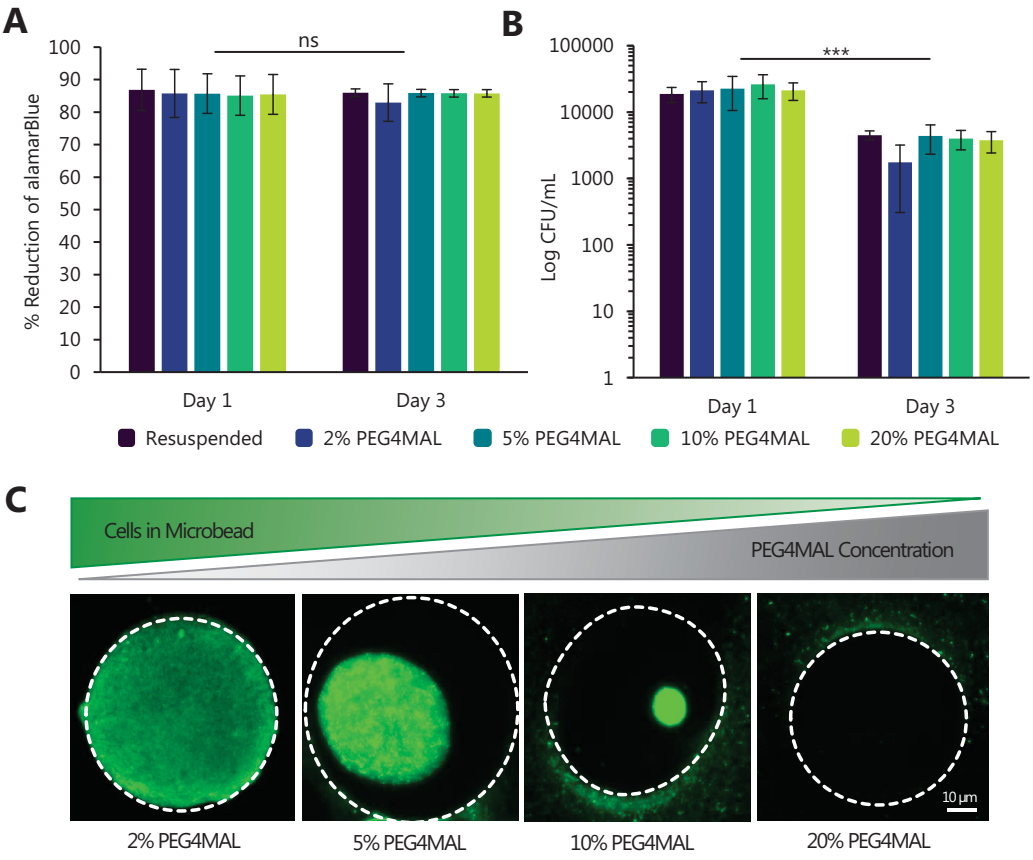

Figure 3.6 The biocompatiblity of PEG4MAL including A) the metabolic activity measured by alamarBlue reagent reduction, $n = 3$ and $p = 0.973$; B) the culturability measured by CFU/mL, $n = 4$ and $p < 0.0001$; C) the behaviour of *E. coli* encapsulated in various PEG4MAL concentrations after a one day incubation

3.3.3 Viability

In addition to robust cell confluency within the microbead, encapsulated bacteria maintained high viability with around 90% viability observed after 3 days in culture for 5 measured microbeads (Figure 3.7). No observed trends of a necrotic core within the microbead indicated sufficient diffusion of nutrients and wastes.

However, the use of PI as an indicator of cell death is not a reliable method for several reasons. Firstly, it has been shown that PI can bind to extracellular nucleic acids, thus emitting a false positive for cell death. To account for this non-selective staining, the image exposure time and thresholding was optimized to a negative control. This was achieved by complete cell killing through 70% ethanol exposure and subsequent PI staining. Optimal exposure times and post-processing thresholding levels were defined by the negative control and applied to subsequent images for analysis.

Additionally, the PI stain is non-permeable to the cell membrane; therefore, the underlying assumption is that cells with a damaged membrane are dead, and thus fluoresce in the presence of PI. However, cell membranes are compromised during cellular division, and as a result, can lead to additional false positives. Other fluorescent probes can offer reduced bias but require significant time for validation and optimization (Tian, Ma & Lin, 2019). The current 2D area-based approach to analysis underestimates overall cell quantity and viability within the 3D microbead; however, it is used as an approximation for overall cell behaviour.

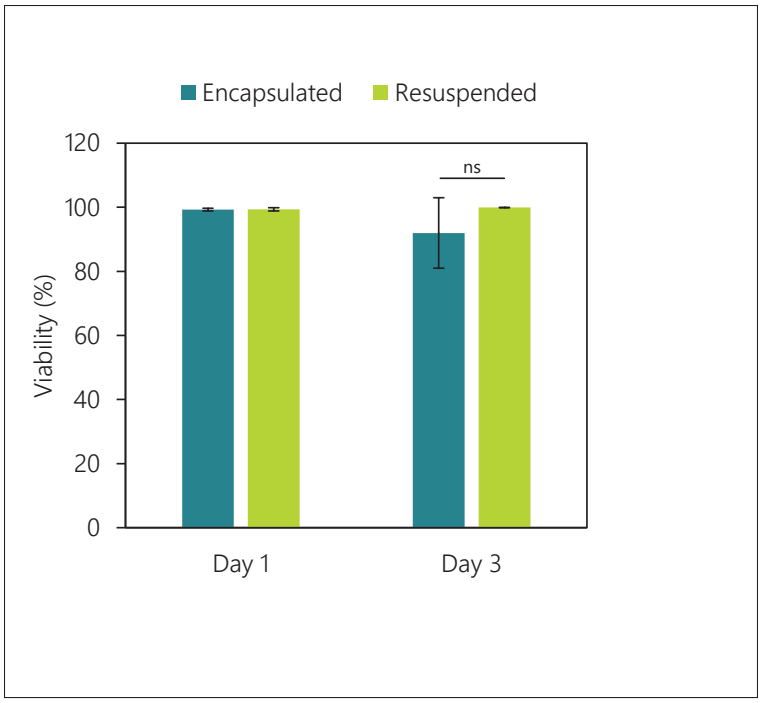

Figure 3.7 The viability of *A. muciniphila* encapsulated in 5% PEG4MAL microbeads, $n = 5$ and $p = 0.55$

3.3.4 Colony Formation

The role of single-cell encapsulation is important for future GI-derived community cultures to spatially isolate individual cells, which may lead to improved growth and ease of sorting (Figure 3.8 A, B). Through microfluidic encapsulation and subsequent incubation, it was observed that single-cell-laden microbeads led to the formation of an individual colony/cell aggregation under both aerobic and anaerobic conditions (Figure 3.8 C, D, E, F). To quantify the abundance of cells within the microbead, a 2D visual analysis enabled a comparison between cellular aggregation within the microbead to that of a resuspended culture. A significant increase in bacterial aggregation was observed for encapsulated *E. coli* compared to resuspended culture for at least 71 measured microbeads (Figure 3.8 C, D). Additionally, the same significant increase was observed for encapsulated *A. muciniphila* incubated under anaerobic conditions for at least 24 microbeads (Figure 3.8 E, F). Although the doubling time for *A. muciniphila* is significantly less than *E. coli*, the rapid proliferation of encapsulated *A. muciniphila* suggests preferential growth within a substrate as opposed to in suspension. In the GI tract, *A. muciniphila* primarily resides within the mucosal layer and is well-characterized for its mucin-degrading properties. It is hypothesized that matrix encapsulation contributed to an altered phenotype for rapid cell division, which may be a promising approach to culture mucosal layer-derived gut bacteria *in vitro*.

In some instances, the rapid colony expansion near the surface of the microbead leads to outward protrusions and eventually to bacterial growth extending outside the microbead (Figure 3.9 A, B). The change in microbead morphology indicates the material's ability to adapt to the evolving colony size, where a more uniform enlargement was observed for centrally dispersed colonies. Additionally, this high-density growth of difficult-to-culture cells within the microbead facilitates the collection of higher biomass samples, thereby enabling rapid sorting and reduced analytical bias.

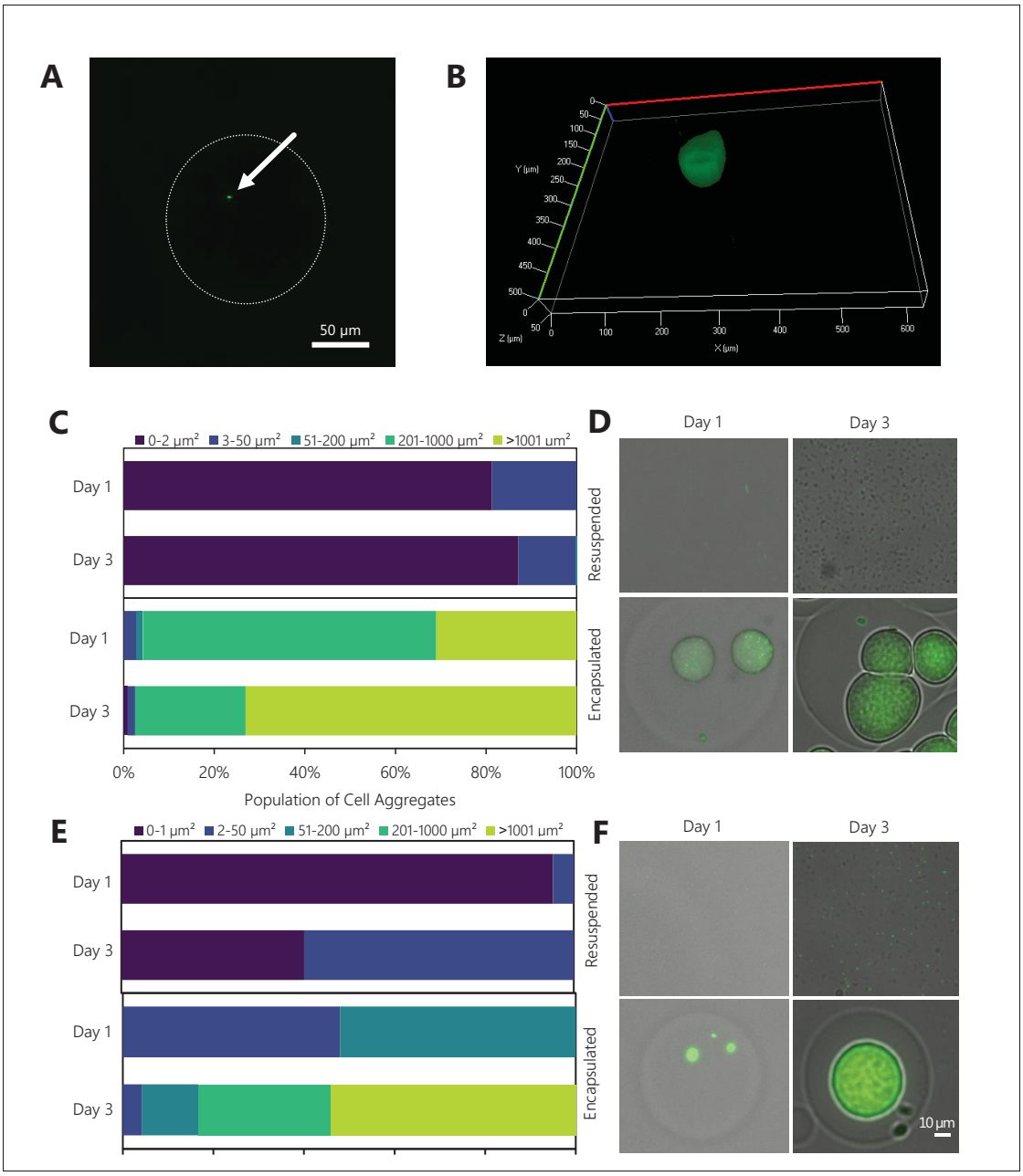

Figure 3.8 The colony formation in PEG4MAL microbeads characterized by A) a 5% PEG4MAL microbead containing a single cell of *E. coli* at day 0 prior to incubation; B) Z-stacking of *A. muciniphila* encapsulated in 5% PEG4MAL after a 3 day incubation at 0.2 μm slice thickness; C) the area of *E. coli* aggregation in microbeads vs. resuspended cells, $n \geq 71$ and $p < 0.0001$; D) encapsulated and resuspended *E. coli* after 1 and 3 days incubation; E) the area of *A. muciniphila* aggregation in microbeads vs. resuspended cells, $n \geq 24$ and $p < 0.0001$; F) encapsulated and resuspended *A. muciniphila* after 1 and 3 days of incubation

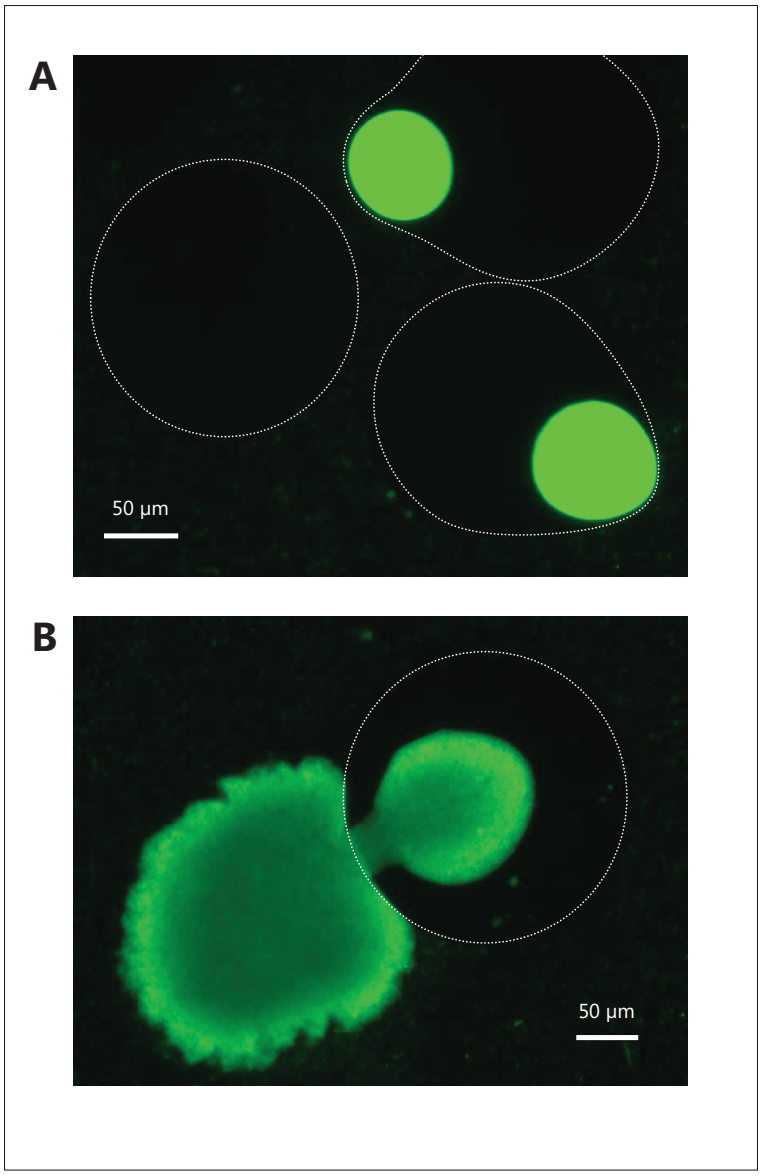

Figure 3.9 The morphological microbead response to encapsulated cell proliferation by A) the morphological changes of the microbead in response to colony growth vs. an empty microbead; B) the cell expansion outside of the microbead

3.4 Modification of PEG4MAL with RGD

RGD is a bioactive peptide motif found within the mammalian extracellular matrix and is widely studied in PEG4MAL-based encapsulation systems for its ability to mediate mammalian cell adhesion. While its use in microbial encapsulation, RGD was selected here as a proof of concept to explore the impact of a peptide-based plasticizer. Additionally, the constituent amino acids of the RGD sequence may be utilized by encapsulated bacteria to support metabolic or cellular processes.

Low concentrations of PEG4MAL, namely 2%, offered enhanced cellular response to encapsulation; however, practical limitations impeded its application. The inclusion of the bioactive RGD peptide into the PEG4MAL precursor was assessed to understand its impact on fluid and mechanical properties in 5% PEG4MAL. No differences were observed in the viscosity of RGD containing 5% PEG4MAL compared to 5% PEG4MAL, which is assumed to be a result of the flexibililty of the RGD peptide (Bogdanowich-Knipp, Jois & Siahaan, 1999). Regarding the interfacial tension, there were significant differences observed as RGD acted as a surfactant to reduce the interfacial tension to a magnitude similar to 2% PEG4MAL. The lower interfacial tension of the 5% PEG4MAL with RGD results in a higher degree of microfluidic droplet generation.

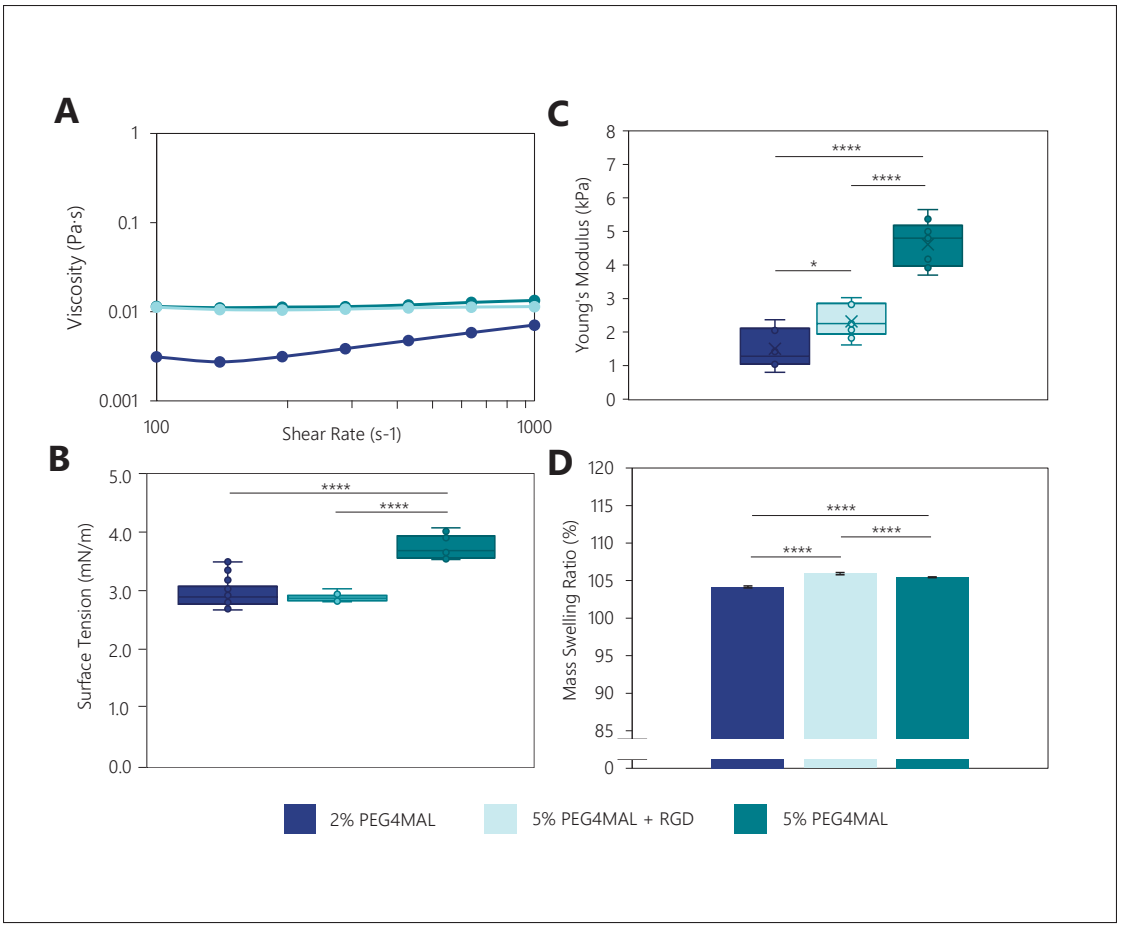

Figure 3.10 The fluid and mechanical properties of PEG4MAL with RGD including; A) the viscosity; B) the interfacial tension; C) the Young's modulus; D) the mass swelling ratio

For the mechanical properties, a notable decrease in the material stiffness was observed with the inclusion of RGD into the crosslinked 5% PEG4MAL network. It is presumed that the RGD peptide reduces the crosslinking efficiency, thus producing a greater degree of porosity and a less dense microstructure. Lastly, due to the greater intra-microstructure space available for cell growth, a significant increase in cell aggregation was observed compared to 5% PEG4MAL. It is hypothesized that the external environment surrounding the microbial cells during culture influences their fitness and expression, which results in differences in the observed cell behaviour.

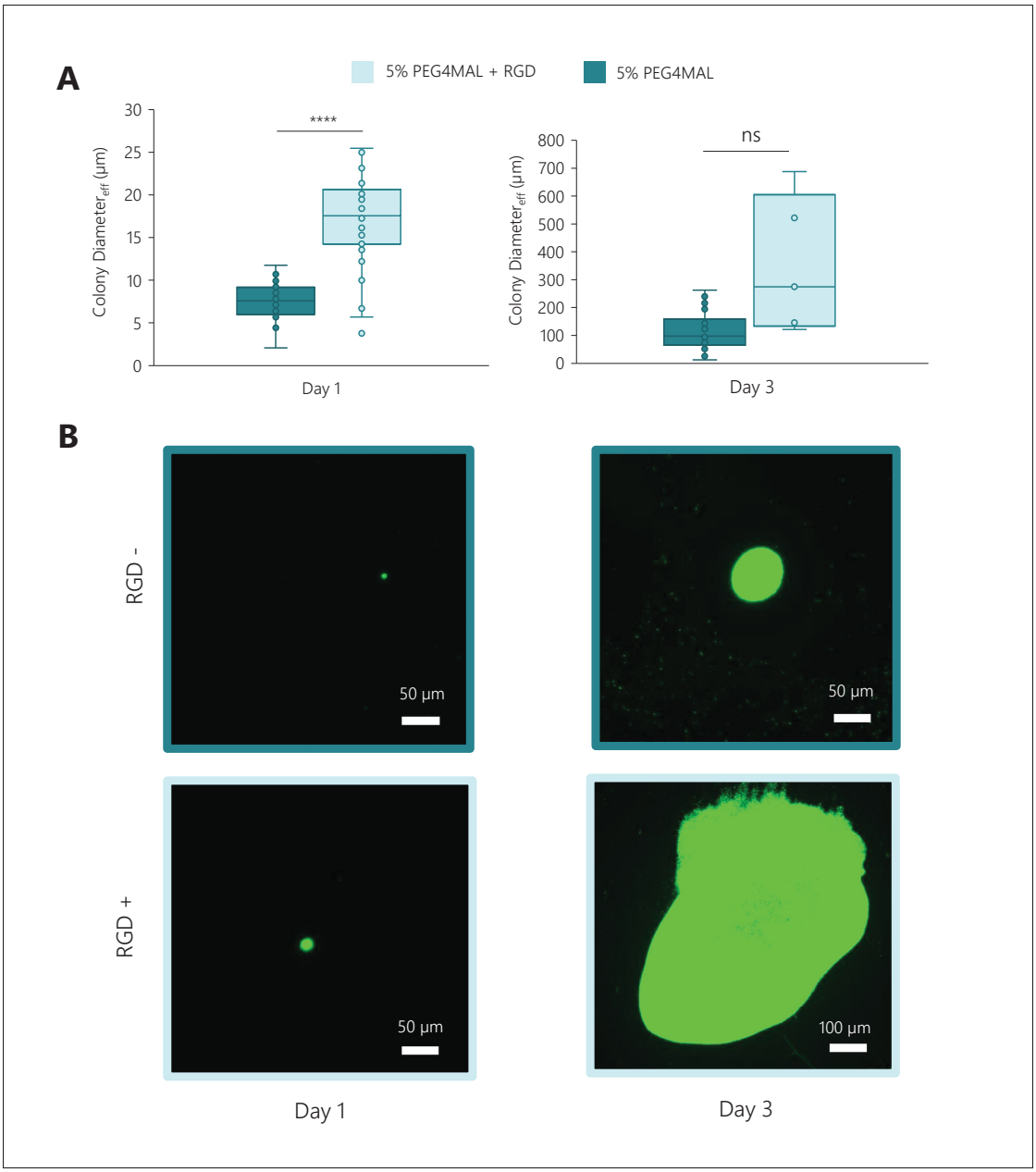

Figure 3.11 The cellular response to encapsulation in PEG4MAL with RGD by A) assessment of the effective colony diameter of encapsulated cells; B) representative images of colony containing microbeads after day 1 and day 3 of incubation

CONCLUSION AND RECOMMENDATIONS

This work offers a commercially available approach to uncover bacteria present in the GI tract that have evaded detection or culture through conventional microbiology techniques thus far. It was demonstrated that droplet generation within a microfluidic chip could be easily manipulated due to the low viscosity of the selected PEG4MAL biomaterial and the low interfacial tension between the dispersed and continuous phases. The osmolality of low PEG4MAL precursor concentrations prevented osmotic shock for the suspended microbial cells prior to forming uniformly dispersed crosslinked microbeads. Regarding mechanical properties, it was observed that at low PEG4MAL concentrations, the stiffness approached that of the gut mucosal layer, a key environment of microbial colonization. Additionally, the higher degree of swelling observed at low concentrations of PEG4MAL was hypothesized to enable a better temporal biomaterial response to intra-bead colony expansion. The crosslinked PEG4MAL biomaterial exhibited a high degree of biocompatiblity in terms of both metabolic activity and culturability of the microbial cells post-exposure. It was also demonstrated that the concentration of PEG4MAL influenced encapsulated cell behaviour, with low concentrations of PEG4MAL more desirable for bacteria confluency within the microbead. Over time, significant colony formation within the microbead was observed compared to standard resuspended culture, which translates to sufficient sequencing biomass using only 1-2 microbeads. Further enhancement of microbial proliferation within the microbead could be achieved by modifying the PEG4MAL structure using RGD. Overall, it was demonstrated that PEG4MAL is a desirable biomaterial for microfluidic encapsulation of gut bacteria due to its high degree of biocompatibility and tunable physical and mechanical properties. The mechanostimulation from the surrounding PEG4MAL network was shown to impact the proliferation of cells within the microbead. However, this work is limited by the study of only *A. mucinphila* and does not provide a comprehensive assessment of the impact of encapsulation for all dominant phyla present within the GI tract. Additionally, this work lacks investigation into other PEG4MAL concentrations between 2% and 5%, nor has it explored the

use of other small molecules, besides RGD, that may influence changes in mechanical and fluid properties of PEG4MAL.

Future work is recommended to better characterize the importance of mechanostimulation of the gut microbiota during *in vitro* culture. Understanding the role of peristaltic forces within the GI tract and mechanical properties of the mucosal layer may be key to unlocking future discoveries within the gut and advancing opportunities for *in vitro* experimental models. This work provides foundational evidence of the benefits of GI microbiota encapsulation; however, future investigation is required to examine the impact of community-based encapsulation and co-culture. By leveraging microfluidic encapsulation of the gut microbiota, future community and co-culture based-studies may provide new insights into inter-microbial interactions within the GI tract as well as the production of novel bioactive compounds important for human health. As it stands, this approach is well-suited for integration with *in situ* culture during passage through the GI tract to reassimilate bacteria to the natural environment and permit access to nutrients and chemical signaling that are difficult to recreate *in vitro*. The impacts of this work will be further amplified with improved efforts to achieve targeted sampling of all previously uncharacterized niches of growth within the GI tract. Collectively, this work will arm researchers with the tools to overcome current limitations in the search for answers within the gut and change the uncultured majority into a minority.

APPENDIX I

FLOW CYTOMETRY FOR CELL CONCENTRATION CALIBRATION

Flow cytometry was used to create a calibration curve between optical density and the concentration of cells within a culture (Figure I-1, I-2, I-3 A, B). Due to the differences in cell size and morphology, distinct calibration curves were required for each species of interest.

For this experiment, *E. coli* was grown overnight from frozen stock at 37 °C in BHI media under aerobic conditions. Additionally, *A. muciniphila* was grown over 72 hours from frozen stock at 37 °C in BHI media under anaerobic conditions. Each of the cultures were independently diluted to achieve a range of optical density measurements. Each sample was centrifuged at 9500 x g for 5 minutes. The supernatant was carefully removed, and the cell pellet was resuspended in PBS. The centrifugation and resuspension was performed twice for each sample. After washing, the samples were diluted by a ratio of 1:100 in PBS, and a 500 μL aliquot was added to a round-bottomed 5 mL tube. For quantification of cells, 50 μL of CountBright Absolute Counting Beads (Thermofisher Scientific, CA) and 0.5 μL of SYBR green were incorporated into the sample via vortexing and protected from light.

Samples were assessed on FACSCanto (BD, US) and population gating was performed on the FlowJo software. The concentration of cells,

$$\text{Absolute Count} = \frac{\text{Cell Count} \times \text{Counting Beads Volume}}{\text{Counting Beads Count} \times \text{Cell Volume}} \times \text{Counting Beads Concentration}, \quad \text{(A.1)}$$

was determined by the number of hits of SYBR green-labelled cells and counting beads within the gated populations.

Unfortunately, the magnitude of cell concentration was below the expected values at the given optical densities. It is hypothesized that this was driven by excessively high centrifugation. After imaging a sample, which was exposed to similar experimental conditions, significant cell

clumping was observed (Figure I-3 C). The presence of cell aggregates could be a leading cause of the low cell counts, as there is a higher likelihood of doublets.

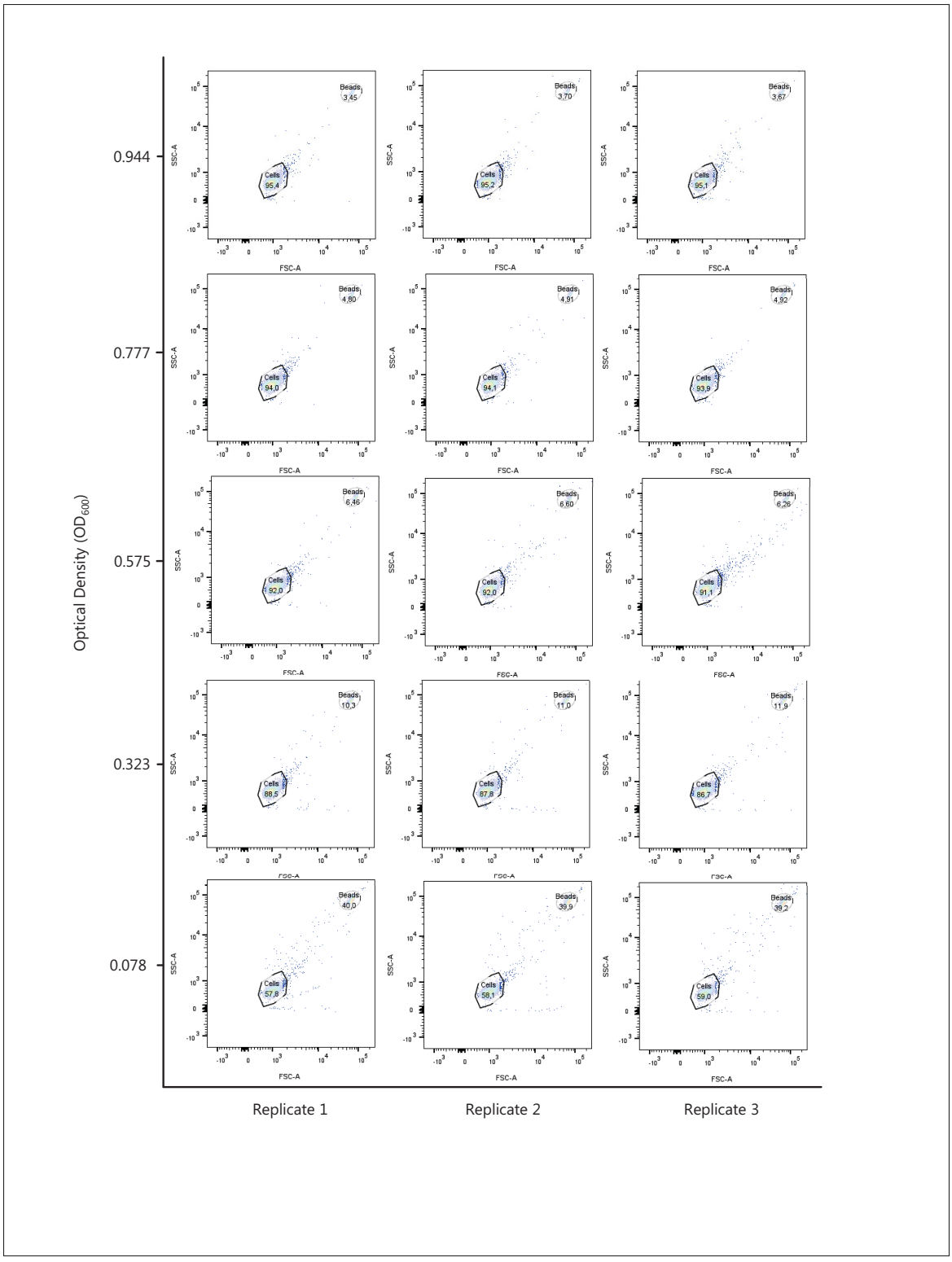

Figure-A I-1 The concentration of *E. coli* detected by flow cytometry at various optical densities

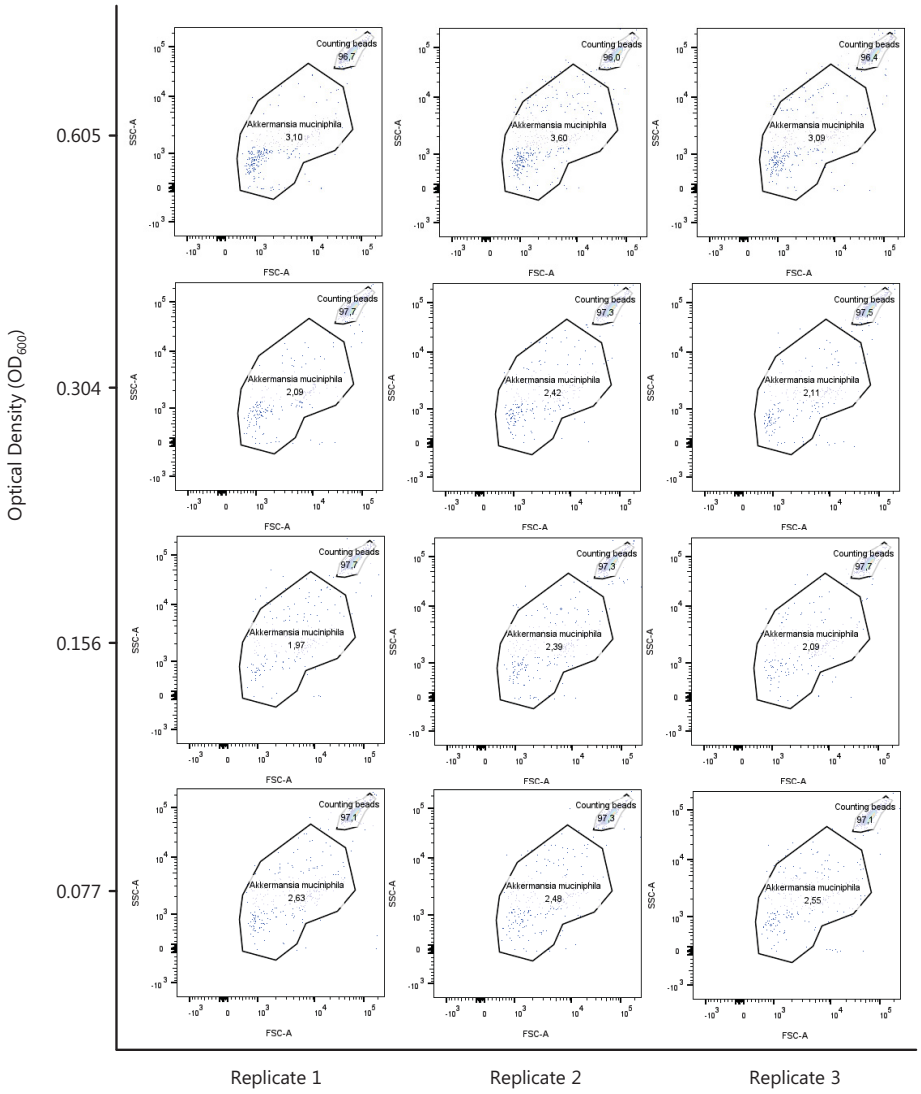

Figure-A I-2 The concentration of *A. muciniphila* detected by flow cytometry at various optical densities

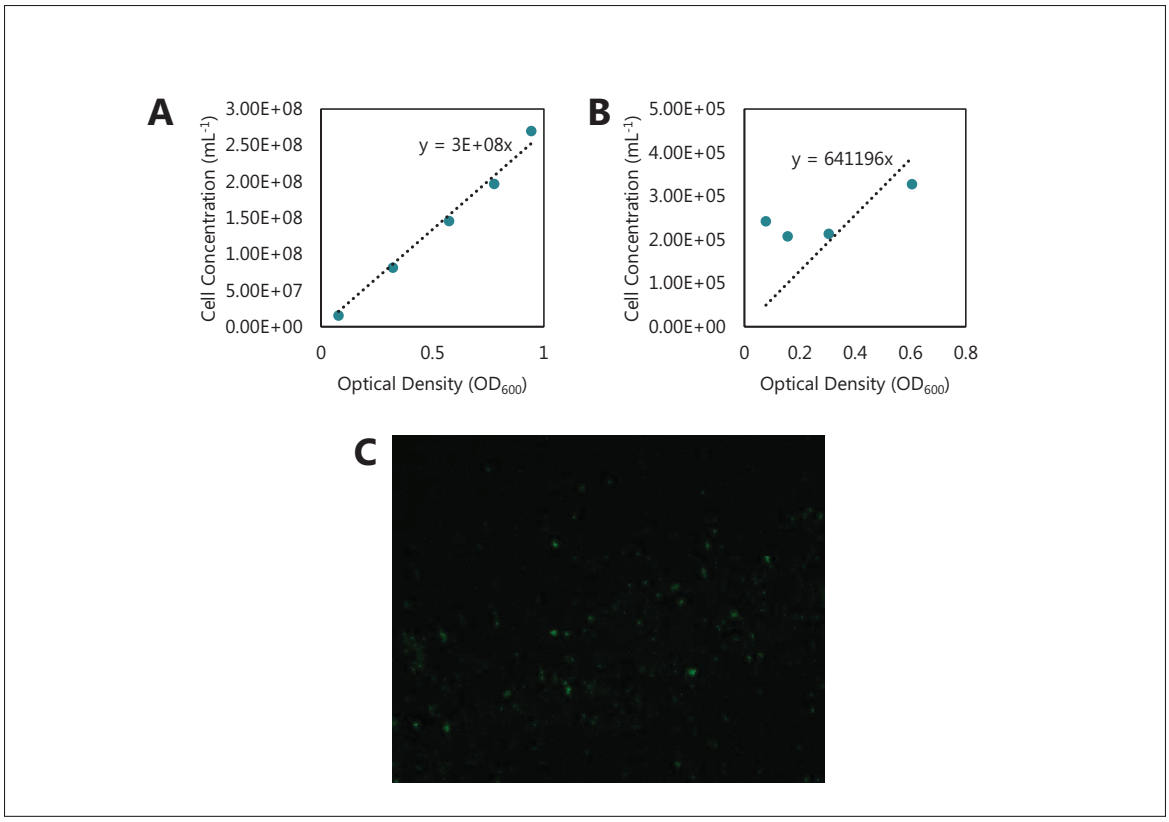

Figure-A I-3 The cell concentration calibration curves of A) *E. coli* and; B) *A. muciniphila*; an image of cells post flow cytometry preparation

APPENDIX II

PEG4MAL DEGRADATION ASSESSMENT

Understanding the stability of PEG4MAL under different physiologically relevant pH conditions was important to ensure culture compatibility for GI-associated bacteria. To assess stability, 5 crosslinked droplets of 2, 5, 10, and 20% PEG4MAL were prepared as previously described and subjected to different conditions to mimic passage through the GI tract (Nguyen *et al.*, 2020; Liu *et al.*, 2021; Verhoeckx *et al.*, 2015). Simulated gastric and intestinal fluids were prepared using hydrochloric acid (VWR, CA) and sodium hydroxide (VWR, CA). The first condition consisted of a normal control, and these droplets were directly freeze-dried (HarvestRight, US) for 8 hours. The following samples were submerged in simulated gastric conditions consisting of a pH 2 solution for 3 hours. There were two simulated intestinal conditions where droplets were exposed to pH 7 or pH 6 solutions for 2 or 12 hours, respectively. Finally, the last set of droplets was subjected to a sequential exposure of the gastric and intestinal conditions. Following submersion, the simulated fluids were removed and the samples were rinsed and freeze-dried as previously described.

Prior to imaging, the samples were sputter coated with a 20 nm layer of gold nanoparticles using a K550X Sputter Coater (Quorum, UK) and imaged with a TM3000 scanning electron microscope (Hitachi, US). Degradation was assessed visually by measuring the void fraction (ϕ) of 2D cross-sectional images for the exposed conditions (ϕ_{final}) and the control condition (ϕ_{initial})(Fonseca & Scherer, 2015).

Stability was assessed by characterizing the 2D void fraction of the PEG4MAL microstructure, revealing limited differences in surface porosity-based degradation (Figure II-1 A, B). Some significant differences were observed and assumed to be driven by the heterogeneous microstructure. The lack of degradation suggests that PEG4MAL microbeads could withstand passage through the GI tract, although future studies are needed to assess the impact of enzymatic activity on PEG4MAL stability.

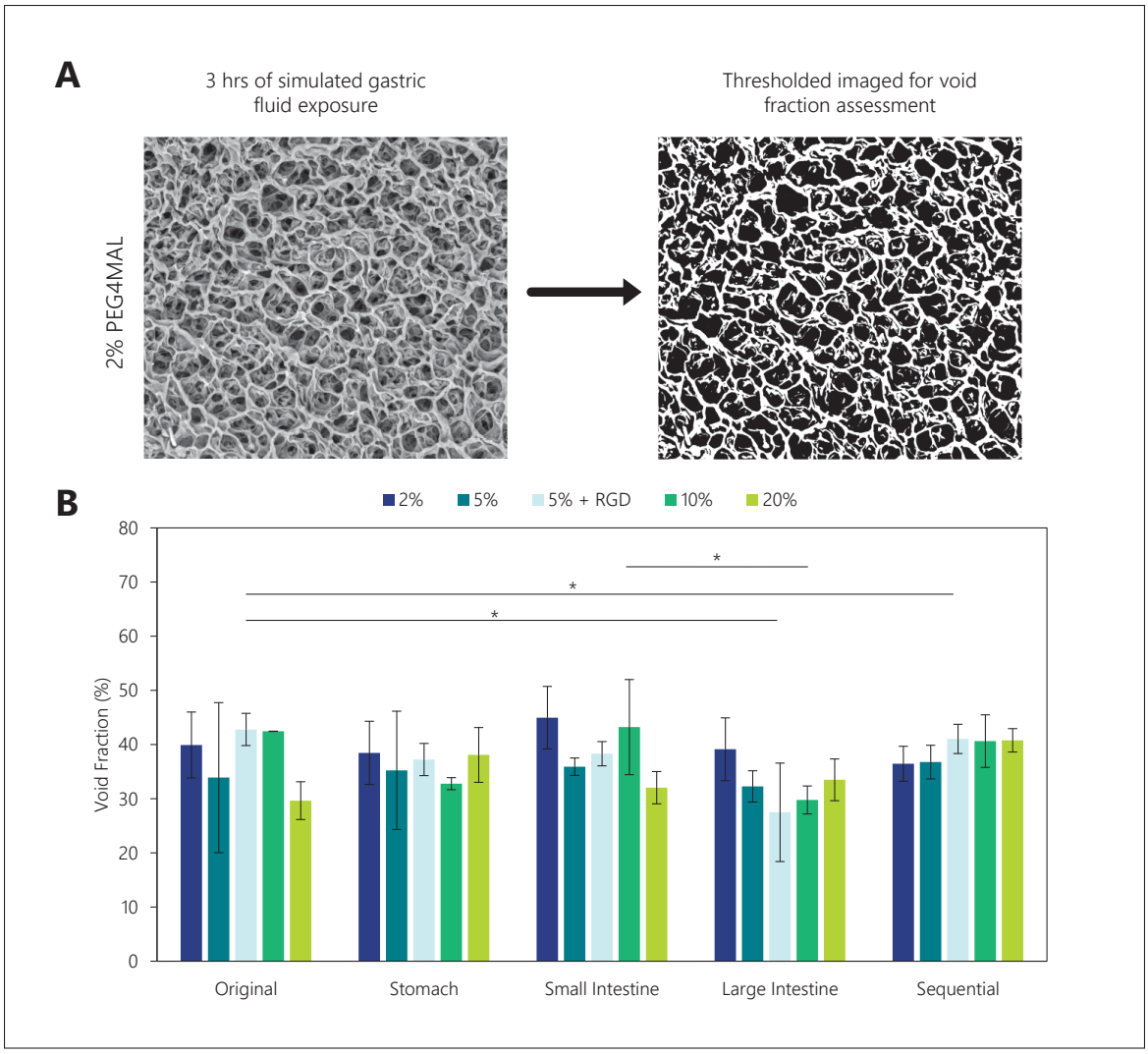

Figure-A II-1 PEG4MAL degradation post-exposured to simlated gastric and intestinal fluids by A) imaging the surface porosity of PEG4MAL via SEM and; B) the void fraction of the samples following image processing

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