Universidade de Aveiro Departamento de Biologia
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ESTRATÉGIAS PARA PROMOVER A VIABILIDADE E ESTABILIDADE DE *Akkermansia muciniphila* SOB CONDIÇÕES DE STRESS.

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Tese apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Microbiologia, realizada sob a orientação científica da Doutora Ana Cristina Cardoso Freitas Lopes de Freitas, Investigadora Principal do Centro de Biotecnologia e Química Fina (CBQF) – Laboratório Associado da Escola Superior de Biotecnologia, Universidade Católica Portuguesa e co-orientação da Doutora Sónia Alexandra Leite Velho Mendo Barroso, Professora Auxiliar com Agregação do Departamento de Biologia da Universidade de Aveiro e Doutora Ana Maria Pereira Gomes, Professora Associada da Escola Superior de Biotecnologia, Universidade Católica Portuguesa.

This work was supported by national funds through FCT/MEC (PIDDAC), project reference IF/00588/2015 and by Operational Program Competitiveness and Internationalization in its FEDER component and by the budget of the Foundation for Science and Technology, I.P. (FCT, IP) in its OE component, project reference POCI-01-0145-FEDER-031400 with scientific collaboration of CBQF under the FCT project UID/Multi/50016/2013.

Dedico este trabalho a toda a minha família, em especial à minha prima Andreia e tia Natália, porque a coragem nem sempre é um rugido.

"*I have not failed. I've just found 10.000 ways that won't work."*

Thomas Edison

"Magic happens when you don't give up, even though you want to. The universe always falls in love with a stubborn heart."

J. M. Storm

o júri

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agradecimentos

À minha orientadora, Doutora Ana Freitas, pelo voto de confiança, disponibilidade, orientação, mas acima de tudo, pela amizade, paciência, incansável apoio e palavras de encorajamento que me acompanharam ao longo deste "longo" percurso.

Às minhas co-orientadoras, Doutora Sónia Mendo, pela oportunidade concedida, bem como pelo acompanhamento desta fase da minha jornada científica e Doutora Ana Gomes, pelas sessões de brainstorming, pelo input critico e científico e, pela preocupação e carinho que sempre teve para comigo.

À Doutora Daniela Machado, pela supervisão, pelos longos dias de experiências com almoços tardios, e fins-de-semana de trabalho intenso, pela amizade, paciência e preocupação.

A todo o pessoal do laboratório do CBQF que tive prazer de conhecer, pelo bom ambiente de trabalho, pelas gargalhadas de corredor, pelas *small talks* entre experiências que foram capazes de amenizar os dias mais complicados. Em especial à Dina, pela amizade, pelo apoio incondicional e pelas *late nights* no laboratório e à Sara e ao Eduardo, pelo acompanhamento e apoio prestados.

À Catarina, Mariana, Leandro e Feliciana que, quando cansada e desanimada, sempre me providenciaram com momentos de alegria e distração. Pelos "só mais 5 minutos ao sol por favor!"

Aos meus colegas de mestrado, pelo companheirismo que nos permitiu superar obstáculos, pelas longas viagens de comboio, pelas conversas que "não lembram a ninguém", pelas risadas de fazer doer a barriga. Pela família criada da qual nunca me vou esquecer.

À Joana, pela presença constante desde o inicio desta caminhada. Pelo apoio, amizade. Pelo "se tu fores eu também vou".

À Ju e ao Pedro, pela amizade de muitos "anos e troca-o-passo", pelas sessões de trabalho, pelas conversas sem necessidade de palavras, pelo apoio sem fim que sempre demonstraram.

Aos meus amigos mais próximos, pela amizade, preocupação e incansável apoio.

À minha família, que apesar de todas as adversidades, me mostra todos os dias que na união há força, que só com amor alcançamos vitória e que o sorriso é a nossa divisa. Acima de tudo, aos meus pais e irmãos, pela paciência, encorajamento e amor manifestado. Pela compreensão demonstrada nos meus piores dias. Não há preço neste mundo que pague os sacrifícios que fizeram por mim.

palavras-chave

Akkermansia muciniphila, Probióticos de Nova Geração, disbiose, formulação, microencapsulação, simulação gastrointestinal

resumo

Nos últimos anos, a comunidade científica tem vindo a reunir um maior conhecimento das dinâmicas que estão na base dos distúrbios metabólicos e inflamatórios, muitos dos quais relacionados com a alimentação. O intenso crescimento destes distúrbios está a atingir proporções epidémicas, trazendo novos desafios aos clínicos e investigadores. As funções moduladoras e as propriedades específicas que as bactérias benéficas/probióticas possuem no contexto do ecossistema intestinal, parecem ser a chave para prevenir tais perturbações. Atualmente, *Akkermansia muciniphila* tem emergido como um "probiótico do futuro ou de nova geração" ("Next Generation Probiotics" – NGP), dado o seu potencial na prevenção e tratamento de distúrbios inflamatórios/cardio-metabólicos. Os desafios envolvendo esta bactéria probiótica residem principalmente na sua sensibilidade à atmosfera aeróbia e baixo pH. Por estas razões, esta tese tem como objetivo explorar formulações liofilizadas envolvendo agentes protetores tais como antioxidantes, prebióticos e agentes de volume bem como a microencapsulação como estratégias tecnológicas para aumentar a viabilidade da *A. muciniphila* face à passagem no trato gastrointestinal (GI) e promover a sua estabilidade durante o armazenamento aeróbio.

Primeiramente, uma caracterização fenotípica da estirpe *A. muciniphila* DSM 22959 foi efetuada. Nesta análise, características morfológicas e a coloração face à técnica de Gram, confirmam a sua natureza Gram-negativa e morfologia cocobacilar. Além disso, foi demonstrado que os ácidos miristoleico e pentadecanóico são os principais ácidos gordos presentes na membrana de *A. muciniphila*. Adicionalmente, as suas colónias foram caracterizadas como sendo pequenas, circulares e translúcidas.

A exposição ao ar ambiente revelou a capacidade de sobrevivência de *A. muciniphila* até 60 horas em atmosfera aeróbia, a 37 ºC. Apesar da tendência de declínio na viabilidade, a *A. muciniphila* foi capaz de sobreviver à atmosfera aeróbia durante 60 h.

Também, as propriedades de adesão desta bactéria ao epitélio intestinal foram comprovadas usando duas linhagens epiteliais, nomeadamente Caco-2 e HT29-MTX. Após caracterização fenotípica, formulações liofilizadas e um método de encapsulação foram explorados como estratégias tecnológicas para promover a viabilidade e estabilidade de *A. muciniphila* quando expostas ao trato GI e armazenamento aeróbio. No geral, obtiveram-se valores elevados nos liofilizados com a formulação contendo inulina (10 % m/v), riboflavina (16.5 mM) e glutationa (0.2 % m/v) do que no seu liofilizado homólogo com amido (10.2 vs 6.3 log UFC g-1). Além disso, a adição de amido à formulação conferiu maior estabilidade durante o armazenamento aeróbico. No entanto, em ambas as formulações *A. muciniphila* demonstrou maior suscetibilidade ao trato GI e ao armazenamento aeróbio do que na sua forma não-formulada.

Numa tentativa de reduzir a sensibilidade face ao trato GI e armazenamento aeróbio, *A. muciniphila* foi encapsulada através do método de emulsificação/gelificação interna, numa matriz contendo alginato-Na (4 % m/v), $CaCO_3$ (500 mM) e isolado de proteína de soro de leite desnaturado (DWPI; 10 % m/v). *Akkermansia muciniphila* foi eficientemente encapsulada (95.8 ± 0.01 %), em que o diâmetro das microcápsulas foi menor do que 100 µm. Para além disso, *A. muciniphila* encapsulada demonstrou elevada resistência às condições GI e ao armazenamento aeróbio, uma vez que a sua viabilidade apenas decresceu um ciclo logarítmico após exposição simulada ao trato GI apresentando elevada estabilidade após 7 dias de armazenamento aeróbio, a 4ºC. Em suma, as microcápsulas de alginato-Na:$CaCO_3$:DWPI revelaram ser a melhor estratégia na proteção de *A. muciniphila* contra as condições desfavoráveis do trato GI e de armazenamento em aerobiose.

keywords

Akkermansia muciniphila, Next Generation Probiotics, dysbiosis, formulation, microencapsulation, gastrointestinal simulation

abstract

In recent years, the scientific community has been gathering increasingly more insight on the dynamics that are at play in metabolic and inflammatory disorders many of which are diet-related. These rapidly growing conditions are reaching epidemic proportions, bringing new challenges to clinicians and researchers. The specific roles and modulating properties that beneficial/probiotic bacteria hold in the context of the gut ecosystem seem to be a key strategy to avert such imbalances. Currently, *Akkermansia muciniphila* has emerged as a potential next generation probiotic (NGP) given its demonstrated potential in prevention and treatment of inflammatory/cardio-metabolic disorders. The challenges of this non-conventional native gut bacterium lie mainly on its sensitivity to aerobic environments and low pH conditions. Based on these rationales, this thesis aims to explore freeze-dried formulations involving protective agents such as antioxidants, prebiotics and bulking agents, and microencapsulation as technological strategies to increase *A. muciniphila* viability throughout gastrointestinal (GI) passage and stability under aerobic storage.

Firstly, a comprehensive phenotypic characterization involving *A. muciniphila* DSM 22959 strain was conducted. In this analysis well-known staining and morphological traits namely Gram-negative and coccobacillary-shape were confirmed; furthermore, myristoleic and pentadecanoic acids were demonstrated to be the major membrane fatty acids in *A. muciniphila*. In addition, their colonies were morphologically characterized as being small, circular and translucent. Exposure to ambient air revealed that *A. muciniphila* survived up to 60 hours in an aerobic atmosphere at 37ºC. In addition, the adhesion properties of *A. muciniphila* to gut epithelium were proven, using Caco-2 and HT29-MTX cell lines as in vitro models. Upon phenotypic characterization, freeze-dried formulations and encapsulation methods were explored as technological strategies to enhance viability and stability of *A. muciniphila* when submitted to both GI transit and aerobic storage. Overall, *A. muciniphila* achieved high numbers in freeze-dried powders of the formulation containing inulin (10 % w/v), riboflavin (16.5 mM) and glutathione (0.2 % w/v). In addition, this formulation matrix contained higher number of viable cells than the starch counterpart (10.2 vs 6.3 log CFU g-1), yet the addition of starch to the formulation conferred higher stability during aerobic storage. Nevertheless, in both freeze-dried formulations *A. muciniphila* displayed a higher susceptibility to GI transit and aerobic storage than non-formulated cells.

In an attempt to reduce sensitivity to GI and aerobic storage conditions, *A. muciniphila* was encapsulated, by emulsification/internal gelation method, in a Na-alginate (4 % w/v), calcium carbonate ($CaCO_3$; 500 mM) and denatured whey protein isolate (DWPI; 10 % w/v) matrix. *Akkermansia muciniphila* was efficiently encapsulated (95.8 ± 0.01 %) via such microencapsulation method, where microcapsules size diameter was smaller than 100 µm. Moreover, encapsulated *A. muciniphila* demonstrated high resistance to GI conditions and aerobic storage since their viability only decreased 1 log cycle after simulated GI tract exposure presenting a high stability after 7 days of refrigerated aerobic storage.

In conclusion, Na-alginate:$CaCO_3$:DWPI microcapsules reveal a better strategy to protect *A. muciniphila* against detrimental gastrointestinal transit and aerobic storage conditions.

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List of Abbreviations

2-OG	2-oleoylglycerol
ANOVA	Analysis of variance
$CaCl_2$	Calcium chloride
Caco-2	Caucasian colon adenocarcinoma
$CaCO_3$	Calcium Carbonate
CFU	Colony-forming units
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
DWPI	Denatured WPI
EE	Encapsulation efficiency
FA	Fatty acid
FAME	Fatty acids methyl esters
FBS	Fetal Bovine Serum
FCM	Flow Cytometry
FISH	Fluorescent *in situ* hybridization
FOS	Fructo-oligosaccharide
FSC	Forward-scatter
GI	Gastrointestinal
GLP-1	Glucagon-like peptide-1
GP	Gastric Phase
HT29-MTX	Mucus secreting human colon adenocarcinoma
IFN-γ	Interferon gamma
IP	Intestinal phase
LAB	Lactic acid bacteria
LPS	Lipopolysaccharides
MAMP	Microbial associated-molecular pattern
MUFA	Monounsaturated fatty acids
Na-alginate	Sodium alginate
NGP	Next-Generation Probiotic
OD	Optical density
OP	Oral phase
PB	Phosphate buffer
PBS	Phosphate buffer saline
PI	Propidium iodide
Rpm	Revolutions per minute
rt-PCR	Real-time quantitative polymerase chain reactions
SCFA	Short-chain fatty acids
SFA	Saturated fatty acids
SGF	Simulated gastric fluid
SIF	Simulated intestinal fluid
SSC	Side-scatter
SSF	Simulated salivary fluid
T2D	Type 2 diabetes mellitus
TO	Thiazole orange
VBNC	Viable but not culturable
W/O	Water-in-Oil
WPI	Whey protein isolate

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Scientific outputs

Paper in Peer Reviewed Journals

Almeida D. et al. Submitted. Evolving trends in next-generation probiotics: a 5W1H perspective. Critical Reviews in Food Science and Technology. Submitted at 3rd August. BFSN-2018-3570.

Poster Presentations

Freitas A., Almeida D. et al. 2018. Strategies to increase *Akkermansia muciniphila* viability during simulated gastrointestinal conditions and stability storage. Poster presented in the *6th World Congress on Targeting Microbiota*, Porto, 28-30th October. Abstract in page 75, Book of Abstracts.

Freitas A. Almeida D. et al. 2018. Formulation strategies for enhancing growth of *Akkermansia muciniphila* and its survival through lyophilisation and storage at air ambient. Poster presented in the *12th International Scientific Conference on Probiotics, Prebiotics, Gut Microbiota and Health* – IPC2018, Budapeste, 18-21th June. Abstract in page 71, Book of Abstracts.

1. Introduction

Worldwide, type 2 diabetes mellitus (T2D), obesity and inflammatory bowel diseases (IBD) among others have achieved high proportions, constituting serious public health problems with important social, financial and health systems implications (GBD 2015 Obesity Collaborators et al. 2017; Ogurtsova et al. 2017; Ng et al. 2018) Furthermore, these metabolic and inflammatory conditions have been related with dramatic changes in the human gut microbiota at both quantity and quality (bacterial species diversity) levels. Indeed, the gut microbiota associated with the gut epithelial barrier plays a key role in the regulation of the inflammatory and metabolic host profiles, promoting an overall host cellular homeostasis status contributing to global health and minimizing the triggering of inflammatory mechanisms (Hartstra et al. 2015; Marchesi et al. 2016; Cani 2017). Recently, de Vos and colleagues (2012), described the nature of the relationships between the microbiota profile and associated intestinal diseases suggesting that the intestinal microbiome could be linked to a growing number of over 25 diseases or syndromes (De-Vos & De-Vos 2012).

It is still not clear how the interaction between microbiome and host immunity affects the development of specific diseases, yet it is established that exposure to low bacterial diversity in early life can prevent or delay immune mucosa maturation reducing immunological tolerance and hence increasing the risk of aberrant immune response and allergic disease (Brooks et al. 2013). In addition to the immune modulating properties, the gut microbiota also contributes to the host condition by granting a protective barrier against pathogens, enabling digestion through the breakdown of non-digestible food constituents and producing essential metabolites (Ottman et al. 2012). Since low microbial diversity has been associated with several life-style related non-communicable diseases such as obesity, metabolic syndrome, immune-related, and inflammatory diseases (D'Argenio & Salvatore 2015), a microbial ecosystem with higher diversity can be considered as an indicator of a more healthy status (Jordán et al. 2015), as demonstrated in elderly subjects (Jeffery et al. 2016).

One of the most attainable approaches for modulation of gut microbiota diversity is through dietary interventions. Studies focusing on the negative impact of westernization diet have demonstrated the co-evolution of microbial species and the human host (Cordain et al. 2005; Blaser & Falkow 2009; Quercia et al. 2014). It is important to consider that dietary intake, dependent on its nature and quantity, may promote imbalances in colonic microbial populations leading to the onset of numerous inflammatory conditions that may degenerate into chronic diseases (Celiberto et al. 2015). In addition to certain well-balanced diets, such as the Mediterranean diet

(Garcia-Mantrana et al. 2018), specific food choices such as high-fat dairy products (Bordoni et al. 2017) or dietary fiber (Masuko 2018), associated to pro- (in subjects with milk allergy) and anti-inflammatory effects, respectively, can significantly modulate gut microbiota composition. Furthermore, this relationship is more dynamic than initially thought, as the active “adjustment” of microbial composition - e.g. probiotic and synbiotic supplementation (Plaza-Díaz et al. 2017) - can also elicit anti-inflammatory effects. Given these recent findings, overcoming host genetic predisposition for the development of inflammatory and metabolic conditions through dietary interventions or more actively, via new generation probiotics supplementation, may be the future strategy choice for therapy or, even as a prevention tool to avoid the onset of these chronic diseases, fueling hope for the millions of patients worldwide that experience the taxing consequences of inflammatory/metabolic conditions.

1.1. Probiotics: a new game plan

Upholding the above considerations, new actions are required for the prevention and treatment of such impacting inflammatory diseases. In recent years, several studies have demonstrated that the consumption of selected microbes, named probiotics, are a highly promising therapeutic alternative for treating the gut microbiota dysbiosis (Cani & Van Hul 2015). In 1908, Ellie Metchnikoff introduced the concept of probiotics stating that "the dependence of the intestinal microbes on food makes it possible to adopt measures to modify the flora in our bodies and to replace the harmful microbes by useful microbes" (Metchnikoff 1908). Currently, the most sound-based definition accepted worldwide for probiotics is that they are “live microorganisms that, when administered in sufficient quantities, confer a health benefit on the host” (Hill et al. 2014). In fact, probiotics inhibit pathogen colonization by competitive exclusion and displacement, enhance intestinal barrier function and modulate the immune and neuroendocrine systems, locally and systemically, as evidenced in *in-vitro* and animal trials (Neef & Sanz 2013).

Conventionally, the microorganisms belonging to the *Lactobacillus* and *Bifidobacterium* genera have been employed as probiotics, encountering wide commercial availability in the market (Douillard & de Vos 2014). These microorganisms are usually well-accepted and tolerated by humans (Brodmann et al. 2017). However, these classical probiotics display limited effects on the human gut microbiota, thus calling for a better selection of bacterial strains and formulation of delivery vehicles (Neef & Sanz 2013). In this context, several bacterial species comprising genera other than *Lactobacillus* and

Bifidobacterium with proven efficacy have been considered as potential next-generation probiotics (NGPs), namely strains from the *Akkermansia*, *Eubacterium*, and *Faecalibacterium* genera (Cani & Van Hul 2015; Patel & DuPont 2015; O'Toole et al. 2017; O'Toole & Paoli 2017).

1.2. NGP: *Akkermansia muciniphila* – A new contender

Among the more recent proposals of new species as NGPs, *Akkermansia muciniphila* stands out as an interesting candidate for this category which should possess high potential to avert inflammatory and diet-related disorders (Gómez-Gallego et al. 2016; Cani 2018; Naito et al. 2018). *Akkermansia muciniphila* belongs to the phylum Verrucomicrobia and is a common resident of the human gut, representing approximately 1-3 % of the total gut microbiota (Derrien et al. 2008). Its cells are oval-shaped, non-motile and stain Gram-negative (Derrien et al. 2004). *Akkermansia muciniphila* was originally classified as a strict anaerobe (Derrien et al. 2004), but recently it was demonstrated that it can use oxygen in nanomolar concentrations, being reclassified as an aerotolerant anaerobe (Ouwerkerk et al. 2016).

The particular feature of this bacterial species is the ability to degrade mucin, a high molecular mass glycoprotein, known as the main component of the intestinal mucus and an important mediator of the gut barrier (Derrien et al. 2004; Derrien et al. 2017). The colonic mucus coating the epithelial layer of the gastrointestinal (GI) tract (Figure 1.1), produced by goblet cells, serves as the primary barrier of the colon protecting the host against toxins and pathogens and its thickness reduction increases gut permeability and the risk of endotoxemia as well as bacterial translocation (Johansson et al. 2014). Additionally, the *A. muciniphila* unique ability to utilize mucin as a stable sole source of nitrogen and carbon (Derrien et al. 2004) also provides this microorganism with an ecological advantage as in the case of glycan deficiency (Derrien et al. 2017).

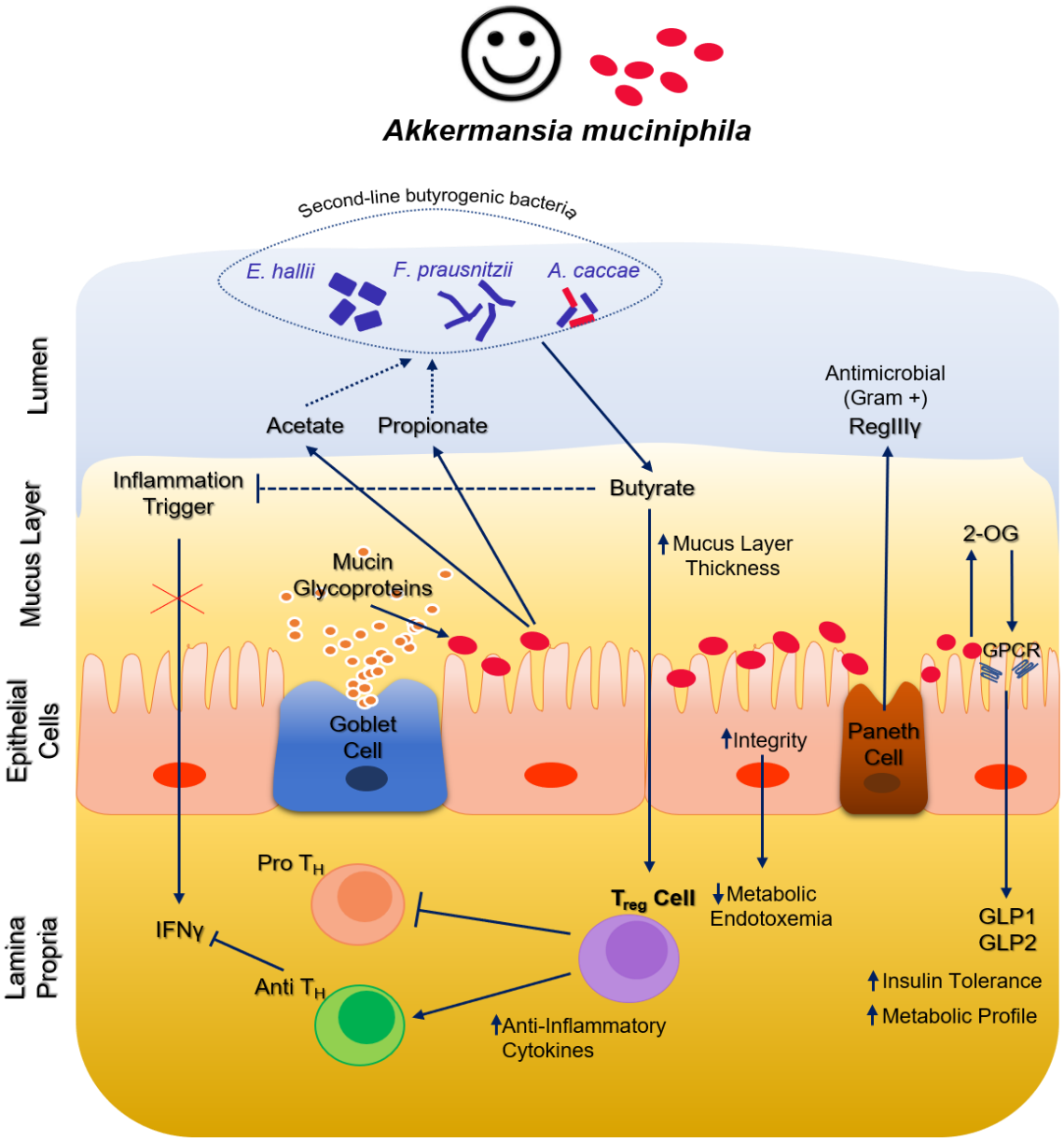

Figure 1.1 - Graphical summary of Akkermansia muciniphila interactions in gut epithelium responsible for maintaining host health. Colonic mucus, produced by goblet cells, is degraded by A. muciniphila leading to the production of bioactive molecules, such as acetate and propionate (SCFAs), and the release of oligosaccharides utilized by non-mucus degrading butyrate – producing bacteria (Anaerostipes caccae, Eubacterium hallii and Faecalibacterium prausnitzii) for butyrate production. Butyrate is linked to distinct beneficial effects on the host improving the intestinal barrier integrity which prevents metabolic endotoxemia (uptake of LPS) a key characteristic of metabolic disorders such as type 2 diabetes and insulin resistance; it also exerts immunoregulatory properties promoting an anti-inflammatory phenotype. Glucose tolerance can be mediated by A. muciniphila through the control of the negative effects of IFNγ and, also through the increase of gut hormone production such as GLP-1 via production of 2-OG (endocannabinoid system). In addition, A. muciniphila induces the production of the antimicrobial peptide RegIIIγ via Paneth Cell synergistically increasing the anti-inflammatory milieu of the gut ecosystem.

Abbreviations: Treg cells, regulatory T cells; Anti TH, Anti-inflammatory cytokines producing T cells; Pro TH, Pro-inflammatory cytokines producing T cells; GPCR, G protein–coupled receptor; GLP1/2, Glucagon-like peptide-1 and 2; IFNγ, Interferon gamma; RegIIIγ, Regenerating islet-derived 3 gamma; 2-OG, 2-oleoylglycerol.

The specialized mucin fermentation results in the production of short-chain fatty acids (SCFAs) such as acetate and propionate (Derrien et al. 2004), as may be seen in Figure 1.1. This phenomenon may also influence gut bacterial composition, by creating a favorable environment for the growth of strict anaerobes, in a syntrophic association, such as non-mucus degrading butyrate-producing bacteria, which could have a synergistic effect on the host (Rios-Covian et al. 2015; Belzer et al. 2017). Moreover, *A. muciniphila* partakes on colonic mucus turnover cycle since the produced SCFAs also stimulate goblet cells mucus production (Shimotoyodome et al. 2000). Hence, *A. muciniphila* cell proportion is correlated with an increase of the number of the gut mucus-producing goblet cells, thus sustaining the intestinal barrier integrity (Shin et al. 2014). Indeed, an *in vitro* model demonstrated that *A. muciniphila* adheres to the gut epithelial cells, fortifying enterocyte monolayer integrity (Reunanen et al. 2015; Chelakkot et al. 2018). In addition to its effect on gut barrier function, *A. muciniphila* is linked to a desirable metabolic profile and to the reduction of fat mass without interfering with total energy intake by the host (Everard et al. 2013; Dao et al. 2016). These host-microbe-overall metabolism interactions are probably mediated by *A. muciniphila* endocannabinoid system since it substantially increases intestinal levels of 2-oleoylglycerol (2-OG), a bioactive lipid involved in gut hormone synthesis and appetite control (Figure 1; Table 1).

1.2.1. *Akkermansia muciniphila* in disease

Recently, it has been reported that *A. muciniphila* can be used as a biomarker of a healthy host metabolic profile. In fact, a reduction of *A. muciniphila* levels in the gut has been related with several metabolic and inflammatory diseases, such as obesity, T2D and IBD (Png et al. 2010; Cani & Everard 2014; Schneeberger et al. 2015). Recent mechanistic studies related with *A. muciniphila* provide insight on what type of physiological routes are at play for the favourable anti-inflammatory and antimicrobial attributes this bacterium offers the host for protection against disease (Table 1).

Unquestionably, and as previously mentioned, one of the most relevant mechanisms for *A. muciniphila* probiotic action is the strengthening of the gut epithelial barrier against the diffusion of potentially immune-activating substances - such as bacteria, endotoxins, and digestion products - from the gut content that, when in contact with intestinal tract immune system, trigger the onset of systemic inflammation (Cani et al. 2007). This process, also termed as metabolic endotoxicity, is due to the damage of the gut barrier integrity such as the breakdown of the intercellular junction between the intestinal mucosal walls, which causes an alteration in intestinal permeability, a

condition known as leaky gut (Michielan & D'Incà 2015). Low grade inflammation is recognized to be one of the triggers involved in the metabolic shifts that occur upon cardio-metabolic diseases such as obesity and T2D (Gomes et al. 2017; Cani et al. 2007). Indeed, it has been demonstrated that the increased levels of circulating lipopolysaccharides (LPS), a component of Gram-negative bacteria, is one of the main factors causative of these conditions (Cani et al. 2007; Reunanen et al. 2015).

An increase in *A. muciniphila* numbers is also linked to an improved adipose tissue metabolism increasing the endogenous production of specific bioactive lipids such as 2-OG. This bioactive lipid stimulates the secretion of glucagon-like peptides, such as glucagon-like peptide-1 (GLP-1) (Everard et al. 2013), and its release results in an interaction with the host endocrine system which in turn influences gut barrier function and energy homeostasis (Everard et al. 2011). More recently, a specific bacterial structural component protein Amuc_1100 found on the outer membrane of *A. muciniphila* and implicated in the formation of pili (Plovier et al. 2016) has been shown to have an important immunomodulatory action in both *in vitro* and *in vivo* models (Ottman et al. 2017; Cani 2018). Indeed, Amuc_1100 protein seems to be involved in the reduction of fat mass development and dyslipidemia, as well as in insulin tolerance improvement (Plovier et al. 2016).

Another important biological property is related to its ability to improve glucose tolerance. In this context, Greer and colleagues showed that *A. muciniphila* can mediate the negative effects of interferon gamma (IFN-γ) on glucose tolerance (Greer et al. 2016).

Table 1 - Summary of beneficial impacts on human health and disease by the NGP *Akkermansia muciniphila*

Target Conditions	Key Finding(s)	Study Type	Refs
Inflammation	Increases enterocyte monolayer integrity by promoting colonic mucus turnover and lowering LPS uptake	*In vitro* (adhesion assay)	(Reunanen et al. 2015; Derrien et al. 2017)
	Amuc_1100 (outer membrane protein implicated in the formation of pili) can recapitulate the beneficial effects of the whole live bacterium; shows stability at temperatures used in pasteurization (via the specific activation of Toll-like receptor 2)	*In vitro*	(Plovier et al. 2016; Ottman et al. 2017)
	Stimulates RegIIIγ (antimicrobial peptide against Gram-positives) production by Paneth cells	Preclinical in mice (*ob/ob* mice model)	(Everard et al. 2013); (Pott & Hornef 2012).
Metabolic disorders (diabetes, obesity)	Negatively correlated with intestinal permeability, metabolic endotoxaemia, inflammatory biomarkers and low grade induced metabolic disorders such as T2D and insulin resistance with additional increased macrophage infiltration into the adipose tissue and hepatic steatosis	Case control studies	(Cani & Van Hul 2015; Dao et al. 2016)
	Mediates IFN-γ (pro-inflammatory cytokine, responsible for the control of intracellular pathogenic infections) adverse effects on glucose metabolism; Abundance negatively controlled by Irgm1 gene (controls *A. muciniphila* levels)	Preclinical in mice (IFN- γ *KO* models)	(Greer et al. 2016)
	Prebiotic administration reduced adiposity, inflammatory markers, insulin resistance, and improved gut barrier (via T regulatory cell induction in adipose tissue and NOD-like receptor pyrin domain containing 6 - NLRP6 - inflammasome assembly)	Preclinical in mice	(Everard et al. 2013; Schneeberger et al. 2015; Anhê et al. 2016)
	Negatively correlated with insulin intolerance	Case control studies	(Zhang et al. 2013)
	Improves metabolic profile and reduced fat mass (increases 2-OG, a bioactive lipid that stimulates the secretion of glucagon-like peptides through the activation of GPR119, via the endocannabinoid system)	Case control study Preclinical in mice	(Dao et al. 2016)
	Higher abundance of *A. muciniphila*-derived extracellular vesicles (AmEVs) in healthy control vs T2D patients. Reduced gut permeability in LPS-treated Caco-2 cells with AmEVs treatment.	Preclinical in mice	(Chelakkot et al. 2018)
Atherosclerosis	Ameliorates metabolic endotoxaemia-induced inflammation (via restoration of the gut barrier endotoxaemia); Reduction of the expression chemokines and the adhesion molecules MCP-1, TNFα, and ICAM-1, along with decreased aortic infiltration of macrophages	Preclinical in mice (ApoE-*KO* model)	(Li et al. 2016)

1.2.2. *Akkermansia muciniphila*: a peacekeeper

In the framework of gut health, the identification of microbiota community members and, more importantly, their role is crucial to better understand the dynamics that are at play to maintain such an ecosystem stable and healthy (Trosvik & de Muinck 2015). One undervalued concept in biology is that of keystone species, initially defined as a species critical for managing the diversity and organization of their ecological communities through biotic interactions with other community members (Paine 1969; Trosvik & de Muinck 2015). The distinct aspect of a keystone species is that considering its relatively low abundance; it has a disproportionately substantial effect on the community. Thus its removal has strong destabilizing impact, resulting in loss of biodiversity which represents a vulnerable point in an ecosystem (Stachowicz & Hay 1999). It should also be noted that a microorganism can even be considered as a keystone member if pivotal to an ecosystem by producing essential metabolites such as SCFAs that set off trophic cascades, strengthening its defenses against pathogenic species, aiding the establishment of beneficial species, and overall helping to preserve a balanced relationship with the host (Laforest-Lapointe & Arrieta 2017). Indeed, research suggests that the abundance of certain keystone species are responsible for the individuality of the human gut microbiome (Fisher & Mehta 2014). Even though this concept may seem not to entirely apply to *A. muciniphila* regarding its relative abundance, it provides a proper context to understand the reasons why these microorganisms are viewed as peacekeeping players and prime targets for maintenance of intestinal health through manipulation of the GI microbiota (Trosvik & de Muinck 2015).

Among keystone species commonly present in gut microbiota, *A. muciniphila* has been highlightned as a potential entrance point for novel diagnostic strategies and therapeutic modulation (El Hage et al. 2017) not only due to its bioactive properties but also due to interesting microbial cross-feeding dynamics and symbiotic relationships that occur at the intestinal mucus layer that support other species growth and maintaining the gut microbiome functioning as a whole unit (De Vuyst & Leroy 2011; Belzer et al. 2017). Cross feeding is a valuable microbial feature, where metabolic products produced, for example from the metabolism of dietary prebiotics by one species serve as a substrate for other species, allowing the retrieval of nutrients and energy through fermentation (Chassard & Lacroix 2013).

As a mucolytic microorganism *A. muciniphila* supplies sugars, via the degradation of complex glycans such as mucin, to butyrate-producing bacteria like *Eubacterium hallii* and *Anaerostipes caccae* (Belenguer et al. 2006; Schwab et al. 2017). Additionally, *A. muciniphila* mucin degradation activity, which also increases acetate and propionate

pool, can also benefit the butyrate-producing *Faecalibacterium prausnitzii* (Rios-Covian et al. 2015). Butyrate has several beneficial effects on the host, including the provision of an energy source for epithelial cells, induction of colonic regulatory T cells, induction of apoptosis in human colonic carcinoma cells, inhibition of inflammatory responses in intestinal biopsy specimens and improvement of metabolic syndrome (Reigstad et al. 2015; Yano et al. 2015; Brodmann et al. 2017). Furthermore, a vitamin B12-dependent syntrophy between *E. hallii* and *A. muciniphila* was observed, providing *A. muciniphila* a necessary cofactor for the production of propionate, benefiting host cell metabolism (Belzer et al. 2017). Overall, cross-feeding interactions evidence broadens our understanding in that modulation of a gut keystone species, such as *A. muciniphila*, impacts dramatically the intestinal microbial ecosystem and the associated host-microbiota equilibrium.

1.2.3. *Akkermansia muciniphila*: friend or foe?

In some cases, the mechanism by which this bacteria aids in the amelioration of metabolism and protects LPS endotoxaemia, namely the mucus turnover cycle (Derrien et al. 2004), can also contribute to the reduction of the gut barrier integrity and function (Sonoyama et al. 2010). Studies proposed that this action can lead to an increase in the uptake of allergenic proteins, such as ovalbumin, in the GI tract (Sonoyama et al. 2010). It is important to note, however, that no evidence was shown supporting that *A. muciniphila* alone imparts pathogenic characteristics. Comparatively, Zheng et al. (2016) demonstrated that the higher amounts of *A. muciniphila* and of pathogenic bacteria belonging to *Enterococcus* and *Shigella* genera, alongside with the decreased abundance of bacterial groups with anti-inflammatory action (e.g. *Bacteroides fragilis* and *Streptococcus salivarius*), may contribute to eczema in infants (Zheng et al. 2016). Moreover, the collapse of the gut barrier integrity can also lead to increased levels of inflammatory microbial associated-molecular patterns (MAMPs) in the blood circulation potentially contributing to neuro-inflammation (Derrien et al. 2017). Additionally, *A.muciniphila* was also positively correlated with colorectal cancer patients, with about 4-fold higher numbers versus the healthy subjects involved in the study (Weir et al. 2013). It is, however, important to mention that patients with cancer usually have decreased food intake, and studies showcase that fasting correlates with increased *A. muciniphila* levels (Remely et al. 2015). Furthermore, this type of cancer is related to increased mucus production and cell proliferation, which in turn can potentiate the boost in this mucus degrading bacterium abundance (Gómez-Gallego et al. 2016). Thus, it appears that the mucin degrading role of *A. muciniphila*, which can

be viewed as a typical pathogen-like behavior, is in fact regarded as a regular process in a self-renewing healthy intestine (Gómez-Gallego et al. 2016). Ultimately, even though the possible downside of its physiological behaviors still needs to be more addressed, the overwhelming current evidence correlating *A. muciniphila* and human health offers the possibility of not only using it as a near future therapeutic approach but also as a diagnostic and prognostic tool for the host's cardio-metabolic conditions.

1.2.4. *Akkermansia muciniphila*: modulation by diet

As previously mentioned, gut microbiota modulation can be achieved through diet. Based on its physiological influence on the host, *A. muciniphila* has been suggested as a prognostic tool to anticipate the success of dietary interventions. Dao et al. (2016) assessed several clinical parameters as well as *A. muciniphila* abundance before and after a 6-week calorie restriction period, followed by a stabilization diet. According to these researchers, it was shown that the higher abundance of *A. muciniphila* at baseline was linked with improvement in blood glucose homeostasis, lipid profile and body fat distribution after dietary intervention (Dao et al. 2016).

In the same way, the consumption of specific prebiotic compounds, defined as non-digestible food ingredients that pass through the upper gut and which are selectively fermented by colonic bacteria, can positively influence health through several mechanisms (Sarao & Arora 2017). Initially, it was demonstrated that prebiotic feeding resulted in improved metabolic syndrome associated conditions such as insulin sensitivity (increased GLP-1) and glucose homeostasis (Kok et al. 1998). These improvements were linked with a modulation of the gut microbiome composition and activity , mostly referring to *Bifidobacterium* spp. and *Lactobacillus* spp increased numbers and activities (Kapiki et al. 2007) but currently these benefits have been extended to other bacterial genera such as *Akkermansia*, *Eubacterium*, and *Faecalibacterium* (Deehan et al. 2017).

Several studies demonstrated that *A. muciniphila* is able to ferment human milk oligosaccharides mainly due to the chemical similarity of these glycans with mucin structure (Collado et al. 2012; Ward et al. 2013; Petschacher & Nidetzky 2016) Furthermore, this bacterium is capable of foraging specific dietary components including prebiotic compounds (Everard et al. 2011) and polyphenols (Roopchand et al. 2015; Anhê et al. 2016) to provide continuous benefits to the host. Among the many prebiotics, dietary fructan inulin and its breakdown product fructo-oligosaccharide (FOS) are particularly well-studied, and evidence supporting their health-promoting

effects, specifically their influence on host gut microbiota is accumulating quickly (Sarao & Arora 2017).

It has been demonstrated that inulin promotes *A. muciniphila* growth, while improving metabolic disorders associated with obesity, including reduction of fat mass and insulin resistance, lower liver steatosis and the gut barrier reinforcement (Van-den-Abbeele et al. 2011; Everard et al. 2011; Cani & Everard 2014; Greer et al. 2016) However, the specific modulation effect of inulin on *A. muciniphila's* abundance appears to be due to an indirect side effect of its activity and not because of its direct use (Van Herreweghen et al. 2017). Inulin fermentation by gut resident strains lowers the pH (Van-den-Abbeele et al. 2011) which, in turn stimulates colonic mucus production (Ten Bruggencate et al. 2004); this relationship is based on the observation that colonization correlates with pH and mucin content, but not with inulin concentration (Van Herreweghen et al. 2017). Thus, it is fair to state that by increasing mucin secretion and maintaining pH homeostasis, inulin consequently promotes the growth of *A. muciniphila*.

FOS is another commonly studied prebiotic that seems to possess similar *A. muciniphila*-promoting activities. In recent *in vivo* studies, supplementation with FOS was found to have a significant prebiotic effect, in particular, demonstrating an increase of *A. muciniphila* abundance and, this increase was accompanied by fat mass reduction and improved glucose control (Everard et al. 2011; Everard et al. 2013; Burokas et al. 2017). However, FOS mechanistic modulation upon the ecology of *A. muciniphila* is still not well understood (Zhou 2017).

Dietary polyphenols are other bioactive compounds that seem to exert prebiotic-like effects on *A. muciniphila*. As natural antioxidants and antimicrobials compounds, polyphenols may create a selective pressure on the intestinal lumen by scavenging free oxygen radicals, favoring anaerobic species like *A. muciniphila* (Anhê et al. 2016; Daglia 2012; Roopchand et al. 2015). Although there is some evidence that dietary polyphenols do affect positively this bacterium abundance, inconsistent results suggest that the *A. muciniphila*-promoting effects are dependent on polyphenols chemical nature and sources (Roopchand et al. 2015; Li et al. 2015; Anhê et al. 2016)..

Overall a more systematic analysis of which specific prebiotics confer positive impact on *A. muciniphila* growth and stability and, by which mechanisms such modulations occur, should be investigated, since it seems to be a more passive or indirect approach. In particular, changes in diet through the introduction of compounds that possess mucin-like structures or prebiotic substrates, as inulin-type fructans and FOS, that stimulate mucus secretion in the gut, seem to provide a stable colonization niche (Moens et al. 2016), which translates into a passive strategy to be implemented in

future dietary approaches or even in probiotic technological applications, such as the inclusion in formulation matrices to support cell viability and stability.

1.3. Strategies for new probiotic carriers

Since modulation of the gut microbiota has demonstrated promising outcomes in the treatment and prevention of several disorders, introducing potential NGPs, such as *A. muciniphila*, in the pharmaceutical and food nutraceutical markets seems an interesting strategy for the prevention and treatment of such dysbiosis-associated diseases (O'Toole et al. 2017). However, in contrast to the fairly oxygen-tolerant probiotics that are currently commercialized, *A. muciniphila´*s high sensitivity to oxygen, requires new and adequate approaches to standardized experimental protocols limiting strain to ambient oxygen exposure.

To ensure oxygen exclusion from processes such as formulation and freeze-drying, strategies using the incorporation of antioxidants for redox potential reduction (Sousa et al. 2012) or the physical protection of the strain by means of encapsulation (Sousa et al. 2015) have been evidenced as useful efforts to promote probiotic viability as well as functional stability (O'Toole & Paoli 2017). The incorporation of protective agents, such as prebiotics, into bacterial formulations appears to positively impact bacterial viability, especially in commonly used processes such as freeze-drying and storage (Sarao & Arora 2017). To date, the most widely used protective agents showing satisfactory impact on cell viability and stability, include: inulin (Mensink et al. 2015), maltodextrin (Fernandes et al. 2014) and trehalose (Fowler & Toner 2005). Bacteria with particular oxygen sensitivity can also be protected against oxygen toxicity during storage, by the addition of oxygen scavengers (e.g., cysteine, glutathione, and ascorbate)(Ross et al. 2005) and through co-culture with oxygen-consuming species such as *Bifidobacterium* spp, *Streptococcus thermophilus* and yeasts (Xie et al. 2012; Ma et al. 2015), reducing oxygen content in the formulation matrix, thereby improving probiotic viability.

Another approach considered to enhance microbial robustness against not only oxygen but other factors such as pH and temperature, is the application of sub-lethal stress treatments which allow the activation of metabolic pathways that permit the re-adaptation to the aforementioned elements, in addition to other processes (freeze-drying and storage), improving the survival during industrial formulation and ultimately in the gut (Borges et al. 2012; Nguyen et al. 2016).

The viability and stability of bacterial cells can be further improved through encapsulation (Šipailienė & Petraitytė 2018). Besides offering a physical barrier against the external environment (storage temperatures and oxygen content), encapsulation also provides a controlled release into the gut lumen, which is particularly important

when it comes to ensuring survival during the passage through the GI system, mainly due to pH values (Martín et al. 2015; Anal & Singh 2007). One of the most widely used probiotic encapsulation agents is alginate, which has been linked to an increase in bacterial survival of up to 95%, depending on the species and strain (Mandal et al. 2006; Lopes et al. 2017). Alginate is a natural occurring heteropolysaccharide, comprised of β-$_D$-mannuronic acid and α-$_L$-guluronic acid (Gacesa 1988), widely used in encapsulation methods due to its low immunogenicity and good biocompatibility (Fang et al. 2007) and its resistance to pH (3 to 7) and enzymatic degradation (Klayraung et al. 2009). Within the various reported encapsulation methods, spray drying (Rodrigues et al. 2011), extrusion/external gelation (Rodrigues et al. 2012) and emulsification by internal gelation (Holkem et al. 2016) are the most well documented. Choice of the most favorable probiotic encapsulation methodology should evaluate the materials and conditions to be used before application. Despite the low cost and elevated production rate, spray drying involves exposure to elevated temperatures and high osmotic pressure, that can lead to a decrease in viability (Rajam & Anandharamakrishnan 2015). Encapsulation by extrusion/external gelation is a simpler method that, even though it enables high cell viability, it produces capsules larger than 500 μm, which can ultimately negatively impact sensorial analyses, impeding successful incorporation into food products (Prisco et al. 2015). Internal gelation, on the other hand, offers the possibility to produce smaller capsules (<100 μm), using a gentler method that is highly efficient in cell viability protection and is more affordable at a laboratory scale, which makes it as one the most promising encapsulation techniques for future applications (Šipailienė & Petraitytė 2018). Both emulsification techniques produce capsules from a water-in-oil (W/O) dispersion (Poncelet et al. 1992; Poncelet et al. 1995), although internal gelation emerged as an alternative to external gelation since it overcomes the difficulty in dispersing calcium chloride ($CaCl_2$; Ca^{2+} source) in the oil phase, which ultimately produces clumped capsules (Poncelet et al. 1995). In this manner, acidification allows the release of Ca^{2+} from an insoluble calcium salt (usually calcium carbonate – $CaCO_3$), which interacts with alginate forming an “egg-box”-like structure, hence producing microcapsules (Poncelet et al. 1992; Poncelet et al. 1995).

Ideally, the combination of different strategies of probiotic delivery systems might offer increased effectiveness in providing sufficient quantities of bacteria to guarantee health benefits for the patient. According to our best of knowledge, only two studies approached the issue of delivery systems for *A. muciniphila*. Van der Ark and colleagues (2017) encapsulated *A. muciniphila* in a double emulsion having achieved a high viability during *in vitro* gastric passage, and Marcial-Coba et al (2018) by extrusion

method reporting acceptable storage stability. The lack of strategies for adequate delivery systems or carriers involving *A. muciniphila* reveals an urgent need for research studies targeting new approaches using proper chemical agents and materials that offer probiotics protection against GI conditions and simultaneously promote their viability and stability during storage.

1.4. Thesis aim

Keystone species *A. muciniphila* demonstrates a great potential in the prevention and treatment of dysbiosis-associated diseases. Given its sensitivity to oxygen, preservation strategies are paramount for the introduction of this NGP into the health market. The protective effects of some technological solutions for enhancing viability and stability of probiotics, such as *Bifidobacterium* spp, *Lactobacillus* spp and some yeasts, upon harsh GI and storage conditions, have been shown. Based on the above rationale, the main goal of the present work was the design and study of technological strategies capable of accomplishing a protective impact on *A. muciniphila* when exposed to detrimental GI conditions as well as to storage at ambient air.

Considering the main objective of the work, the study was divided into two parts with different specific objectives:

Part I – Phenotypic characterization of *A. muciniphila* DSM22959 through study of several physiological properties:

i) Study of growth and death kinetics of *A. muciniphila* DSM22959 exposed to different conditions;

ii) Evaluation of *A. muciniphila* viability and stability during exposure to simulated GI transit conditions;

iii) Evaluation of *A. muciniphila* ability to adhere to *in vitro* intestinal epithelium models.

Part II – Design and study of technological strategies based on freeze-dried formulations and encapsulation.

iv) Design, preparation and freeze-drying of formulations incorporating *A. muciniphila* therein and evaluation of viability and stability of formulated bacteria under simulated GI transit and throughout aerobic storage under different conditions.

v) Encapsulation of *A. muciniphila* via emulsification/internal gelation method and evaluation of viability and stability of encapsulated bacteria under simulated GI transit and throughout aerobic storage under different conditions.

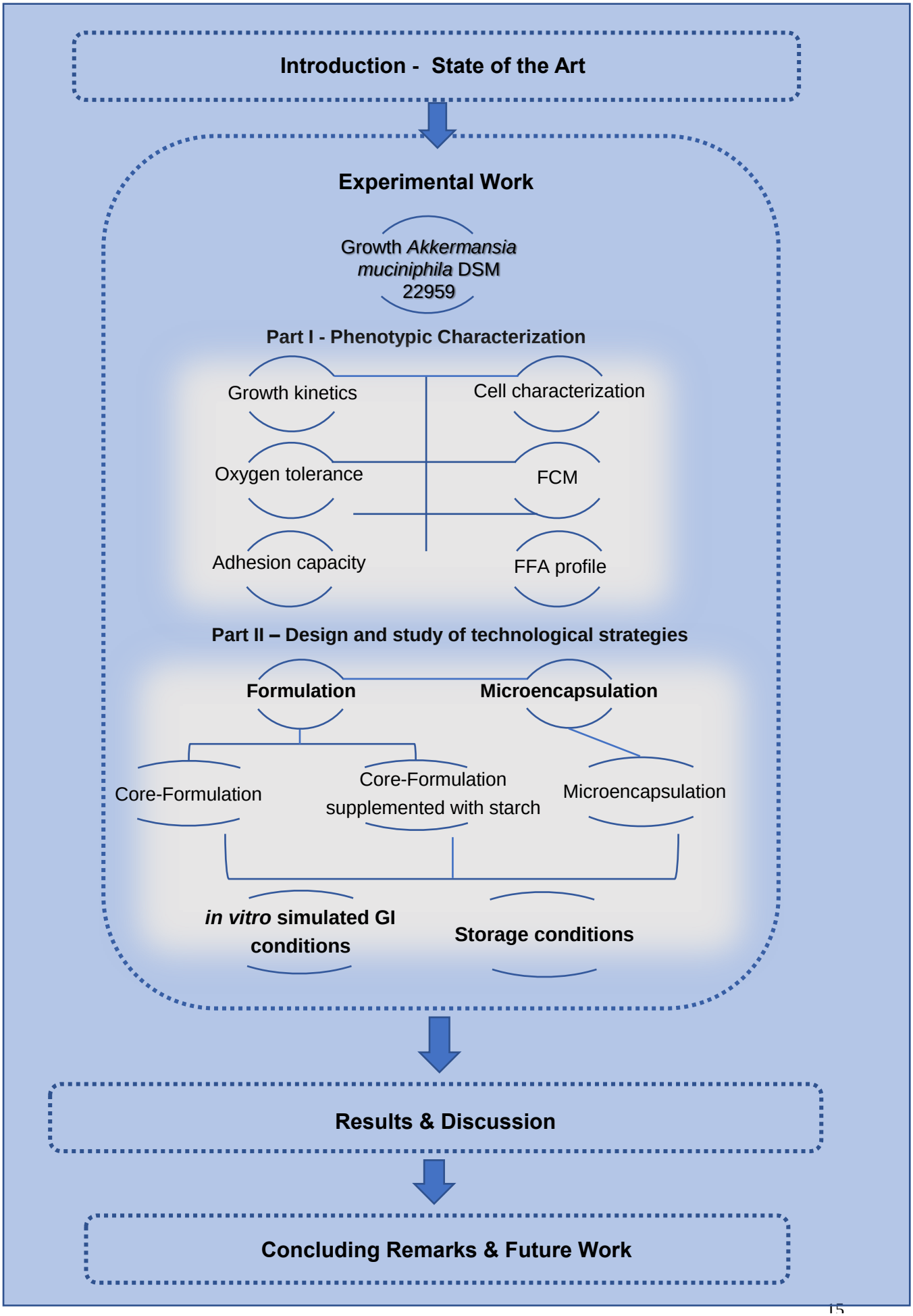

Figure 1.2 - Schematic flow chart of thesis outline.

2. Material and Methods

2.1. *Phenotypic characterization of A. muciniphila DSM22959*

2.1.1. *Akkermansia muciniphila* cultivation under reference conditions

Akkermansia muciniphila strain DSM 22959 from DSMZ collection (Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures, Germany) was used in the present work. The bacterial strain was kept at -80 ºC in PYGM broth (Table 2.1) with 20 % (v/v) glycerol (Fisher Chemical, UK) until phenotypic analyses and technological studies, based on freeze-dried formulations and encapsulation, were performed.

For each analysis and study, *A. muciniphila* DSM 22959 was reactivated from the frozen state in PYGM broth, in Hungate culture tubes sealed with butyl rubber septa (Bellco Glass, USA), incubated for 17±2 h at 37 °C under anaerobic atmosphere (80 % N_2, 10 % H_2 and 10 % CO_2) in an anaerobic chamber (Whitley DG250 Anaerobic Workstation, UK), with at least two subsequent culturing steps at the same growth conditions.

The PYGM broth was prepared as described in table 2.1 with pH adjusted to 7.2 using 8 N NaOH (Labor Spirit, Portugal). PYGM agar was prepared with addition of 1.5 % (w/v) agar (Liofilchem, Italy). Both PYGM broth and PYGM agar media were then autoclaved at 121 ºC for 20 min. Upon sterilization, the absence of oxygen in the media was verified by its color; if anaerobic conditions prevail a brown color is achieved, if exposed to aerobic atmosphere, the medium becomes pink.

Table 2.1 - Composition of Akkermansia muciniphila reference growth medium (PYGM broth) according to DSMZ.

Ingredients	Concentration	Manufacturer
Trypticase peptone	5 $g.L^{-1}$	VWR Chemicals, EC
Peptone	5 $g.L^{-1}$	VWR Chemicals, Belgium
Yeast extract	10 $g.L^{-1}$	VWR Chemicals, Belgium
Beef extract	5 $g.L^{-1}$	VWR Chemicals, India
Glucose	5 $g.L^{-1}$	Sigma-Aldrich, USA
Mucin	0,5 $g.L^{-1}$	Sigma-Aldrich, USA
K_2HPO_4	2 $g.L^{-1}$	Sigma-Aldrich, USA
Tween 80	1 $mL.L^{-1}$	VWR Chemicals, France
Cystein-HCl	0,5 L^{-1}	Alfa Aesar, Germany
Resazurin	1 $mg.L^{-1}$	Sigma-Aldrich, USA
Salt Solution [1]	40 $mL.L^{-1}$	NA
Hemin Solution [2]	10 $mL.L^{-1}$	NA
Vitamin K_1 Solution [3]	200 $\mu L.L^{-1}$	NA
dH_2O	0,95 L	NA

[1] Salt solution composition according to table 2.2.
[2] Hemin solution: 50 mg of hemin (Alfa Aesar, Germany) in 1 mL of 1 N of NaOH and diluted into 100 mL of dH_2O.
[3] Vitamin K_1: 0.1 mL of vitamin K_1 (Sigma-Aldrich, USA) in 20 mL of 95 % (v/v) ethanol filtered by 0.22 µm sterile filter (Millipore, USA).
NA – Not applicable

Table 2.2 - Composition of salt solution included in Akkermansia muciniphila reference growth medium according to DSMZ.

Ingredients	Concentration	Manufacturer
$CaCl_2.2H_2O$	0.25 $g.L^{-1}$	Carlo Erba Reagents, Italy
$MgSO_4 \cdot 7H_2O$	0,5 $g.L^{-1}$	Merck, Germany
K_2HPO_4	1 $g.L^{-1}$	Sigma-Aldrich, USA
KH_2PO_4	1 $g.L^{-1}$	Sigma-Aldrich, USA
$NaHCO_3$	10 $g.L^{-1}$	Merck, Germany
NaCl	2 $g.L^{-1}$	Labchem, USA
dH_2O	1 L	NA

2.1.2. Growth kinetics and cellular characterization

Determination of growth kinetics. To obtain the growth curves, two replicas were performed. For each replica, 45 mL of PYGM broth was inoculated with 5 mL of *A.*

muciniphila grown according to procedures described in section 2.1.1 and incubated at 37 ºC under anaerobic conditions (80 % N_2, 10 % H_2 and 10 % CO_2) for 24 h. Growth curves were determined by measuring optical density (OD) at 600 nm in a spectrophotometer (UV mini-1240 Shimadzu, Japan), throughout time and simultaneously by quantification of colony-forming units (CFU) of *A. muciniphila* in some sampling points. For viable cell numbers quantification, ten-fold serial dilutions of bacterial suspension were performed in phosphate buffer saline (PBS x1; VWR, USA). Then, 10 µL of each dilution was spotted, in triplicate, on PYGM agar plates subsequently incubated for 7 d at 37 ºC under anaerobic conditions, and results were expressed as CFU per milliliter.

Cellular morphology and Gram stain. The cellular morphology and Gram test of 17±2 h grown *A. muciniphila* cells were determined after Gram staining as described by Bartholomew & Mittwer (1952) and examination by optical microscopy at 100x magnification.

2.1.3. Oxygen tolerance

To assess *A. muciniphila* tolerance to oxygen, fully grown cultures at the early stages of stationary phase (17 ±2 h growth) were exposed to ambient air atmosphere at 37 ºC with and without agitation (140 rpm) over a period of 60 h. A positive growth control was included and consisted in bacterial growth at 37 ºC under anaerobic conditions as described in section 2.1.1. For each sampling point, the total viable cell numbers of *A. muciniphila,* grown in each atmosphere, were determined by plating ten-fold serial dilutions of bacterial suspension, in triplicate, on PYGM agar plates according to procedures described in 2.1.2. Two replicas were performed for each atmosphere condition tested.

2.1.4. Adhesion assays to intestinal epithelium cells

2.1.4.1. Epithelial Cell Lines

The caucasian colon adenocarcinoma (Caco-2) and mucus secreting human colon adenocarcinoma (HT29-MTX-E12) cells were obtained from the European Collection of Authenticated Cells Cultures (ECACC 8601020 and 12040401, respectively) through Sigma-Aldrich (ECACC, USA) (references 09042001 and 12040401, correspondingly). The cell lines were grown at 37 °C under a humidified atmosphere (95%) in an incubator supplemented with 5 % CO_2. Except when stated otherwise, the cells were grown using high glucose (4.5 % (w/v)) Dulbecco's Modified Eagle's Medium (DMEM;

Lonza, Basel, Switzerland) supplemented with 10 % (v/v) heat inactivated Fetal Bovine Serum (FBS; Biowest, France), 1 % (v/v) Pen-Strep (Lonza, Basel, Switzerland) and 1 % (v/v) of non-essential amino acids (100x stock solution) (Lonza, Basel, Switzerland).

2.1.4.2. Adhesion assessment of *A. muciniphila*

To assess *A. muciniphila* DSM22959 capacity to adhere to intestinal cells, *A. muciniphila* cells (17±2 h growth) were recovered by centrifugation at 5000 rpm for 20 min, washed once with sterile PBS and suspended to a concentration of 10^6 CFU mL^{-1} in DMEM medium. Short and long adhesion assays of *A.muciniphila* to Caco-2 and HT29-MTX intestinal cells were performed according to Veiga (2018) as follows: aliquots of 100 µL of a cell suspension (10^6 cells mL^{-1}) were seeded in 96 well microplates (Nucleon Delta Surface, Thermo Scientific, Denmark) and after 24 h incubation, the medium was replaced with a 1:1 mixture of medium and bacteria, with three replicates for each intestinal cell model per time point. The mixture was left in contact with the intestinal cells for 15, 30 and 60 min, for the short-term assays and, 60, 120, 180 and 240 min, for the long-term assays, at 37 °C under a humidified atmosphere in an incubator supplemented with 5% CO_2. After these periods, the total viable cell numbers of *A. muciniphila* were determined by plating ten-fold serial dilutions of bacterial suspension, in triplicate, on PYGM agar plates according to procedures described in 2.1.2. The relative adhesion % (defined as the viable cells (log CFU mL^{-1}) at the sample point in comparison to the viable cells (log CFU mL^{-1} in the adhesion inoculum) was calculated at each timepoint.

2.1.5. Cell membrane lipid extraction and fatty acids methyl esters (FAME) analysis

Culture conditions. *Akkermansia muciniphila* DSM22959 suspension was prepared as aforementioned (section 2.1.1). The bacterial pellets were collected after centrifugation at 5000 rpm for 20 min at 4 ºC and washed twice with 1 mL of sterile PBS. Afterwards, the resultant pellets were stored at -80 ºC for further analysis.

Fatty acids derivatization and analysis. Three bacterial pellets (replicas) were derivatized according to the previously described method by Pimentel et al. (2015). Briefly, a 50 mg of pellet was added with 100 µL of tritridecanoin (Nu-Chek Prep, USA; 1.64 mg/mL) prior to derivatization. Then 2.26 mL of methanol (VWR Chemicals, USA) were added, followed by 1 mL of hexane (VWR Chemicals, USA) and 240 µL of sodium methoxide (Sigma-Aldrich, USA). Samples were vortexed and incubated at 80

ºC for 10 min. After cooling in ice, 1.25 mL of dimethylformamide (VWR Chemicals, USA) and 1.25 mL of sulfuric acid (VWR Chemicals, USA) were added. Samples were vortexed and incubated at 60 ºC for 30 min. Upon cooling, samples were vortexed and centrifuged (1250 g; 18 ºC; 5 min.) and the upper layer containing methyl esters (FAME) was collected for further analysis. All experiments were carried out in triplicate.

Chromatography conditions. FAMEs were analyzed in a gas chromatograph HP6890A (Hewlett-Packard, USA), equipped with a flame-ionization detector (GLC-FID) and a BPX70 capillary column (60 m × 0.32 mm × 0.25 µm; SGE Europe Ltd, France), according to conditions previously described (Fontes et al. 2018). In short, analysis conditions were as follows: injector temperature 250 °C, split 25:1, injection volume 1 µL; detector (FID) temperature 275 °C; flow rate of 1 mL/min. The oven temperature was initially at 60 ºC and then increased to a final temperature of 225 ºC. Supelco 37, FAME mix (Sigma-Aldrich, USA) were used for identification of fatty acids. GLC-Nestlé36 (Nu-Chek Prep, USA) was assayed for calculation of response factors and detection and quantification limits (limits of detection and quantification were 0.79 ng FA/mL and 2.64 ng FA/mL, respectively).

2.1.6. EOS bacterial enumeration using Flow Cytometry

For preliminary assays, cells of *A. muciniphila* were grown in hungate tubes according to reference conditions (2.1.1). Then, each bacterial suspension was diluted at least 10x in staining buffer to a concentration between 10^6 to 10^7 bacteria mL^{-1}. Staining buffer was prepared using sterile PBS as diluent, EDTA at 1 mM (Sigma-Aldrich, MO), 0.1 % (w/v) sodium azide (Sigma-Aldrich, MO) and 0.01 % (v/v) Tween 20 (Sigma-Aldrich, MO). FCM analysis was performed with a BD Accuri™ C6 flow cytometer. For staining, 500 µL *A. muciniphila* previously diluted was incubated for 5 min with 5 µL of thiazole orange (TO) and 5 µL propidium iodide (PI) in a polystyrene tube at 20 ºC ± 0.5. For FCM analysis, *A. muciniphila* cells were gated using forward-scatter (threshold = 500, FSC) and side-scatter (threshold = 200, SSC). Since TO fluoresces mainly in FL1 (detects 533/30 nm laser) and PI fluoresces mainly in FL3 (detects >670 nm laser), for best discrimination between live and dead populations, FL1 vs FL3 (green vs red fluorescence) detector positions were used to quantify the number of events (cells) for each population. Resulting data was treated with BD AccuriTM C6 Software (V. 1.0.264.21).

2.2. *Design and study of technological strategies*

2.2.1. Design and preparation of freeze-dried formulations incorporating *A. muciniphila*

Akkermansia muciniphila suspension was prepared as aforementioned (section 2.1.1). To determine the effect of the selected prebiotic agents inulin and fructooligossacharides (FOS) on *A. muciniphila's* growth, in the last sub-culturing step 30 mL of PYGM broth and 30 mL of PYGM broth supplemented with 2.5 % w/v inulin (Orafti®, Beneo, Germany) or with 2.5 % FOS (Orafti®, Beneo, Germany) were inoculated with 3 mL of *A. muciniphila* suspension. The three inoculated media, replicated twice, were then incubated at 37 ºC for 17±2 h under anaerobic conditions. Optical density measurements at 600 nm were performed for each medium upon incubation coupled with determination of total viable cell numbers of *A. muciniphila* by plating in triplicate on PYGM agar plates according to procedures described in 2.1.2.

The technological strategy considering freeze-dried formulations was based on the procedures described by Khan et. al (2014), with adaptations. Firstly, *A. muciniphila* cells, after growing according to procedures described in 2.1.1, were harvested by centrifugation at 5000 rpm for 20 min at 4 ºC and washed once with sterile PBS. The resultant bacterial pellets were then weighed and re-suspended in a designated core-formulation composed of 200 µL of a 16.5 mM riboflavin (Sigma-Aldrich, MO) solution in PBS and 400 µL of 10% (w/v) inulin solution with or without 0.2% (w/v) cysteine-HCl or glutathione (VWR International, USA), in order to evaluate the effect of each of these antioxidant agents on the freeze-dried formulation. In addition, and to evaluate the role of starch as a bulking agent, and as a possible growth enhancer/stabilizer, re-suspension of the obtained bacterial pellets was performed with core-formulation supplemented with 0.5 g of starch (Fluka, Germany). For each formulation 15-20 replicas were performed in order to characterize their impact on viability and stability of *Akkermansia muciniphila* during storage and when submitted to GI conditions as described in the following section

All the formulations incorporating *A. muciniphila* were homogenized by vortexing and then frozen at -80 ºC. Frozen samples were freeze-dried for 24 h using a freeze-drier (Christ, Germany) and stored until further characterization of properties.

2.2.1.1. Survival of *A. muciniphila* cells through simulated gastrointestinal: cells in PYGM broth vs incorporated cells in freeze-dried formulations

The potential protective impact of the formulation, and subsequent freeze-drying, on *A. muciniphila* cells survival capacity during passage through simulated GI conditions was performed in comparison to cells in PYGM broth behavior under the same conditions. The cells in PYGM broth obtained from the bacterial growth suspensions used in the formulation procedure (2.2.1), were distributed (45 mL) in sterile falcons and stored at 4ºC. Two replicas of *A. muciniphila* cells in PYGM broth or formulation-incorporated cells were prepared and analyzed at each sampling time, namely upon freeze-drying process and after 7 d of storage at 4 ºC. For each replica, either 1 mL of cells in PYGM broth or 1 gram of freeze-dried formulation containing *A. muciniphila*, were exposed to simulated GI conditions according to the procedure described by Minekus et al. (2014), with minor modifications The typical conditions prevailing in the mouth, esophagus-stomach and duodenum-ileum were chemically simulated as described in table 2.3.

Table 2.3 - Composition of electrolyte stock solutions for each phase of the GI protocol according to Minekus et.al. (2014).

Simulated solutions *		**SSF**	**SGF**	**SIF**
		pH (7)	pH (3)	pH (7)
Chemical Compound	Stock Concentration	mmol L^{-1}	mmol L^{-1}	mmol L^{-1}
KCl	37.3 $g.L^{-1}$	15.1	6.9	6.8
KH_2PO_4	68 $g.L^{-1}$	3.7	0.9	0.8
$NaHCO_3$	84 $g.L^{-1}$	13.6	25	85
$MgCl_2.6H_2O$	30.5 $g.L^{-1}$	0.15	0.1	0.33
$(NH_4)_2.CO_3$	48 $g.L^{-1}$	0.06	0.5	-
NaCl	117 $g.L^{-1}$	-	47.2	38.4
$CaCl_2$	44.1 $g.L^{-1}$	1.5	0.15	0.6[§]

[§] *Simulated salivary fluid (SSF), simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) are comprised of the respective fluids, enzymes, and water. The enzymes, bile salts, and $CaCl_2$ were not added directly to the simulated fluids but to the final mixture of the digestive solution

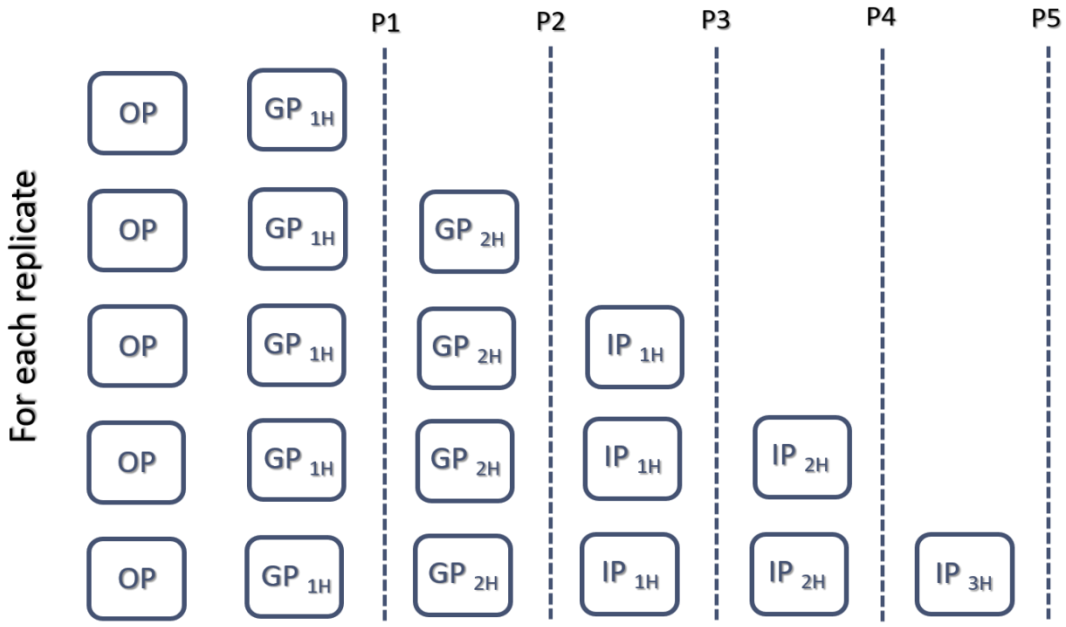

Figure 2.1 - Schematic representation of in vitro GI procedure based on Minekus et al.(2014) *with some modifications. Abbreviations: OP, Oral Phase; GP, Gastric Phase; IP, Intestinal Phase; P1, 60 minutes into gastric phase; P2, 120 minutes into gastric phase;*

For each experiment, all enzyme solutions were freshly prepared. To replicate the temperature and peristaltic movements that prevail during human gastrointestinal transit an orbital shaker incubator (Wiggen Hauser, Germany) was used at 37 ºC at 140 rpm. In the oral phase (OP), each sample was exposed to 2 mL of SSF, with pH adjusted to 7, for 2 min. For the esophagus-stomach step (gastric phase, GP), samples were exposed to 4 mL of SGF with pepsin (2000 U/mL – from porcine gastric mucosa; Sigma Aldrich, USA) for 120 minutes and, pH was adjusted to 3 using 6 M HCl. Finally, intestinal conditions (intestinal phase, IP) were simulated for an additional 180 min with 8 mL of SIF comprised of pancreatin (based on the trypsin activity at 100 U mL^{-1} in the final mixture; Sigma Aldrich, USA) and bile salts (10 mM; Oxoid, IE) and pH was adjusted at 7 with 6 M HCl. Sampling points were selected in order to follow the simulated gastric and intestinal effects and those were: upon 60 (P1) and 120 (P2) min in the gastric phase and upon 60 (P3), 120 (P4) and 180 (P5) min in the intestinal phase (Figure 2.1). At each pre-defined sampling point, the total viable cell numbers of *A. muciniphila,* in both PYGM broth and freeze-dried formulation-incorporated forms, were determined by plating, in triplicate, on PYGM agar plates according to procedures described in 2.1.2. Total viable cell numbers that survived the simulated GI conditions were expressed as CFU mL^{-1} or CFU g^{-1}, for the *A. muciniphila* cells in PYGM broth and freeze-dried formulation-incorporated cells, respectively. The GI protocol was

performed under aerobic conditions, whilst the enumeration of viable cells steps, were performed under anaerobic conditions.

2.2.1.2. Survival of *A. muciniphila* cells throughout storage: cells in PYGM broth vs incorporated cells in freeze-dried formulations

The evaluation of the impact of the formulation, and subsequent freeze-drying, on *A. muciniphila* cells viability throughout different storage conditions was performed in comparison to untreated cells in PYGM broth behavior submitted to similar conditions. Both *A. muciniphila* cells in PYGM broth and freeze-dried formulation powders were stored at -20 ºC, 4 ºC, and room temperature up to 7 d in an aerobic environment. At each pre-defined sampling point (0 and 7 d), the total viable cell numbers of both cells in PYGM broth or freeze-dried formulation-incorporated forms were determined by plating, in triplicate, on PYGM agar plates according to procedures described in 2.1.2. Results were expressed as CFU mL^{-1} or CFU g^{-1}, for the cells in PYGM broth and freeze-dried formulation-incorporated forms, respectively.

2.2.2. Establishment of novel microencapsulation strategy to increase the viability/stability of EOS and microaerophilic bacteria

2.2.2.1. Solutions used in emulsification/internal gelation microencapsulation

Denatured whey protein isolate (DWPI, 10 % w/w) solution was prepared as previously described (Hébrard et al. 2010). Briefly, whey protein isolate (WPI) powder was rehydrated in dH_2O by gentle magnetic stirring for 1 h at room temperature (22 ºC) and then allowed to rest for 2 h to ensure complete hydration of proteins. WPI solution was adjusted at pH 7.0 with NaOH 6 M, heated and maintained at 80 ºC for 40 min to denature proteins completely (DWPI). The solutions were cooled overnight at room temperature. Sodium alginate (Na-alginate) solution (4.0 % w/w; Sigma-Aldrich, USA) was prepared in sterile dH_2O and stirred gently overnight at room temperature Both Na-alginate and DWPI solutions were supplemented with 0.5 g L^{-1} of L-cysteine–HCl. Ultrafine $CaCO_3$ (Omya, Switzerland; 500 mM Ca^{2+} equivalents), and $CaCl_2$ (0.05 M) solutions were prepared in sterile dH_2O. Phosphate buffer (PB) 0.1 M was prepared by adding 12.67 g of $Na_2HPO_4.2H_2O$ (Sigma-Aldrich, USA) and 1.95 g of $NaH_2PO_4.H_2O$ (Merck, Germany) to 500 mL of sterile dH_2O and, adjusting pH to 7.5.

2.2.2.2. Inoculum preparation

Akkermansia muciniphila suspensions were prepared as aforementioned in section 2.1.1 and 2.2.1 respectively but, at the final sub-culturing step, cultures were inoculated in 200 mL of fresh media at 5 % (v/v), and then incubated at 37 ºC under anaerobic conditions. Cells were then harvested by centrifuging at 5000 rpm for 20 min at 4 ºC and washed once in sterile PBS. Biomass pellets were then re-suspended in 10 mL of 0.85 % (w/v) NaCl solution for microencapsulation procedure.

2.2.2.3. Microencapsulation by emulsification/internal gelation method

The emulsification/internal gelation technique used to produce alginate microparticles was based on Poncelet *et al*. (1992) but with modifications supported by published research works of Zou et al. (2011) and Holkem et al. (2016) in order to encapsulate *A. muciniphila* (Figure 2.3). All the mixing stages of the method were performed with a mechanical head stirrer with a three-blade impeller. The continuous phase, composed of 60 mL of vegetable oil and 1 mL surfactant Span 80 (Sigma-Aldrich, USA), was transferred to a glassware reactor and dispersed for 5 min at 150 rpm. The discontinuous phase was prepared by mixing 20 mL of Na-alginate (4% w/v), 10 mL of bacterial suspension, 10 mL DWPI solution (10 % w/v) and 2.8 mL $CaCO_3$ in a syringe. The content was carefully homogenized and added to the continuous phase in the glassware reactor and mixed for 15 min at 300 rpm to form a uniform water-in-oil-emulsion. Subsequently, a hydrophobic mix constituted of 20 mL of vegetable oil and 1.5 mL glacial acetic acid (Fisher Scientific, UK) was added in a dropwise fashion with continuous stirring at 300 rpm in order to have the emulsion initiate internal gelation by acidification. After the dropwise addition, the stirring came to a standstill, and the resulting microcapsules were solidified by adding 0.05 M $CaCl_2$ solution. The phases were allowed to separate, and, after complete deposition of microcapsules, the supernatant was discarded, and the step repeated to ensure complete solidification. A second washing process was carried out twice with $CaCl_2$ solution (0.05 M) prepared in 0.5% (v/v) Tween 80. Finally, the microcapsules were re-suspended in 0.85 % (w/v) NaCl and harvested upon centrifugation for 5 min at 1100 rpm. One gram of the resulting microcapsules was used to assess encapsulation efficiency (% EE – see section 2.2.2.6) and the remainder microcapsules were stored at 4 ºC under aerobic conditions until further analysis.

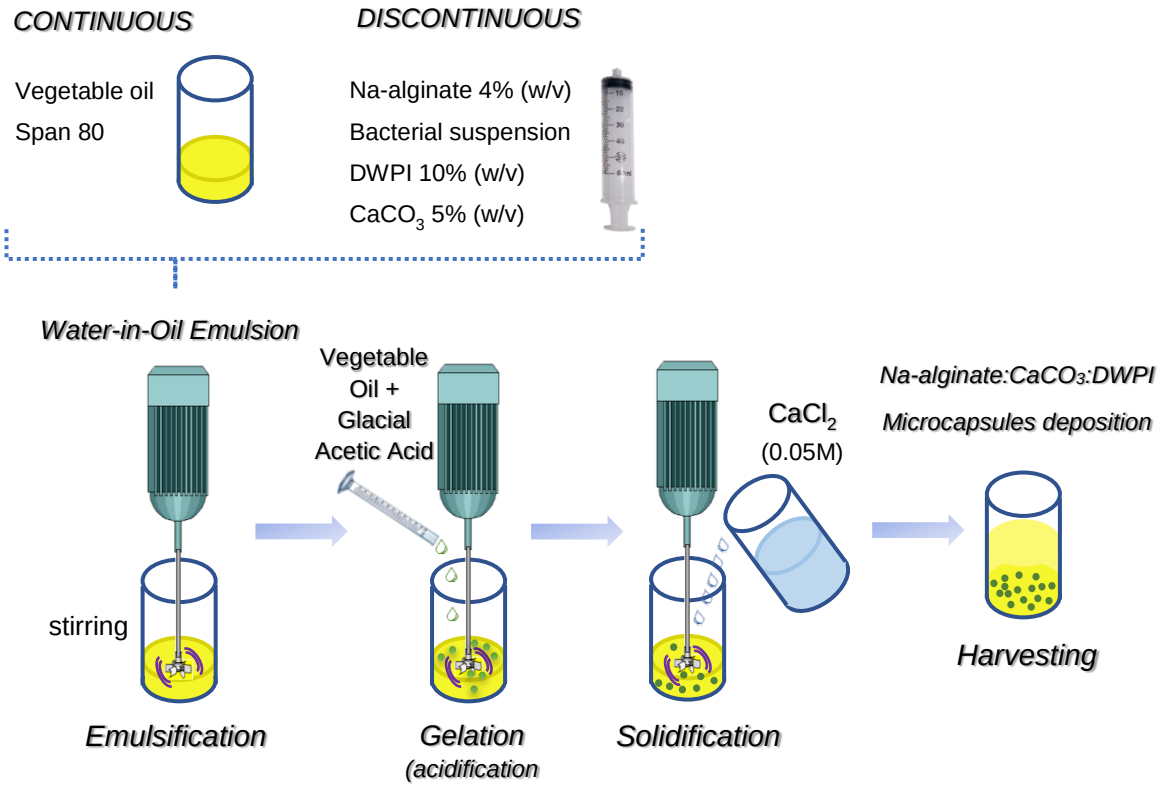

Figure 2.2 - Schematic overview of microencapsulation of Akkermansia muciniphila by emulsification/internal gelation method.

2.2.2.4. Microscopic examination of Na-alginate:$CaCO_3$:DWPI microcapsules

Microcapsules characterization was achieved by morphology and size determination. After microencapsulation, optical images were obtained using an inverted microscope (Nikon Eclipse, TE2000U, EU) equipped with a digital camera (Nikon, DXM1200F, EU), using ACT-1 software (Nikon) to take digital images. Microcapsule size was measured using ImageJ program, version 1.52a (https://imagej.nih.gov/ij/), resulting from average of 15 individual measurements.

2.2.2.5. Enumeration of microencapsulated *A. muciniphila* cells

Encapsulated *A. muciniphila* cells were released from the capsules according to the methods of Sheu and Marshall (1993) with minor modifications. Briefly, 1 g of microcapsules was re-suspended in 9 mL of sterile PB (0.1 M, pH 7.5) and placed in a stomacher at 230 rpm for 7 min, in order to disintegrate the capsules. The successful release of *A. muciniphila* cells was confirmed by observation of the ruptured capsules by optical microscopy (data not shown). For enumeration of viable cells, the resulting solution was then treated like the cells in PYGM broth and decimal dilutions were performed in sterile PBS, and 10 µL of each dilution was spotted, in triplicate, on

PYGM agar plates for *A. muciniphila* and incubated at 37 ºC for 7 days under anaerobic conditions. Results were expressed as CFU g^{-1}.

2.2.2.6. Encapsulation efficiency (% EE)

Viable cells count was performed as described in section 2.2.2.5. The encapsulation efficiency (% EE), which is a combined measurement of the efficacy of entrapment and survival of viable cells during the microencapsulation process, was calculated according to the following equation (1) (Martin et al. 2013):

$$\% \text{ EE} = (\log N / \log N_0) \times 100 \quad (1)$$

where N is the number of entrapped viable cells (CFU g^{-1}) released from the microcapsules and N_0 is the number of viable cells (CFU g^{-1}) in the cell biomass immediately before the microencapsulation process.

2.2.2.7. Survival of *A. muciniphila* cells through simulated gastrointestinal tract: free vs microencapsulated cells

The evaluation of the protective impact of microencapsulation on *A. muciniphila* survival after exposure to simulated GI conditions was compared to free untreated cells. The free cells obtained from the bacterial growth suspensions used in the microencapsulation procedure (2.2.2.2), were distributed (45 mL) in sterile falcons and stored at 4 ºC for 7 days. Two replicas of free or microencapsulated *A. muciniphila*, obtained immediately upon microencapsulation and after 7 d of storage at 4 ºC, were exposed to simulated GI conditions according to the procedures described in section 2.2.1.2 with similar sampling points (P1 to P5). Viable cell numbers of both free and encapsulated cells at each GI sampling point were determined according to 2.2.2.5. Results were expressed as CFU mL^{-1} or CFU g^{-1}, for the free and microencapsulated cells, respectively.

2.2.2.8. Survival of *A. muciniphila* cells throughout storage: free cells vs microencapsulated cells

The evaluation of the impact of microencapsulation on *A. muciniphila* cells viability and stability was performed in comparison to free cells stored at 4 ºC up to 14 d in an aerobic environment. At each pre-defined sampling time (0, 7 and 14 d), the total viable cell numbers of free and microencapsulated *A. muciniphila* cells were determined by plating, in triplicate, on PYGM agar plates according to procedures described in 2.1.2. and 2.2.2.5, respectively. Results were expressed as CFU mL^{-1} or CFU g^{-1}, for the free

and microencapsulated cells, respectively. Determination of survival rate throughout storage of encapsulated *A. muciniphila* was calculated for each sampling time by applying equation (2):

$$\text{Survival Rate} = (\log N_S / \log N_{PMC}) \times 100 \quad (2)$$

where N_S is the number of entrapped viable cells (CFU g^{-1}) released from the microcapsules at the evaluated storage time point and N_{PMC} is the number of viable cells (CFU g^{-1}) immediately after the microencapsulation process (post-microencapsulation).

2.3. *Statistical analysis*

Analysis of variance (ANOVA) two-way were carried out with SigmaStat (Systat Software, Chicago, IL, USA), to assess whether each factor (ex. the presence or absence of starch in the core formulation, cells in PYGM broth in comparison to those incorporated in freeze-dried formulations or in microcapsules) was a significant source of variation throughout GI or storage time at a significance level of P=0.05. One-way ANOVA was also applied whenever only one factor was under evaluation. The Holm-Sidak method was used for pairwise comparisons, also at the P = 0.05 level of significance.

ANOVA is valid provided that the experimental errors are independently and normally distributed and possess a constant variance. Whenever data did not meet these criteria a Kruskal-Wallis One Way Analysis of Variance on Ranks were applied at the same level of significance.

3. Results & Discussion

This thesis has as its main objective the design and study of technological strategies capable of accomplishing a protective impact on *A. muciniphila* when exposed to detrimental GI conditions as well as to storage at ambient air ultimately targeting the possibility of using such sensitivity to oxygen bacterium in therapeutic or probiotic applications. In order to do so, it is important to first validate growth and adhesion capacities in the specific media since it is well known that such physiological traits may vary according to composition and environmental conditions given their diverse metabolic pathways. *Akkermansia muciniphila* is the first cultured representative of the *Verrucomicrobiae* phylum found in the gut, having been described in the literature different culture media for its biomass production. It is important to have a culture medium capable of promoting a good growth, yet the presence of different compounds may influence growth kinetics, cell morphology and adhesion properties. In this study the PYGM reference growth medium according to the strain (under study) supplier DSMZ was used and impact on physiological traits was assessed as described in the following sections.

3.1. *Phenotypic characterization of A. muciniphila DSM22959*

3.1.1. Growth Curve and cell/colony morphology

Growth kinetics. The growth curve of *A. muciniphila* strain DSM 22959 displayed in Figure 3.1 was obtained by following the variation of cell density at 600 nm over 28 h of incubation under the optimum conditions indicated by DSMZ as described in 2.1.1. The *A. muciniphila* growth curve comprises a lag phase of approximately 5 h, followed by an exponential phase of approximately 15 h duration characterized by a specific growth rate of 0.150 h^{-1} with a generation time of 4.61 h. A stationary phase plateau is observable after approximately 20 h of incubation. Lower values (0.056 to 0.122 h^{-1}) of specific growth rate of *A. muciniphila* DSM 22959 were obtained by Van der Ark et al. (2018) in their study to define a minimal medium for these bacteria with L-threonine and N-Acetylglucosamine or N-Acetylgalactosamine as essential components for their growth. However, the addition of soy protein hydrolysate to the medium increased the growth rate five-fold to 0.53 h-1 (van den Ark et al., 2018). Growth of *A. muciniphila* in a mucin-containing medium generated a growth rate of 0.41 h^{-1} (Derrien et al., 2004; Ottman et al., 2017). In this study, a full-grown *A. muciniphila* culture can be obtained

after 16-18 hours of incubation with a correlation between OD at 600 nm and viable cell numbers of: 8,99 ± 0,02 log CFU mL^{-1} for an OD_{600} of 0.454 ± 0.016.

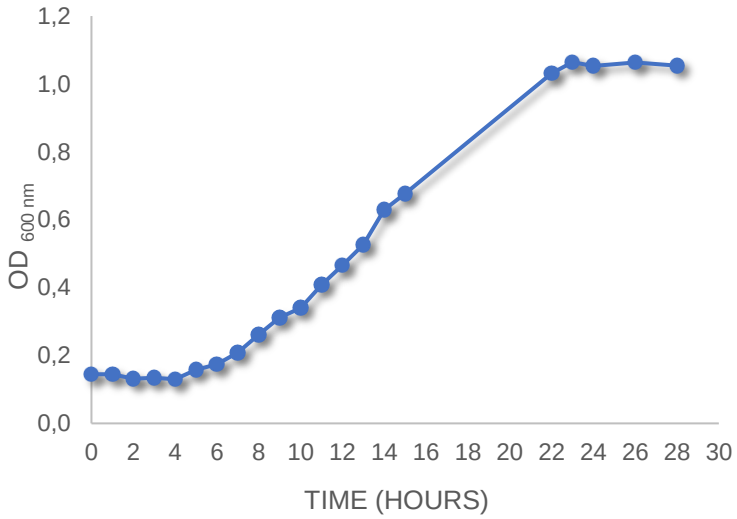

Figure 3.1 - Growth curve of Akkermansia muciniphila DSM22959 incubated at 37ºC under anaerobic conditions, over 28 h.

Cell/colony morphology. Macroscopically, visible *A. muciniphila* DSM22959 CFU's were obtained in PYGM agar only after 7 d of incubation at 37 ºC under anaerobic conditions. Their colonies are small, circular and appear to be translucent-like (colorless) (Figure 3.2). Time of incubation of 7 d is however in accordance to maximum incubation time suggested by DSMZ. According to Derrien et al. (2004), white-type colonies of the same strain of *A. muciniphila* were also obtained after 6 d of incubation at 37 ºC in an agar containing mucin.

Figure 3.2 - Photos of Akkermansia muciniphila DSM 22959 CFUs in PYGM agar after 7 d of incubation at 37 ºC under anaerobic conditions.

Microscopically, cells of *A. muciniphila* strain DSM22959 revealed to be coccobacillary and gram-negative occurring mostly in pairs or short chains (Figure 3.3).

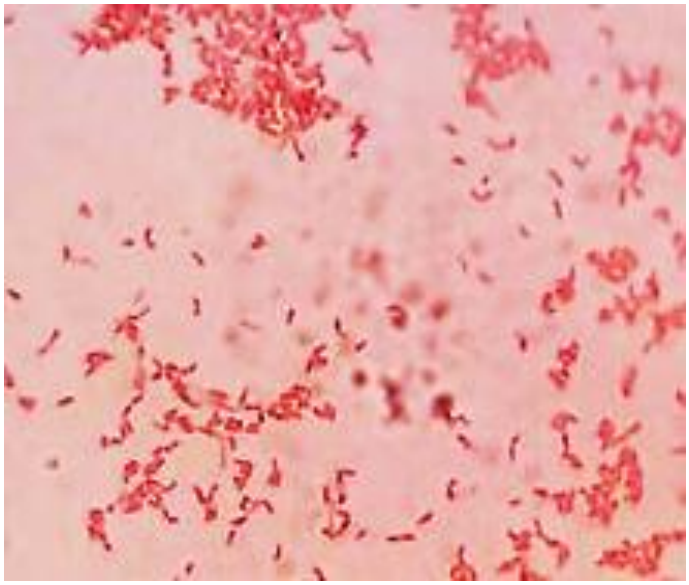

Figure 3.3 - Cells of Akkermansia muciniphila DSM22959 after Gram stain visualized under optical microscope (100 x).

The microscopy-related characteristics are in accordance with the description provided by Derrien *et al*. (2004), when they first isolated and characterized *Akkermansia muciniphila* strain Muc^T (=ATCC BAA-835^T = DSM22959) from intestinal tract (feces). They were able to assess further physiological traits, such as the cellular size range (long axis of cell is 0.6-1 µm), the absence of spore production and motility, and the presence of a capsule (Derrien et al. 2004).

3.1.2. Oxygen tolerance

The evolution over time in viable cell numbers of *A. muciniphila* DSM 22959 when exposed to different atmosphere conditions is displayed in Figure 3.4. In the present study, a fully-grown (at early stationary phase) *A. muciniphila* culture containing 8.80 ± 0.22 log CFU mL^{-1} in PYGM broth when exposed to aerobic conditions with or without agitation, showed a significant decrease in viable cell numbers after 60 h of incubation ($p<0.05$) when compared to the same *A. muciniphila* culture placed under anaerobic conditions. The variation in viable cell numbers over the 60 h exposure observed depicts, three phases with different decrease patterns associated:

i) Phase I (0 to 12 h) - The decrease pattern was similar, independently of aerobic or anaerobic conditions (with specific decrease rates of -0.091 and -0.094 h^{-1} under anaerobic and aerobic conditions without agitation, respectively) although a slightly sharper decrease is observed in culture placed under aerobic conditions with agitation

specific decrease rate of (-0.111 h^{-1}). These trends evidence potential temperature effect on the fully-grown cells of *A. muciniphila* associated with a detrimental oxygen effect for those under aerobic conditions with agitation.

ii) Phase II (12 to 36 h) - This phase is characterized by a plateau between 12 and 24 h followed by a small decrease until 36 h for the three atmospheres tested. Under aerobic conditions and agitation, the lower viable cell numbers reported, between 12 and 36 h, is a consequence of the detrimental effect of oxygen in the first 12 h of incubation causing a higher decrease in viable cell numbers with a specific decrease rate of -0.031 h^{-1} in comparison to values of -0.016 to -0.018 h^{-1} obtained under anaerobic and aerobic conditions without agitation.

iii) Phase III (36 to 60 h) – This phase evidences again the detrimental effect caused by oxygen on the viable cells of *A. muciniphila* in comparison to those maintained under anaerobic conditions. For this period, the highest specific decrease rates for *A. muciniphila* cells under aerobic conditions with or without agitation were obtained of -0.067 h^{-1} and -0.054 h^{-1}, respectively.

An approximate 1000-fold reduction, i.e. 3.7 and 2.8 logarithmic cycles, was observed in terms of viable cell numbers of *A. muciniphila* after 60 h of exposure to aerobic conditions with or without agitation, respectively, corresponding to a 57.4% and 68.9% bacteria survival.

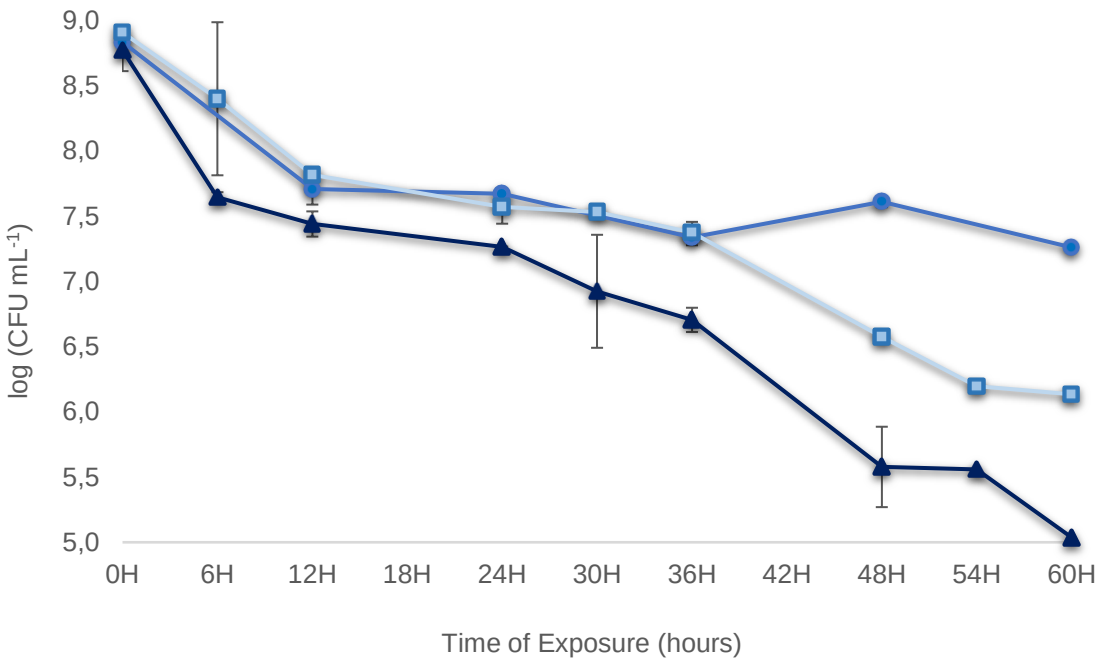

Figure 3.4 - Variation of viable cells (log CFU mL-1) of A. muciniphila in PYGM broth exposed to aerobic conditions (ambient air) without agitation (■) or under agitation (▲) and to anaerobic conditions (●), at 37 ºC.

These differences are naturally related with higher oxygen dissolution in the broth media due to the agitation increasing its concentration therein provoking a higher drop in cell viability when compared to anaerobic conditions counterpart.

Remarkably, these results indicate that the previously designated strict anaerobic or extreme sensitive, *A. muciniphila* susceptibility to oxygen is less than that which has been reported (Derrien et al. 2004). A plausible explanation for the unexpected tolerance might lie on the possibility that *A. muciniphila* is a capsule-possessing bacteria (Derrien et al. 2004). Capsular involucre, although commonly associated with a pathogenic behaviour, can protect bacterial cells from external aggressions, such as oxygen exposure (Rendueles et al. 2017).

In a previous dual staining *in vitro* assay, incubation under different atmospheres led *A. muciniphila* to showcase some tolerance to O_2, since 1 hour of exposure to air atmosphere, aerobiosis supplemented with 5% CO_2 and anaerobic conditions (5% H_2, 10% CO_2, and 85% N_2) all displayed similar staining patterns and, the subsequent binding to Caco-2 and HT-29 human colonic cell lines was not affected (Reunanen et al. 2015). Additionally, it was previously reported that indeed one of the peculiarities of *A. muciniphila* contrasting with some strict anaerobes is its ability to profit from the presence of nanomolar concentrations of oxygen in liquid medium (Ouwerkerk et al. 2016). These authors performed transcriptome analysis which demonstrated that, under aerobic conditions, the subunit cytochrome bd complex (related to respiration) coupled with an unidentified NADH dehydrogenase could use oxygen as a final electron acceptor; this causes a deviation in the metabolic course resulting in a higher acetate-to-propionate ratio yielding more ATP and NADH, and ultimately enhancing growth. This strategy might clarify why *A. muciniphila* can outcompete strict anaerobes in the mucus layer (Ouwerkerk et al. 2016). This goes in line with Loesche study (Loesche 1969), which is still currently recognized (Gajdács et al. 2017). According to these authors some bacteria considered strictly anaerobic may grow in the presence of small amounts of O_2 achieving higher levels of viable cell numbers. Interestingly, there is no current information on the presence of enzymes such as superoxide dismutase coded in *A. muciniphila* genome and therefore not enough knowledge exists to explain the tolerance levels observed with *A. muciniphila* in PYGM broth when exposed to aerobic conditions.

Overall, the present results could suggest that by tolerating molecular oxygen, rather than utilizing a continuous anaerobic environment, *A. muciniphila* cultivation could be met applying a mixed environment, namely, an initial bench manipulation at ambient air followed by incubation under anaerobiosis, similarly to some facultative anaerobic bacteria such as *Bifidobacterium adolescentis* and *Bacteroides fragilis*, that remained

viable after 48 h of exposure to ambient air (Rolfe et al. 1978). Nevertheless, molecular evaluations are required to understand if prolonged O_2 exposure could have any deleterious effects on metabolic functions or other pathways that could hamper cell viability and stability.

3.1.3. *Akkermansia muciniphila* capacity to bind to human intestinal epithelial cells

After overcoming the harsh conditions encountered throughout GI transit, bacterial adhesion to host cells is the first step for successful establishment in the gut environment (Dufrêne 2015). Since faecal enumeration of probiotic bacteria is suspected to be speculative, because it generally depicts non adhered microorganisms (Saxelin et al. 1995), *A. muciniphila* biological adhesion capacity was assessed by measuring its binding capacity to Caco-2 and HT29-MTX cell lines. Both cell lines are derived from human adenocarcinoma colon cells, and are commonly used for *in vitro* adhesion assays (Zweibaum et al. 2011). When grown in the adequate conditions, Caco-2 cell lines present high homology to intestinal epithelium (Yee 1997). Conversely, HT29-MTX cell model, obtained by the treatment of HT-29 cells with methotrexate (Lesuffleur et al. 1990), exhibits complete goblet cell-like phenotype thereby producing mucus (Lesuffleur et al. 1993). This feature can act as an adhesion promoting factor for some bacteria, since mucin glycoproteins polymerization allow certain microbes to adhere, yet to others it appears to interfere with the binding process (Gagnon et al. 2013). Therefore, the differences between both models are of interest to study binding capacity because mucus may play a pivotal role in glycosylation dependent adhesion to the intestinal epithelium (Zhang et al. 2015).

In this study a short-term and a long-term contact assays were performed, with the rationale that the short contact periods between cells act as a preliminary screening for the longer contact assay (Veiga, 2018).

In the short-term assay (Figure 3.5) no statistically significant differences were found between adhesion values of *A. muciniphila* to both cell lines ($p = 0.155$). Overall, there was a significant ($p<0.05$) decrease between the initial viable cell numbers (6.18 Log CFU mL^{-1}) and those bound to Caco-2 and HT29-MTX cells after 15 and 30 min of exposure. This trend is in line with previously reported data (Parkar et al. 2008; Veiga 2018). However, adhesion values for the short-term assay fluctuated between 67% and 78%, as may be seen in Table 3.1, with the highest values corresponding to the 60-minute mark, in particular for Caco-2, which are viewed as good binding values (Veiga, 2018).

Table 3.1 - Percentage values of relative adhesion of A. muciniphila to Caco-2 and HT29-MTX cell lines for the short-term assay.

Time (min)	Caco-2	HT29-MTX
15	74%	ø
30	67%	67%
60	78%	71%

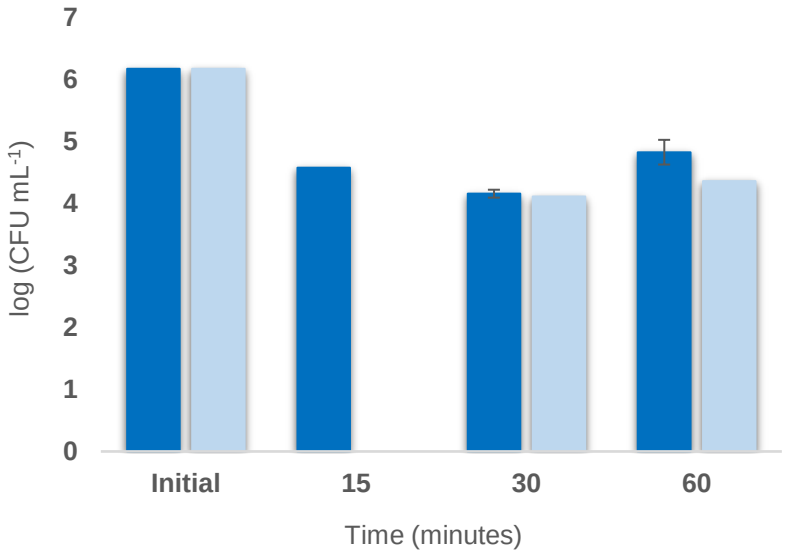

Figure 3.5. Viable cells of A. muciniphila in the adhesion inoculum (Initial) and resulting of the adhesion to Caco-2 (dark blue) or HT29-MTX (light blue) cell lines throughout the short adhesion time assay.

The HT29-MTX cell model has been suggested to be more accurate in depicting the *in vivo* physiological conditions for bacterial interaction with human enterocytes assessment (Gagnon et al. 2013). However, our results demonstrated that, at least for the short term-assays, there were no significant differences between adhesion capacity to Caco-2 and HT29-MTX cell lines. Additionally, due to laboratory constraints at the time, long-term assays with HT29-MTX cell line were not possible to perform. Consequently, long-term adhesion assays were performed, but only with Caco-2 cell model.

According to previous studies, bacterial adhesion strength has been shown to be time-dependent, with binding forces positively correlated with an increase in time (Dufrêne 2015), which supports the higher binding values by *A. muciniphila* to cell lines with extended incubation times. However, compared to the short-term (Table 3.1), the

registered long-term adhesion capacity, of *A. muciniphila* to Caco-2 cell model, appears to be lower (Table 3.2). In fact, the highest adhesion values recorded in the long-term assay were around 66%, at the 3-hour mark (Figure 3.6 and Table 3.3). This is lower to those obtained at the 60 min mark of the short-term assay.

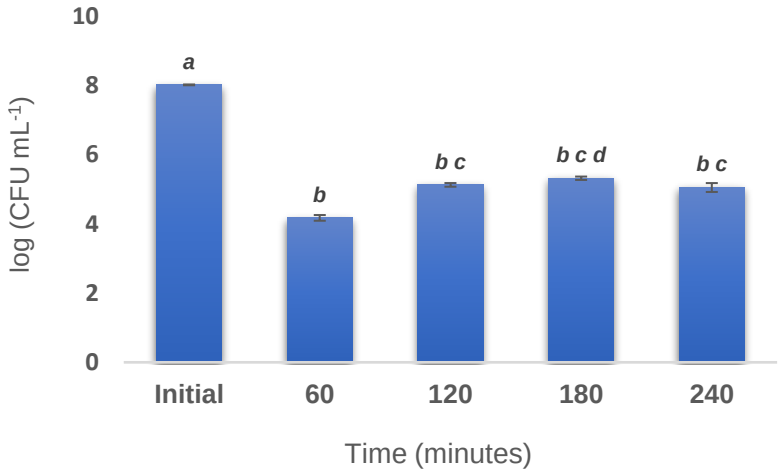

Figure 3.6 - Viable cells of A. muciniphila adhesion inoculum (initial) and resulting of the adhesion to Caco-2 cell line by A. muciniphila DSM 22959 throughout the long-term adhesion assay. Different letters represent the statistically significant differences ($p < 0.05$) found between sampling times.

Table 3.2 - Percentage values of relative adhesion of A. muciniphila to Caco-2 cell line for the long-term assay.

Time (minutes)	Caco-2
60	52%
120	64%
180	66%
240	63%

However, it should be emphasized that percentage of adhesion observed is often very dependent on factors such as the epithelial cell density, the bacterial affinity for the target and time of incubation between both cells (Pedersen et al. 2018). Of special importance are the number of washes (may detach the epithelial cells and consequently, less bacterial adherence) and the multiplicity of infection (MOI). Multiplicity of infection, the ratio of infectious agents to infection targets, is a standard virology concept that lately has been applied in the bacteriology field, becoming the ratio of bacteria to cultured epithelial cells (Pedersen et al. 2018). In this context, it is

important to highlight that the initial bacterial load was not the same for both assays: 10^8 Log CFU mL^{-1} in the long-term assay and 10^6 Log CFU mL^{-1} in the short-term assay, respectively, which in turn translates into different MOI. This can have an impact on adhesion results since higher MOI is associated with higher variability and bacteria might adhere to the adhesion flask instead (Letourneau et al. 2011). In this way, it is imperative to determine the optimal MOI for *A. muciniphila* and to be consistent when performing these assays.

To the best of our current knowledge, only one published study has evaluated *A. muciniphila*'s binding ability to human enterocytes, using Caco-2 and HT-29 cell models (Reunanen et al. 2015). Although the authors tested the same *A. muciniphila* strain (ATCC BAA-835^T = DSM22959), they reported adhesion values below 10%, markedly different from those values observed herein. Conversely, the adhesion ability displayed in these assays by *A. muciniphila* was comparable to those of other bacteria with already established binding ability, such as *Lactobacillus rhamnosus* (Lebeer et al. 2012).

It is also important to highlight that these assays were conducted with undifferentiated cells lines, which does not depict properly the normal intestinal environment, in which a monolayer of differentiated eukaryotic cells would be more representative (Grootaert et al. 2011). Reunanen and co-workers (2015) have assessed *A. muciniphila* BAA-835^T binding behavior to Caco-2 and HT-29 cell lines at different differentiation stages, 3, 8 and 21 d, and found that overall the adhesion levels were similar in both models. Furthermore, given its mucin dependent nature (Derrien et al. 2004), the same authors evaluated *A. muciniphila* binding behavior towards human colonic mucus, finding it to be non-specific, since less than 1% adhesion levels were observed (Reunanen et al. 2015). Again, since these adhesion conditions are not comparable to those assessed herein, more studies are required to validate this hypothesis.

Unquestionably, one of the most relevant mechanisms for *A. muciniphila* contribution toward a healthier metabolic status would be the strengthening of the epithelial barrier (Everard et al. 2013; Reunanen et al. 2015). Reunanen et al. (2015) using their *in vitro* model demonstrated that *A. muciniphila* administration triggered a significant increase in the transepithelial electrical resistance (TER) of cocultures of Caco-2 cells showcasing a positive impact on the monolayer integrity which could lead to a lower uptake of LPS from gram-negative inhabitants, that could otherwise trigger low grade inflammation, one of the causative agents of obesity and related metabolic conditions (Reunanen et al. 2015).

3.1.4. Cell membrane fatty acids of *Akkermansia muciniphila* DSM22959

The fatty acid (FA) membrane composition of the assayed *A. muciniphila* strain is presented in table 3.4. It is well known that bacterial membrane fatty acids not only provide a means for identification but also help in understanding mechanistic responses to stress conditions. Hence, it is important to establish the cell membrane fatty acid inventory to be able to study adaptation mechanisms when bacterium is submitted to oxygen stress conditions.

Table 3.3 – Fatty acid composition (of A. muciniphila DSM22959 cell membrane after 17 ± 2h incubation in PYGM broth.

Fatty Acid	**mg g $^{-1}$ biomass pellet**
C14i	110 ± 8
C14:0	27 ± 1
C14:1	405 ± 29
C15:0	237 ± 16
C15:1	35 ± 2
C16:0	60 ± 4
C16:1 c9	11.2 ± 0.6
C17:0	81 ± 6
C18:0	18 ± 2
C18:1 c9	55 ± 1
Total FA	1040 ± 64
Total SFA	533
Total MUFA	507

i: iso; c9 - cis double bond; FA, Fatty acids; SFA, Saturated fatty acids; MUFA, Mono unsaturated fatty acids

Akkermansia muciniphila DSM22959 was mainly composed of myristoleic (C14:1) and pentadecanoic (C15:0) acids, corresponding to more than 60% of the total fatty acids present. The lowest registered concentrations belonged to stearic (C18:0) and palmitoleic (C16:1 c9) acids. Interestingly, the total membrane fatty acids were almost equally distributed between saturated (SFA) and monounsaturated fatty acids (MUFA), the latter being slightly higher (51% vs 48%, respectively). It has been demonstrated that cell membrane SFA increase in number in Gram-negative biofilm producers compared to their planktonic counterparts (Dubois-Brissonnet et al. 2016). Increase in

SFA leads to higher transition temperatures and phase stability and, more specifically, the long chain SFA might facilitate penetration of FA into the bilayer, favoring its rigidity (Denich et al. 2003). Among the many alterations bacterial cells undergo in order to sustain appropriate membrane fluidity to the environment in which they are integrated, the alteration of ratios of unsaturated to saturated and, branched to non-branched structures, seem to be crucial (Dubois-Brissonnet et al. 2016). In this way, evaluating these specific physiological behaviors may shine some light on the possible mechanisms *A. muciniphila* enforces to improve its survival in harsh conditions such as oxygen stress.

One of the most useful physiological traits to identify microorganism groups are lipid membrane and respective fatty acids (Quezada et al. 2007). The analysis of fatty acid methyl esters (FAME) by gas chromatography for bacterial identification is proven to be fast, efficient and reproducible, since FAME content is a phenotypic parameter and direct expression of cellular genome (Hoffmann et al. 2010)

Quezada *et al*. (2007) demonstrated that microbial communities, in wastewater treatment systems, could be determined based on lipid metabolism. More specifically, FAME chromatographic profiles were related to the terminal electron acceptor utilized by bacteria, in which anaerobic bacteria FAME biomarkers (only found in one group) possessed mainly branched and unsaturated fatty acids. In addition, MUFA are common in gram-negative bacteria (Zelles et al. 1997). This goes in line with the present results in which ca. 48% of the total FA are MUFA, being C14:1 (branched) the main fatty acid in the membrane composition of *A. muciniphila* DSM22959, followed by a ten-fold less concentration of oleic (c18:1 c9), pentadecenoic acid (C15:1) and palmitoleic acids in descending order of magnitude.

In future works it would be necessary to evaluate if conditions or stresses such as laboratory manipulation and passages as well as formulation or encapsulation would induce modifications in the physiological status of *A. muciniphila* and associated lipid profile as an adaptation mechanism to stress.

3.1.5. Flow Cytometry (FCM) as a fast and simple technique for evaluation of viability of EOS bacteria

Flow cytometry (FCM) provides a rapid and reliable method to quantify viable cells in bacterial suspensions (Machado et al. 2017). This methodology can be coupled with commercial kits of fluorescent dyes that facilitate the distinction between live and dead cells such as BD Cell Viability Kit (BD Biosciences).

FCM technique allows multiparametric analysis of both physical and chemical characteristics of thousands of cells per second (Tracy et al. 2010). The different parameters assessed depend on the type of labeling dye that is employed and, can be later associated to specific cellular characteristics (Tracy et al. 2010). Although FCM has been extensively used for enumeration of various bacterial species (Tracy et al. 2010), to the best of our knowledge, there is no published research on the rapid enumeration of EOS bacteria, such as *A. muciniphila*, via FCM technology. Thus, in a tentative proof of concept, an investment was made to optimize a detection protocol that would allow to distinguish between dead and live cells including those that are still viable but not culturable (VBNC) permitting also to bypass the prolonged incubation periods (around 7 d) necessary for conventional viable cell numbers enumeration. Certain stress conditions, such as freeze-drying, can induce bacterial cells to enter a "non-recoverable stage of existence" while still rendering them viable (Xu et al. 1982). It resembles a viability limbo, where injured and stressed bacterial cells that are still viable but not culturable (VBNC), protect themselves from any further taxing conditions by lowering their metabolic demands entering a dormant state, enabling their survival but hampering colony formation on routine bacteriological media (Ramamurthy et al. 2014; Pinto et al. 2015). Quantification of VBNC bacteria can be accomplished via methods such as real-time quantitative polymerase chain reactions (rt-PCR), fluorescent *in situ* hybridization (FISH) and flow cytometry (FCM) (Davis 2014).

In this study, work was focused on the implementation and validity of the ±19344:2015 (E)-IDF 232:2015 (ISO, 2015), a dual staining protocol, in the detection and quantification of *A. muciniphila*. After several tests, exploiting different combinations of threshold values, such as forward-scatter (FSC) and side-scatter (SSC) and staining buffer dilutions, an optimal bacterial cell concentration of 10^7 cells mL^{-1} was identified as the minimum threshold to enable the FCM analysis. An initial suspension with a concentration higher than 10^8 cells mL^{-1} would require further dilutions to remain within the instrumental range for *A. muciniphila*. These values are in agreement with Pane (2018) who indicates that for products with a concentration above 9 log cells g^{-1}, it is necessary to further dilute it in order to stay in the optimal instrumental range.

Figure 3.8 depicts the results of some of the preliminary FCM analyses for *A. muciniphila*. After being able to define gate bacterial population using FSC and SSC (Figure 3.8a) and single-parametric histograms representing the non-stained population on scale (Figure 3.8b-c) the staining with TO (stains DNA), with the threshold set at the point where the noise level is just starting to increase (red arrow), allowed to distinguish whole population from background noise (Figure 3.8d-e). Finally, unstained *A. muciniphila* gated on the bacterial region placed in FL1 vs FL3 dot plot

(live vs dead cells) (Figure 3.8f) allowed the comparison with a representative FL1 vs FL3 dot plots of *A. muciniphila* stained with TO (Figure 3.8g). The live and dead populations do not line up in a rectilinear fashion and analysis is best accomplished by drawing regions in the appropriate areas (quadrants; Figure 3.8f-g).

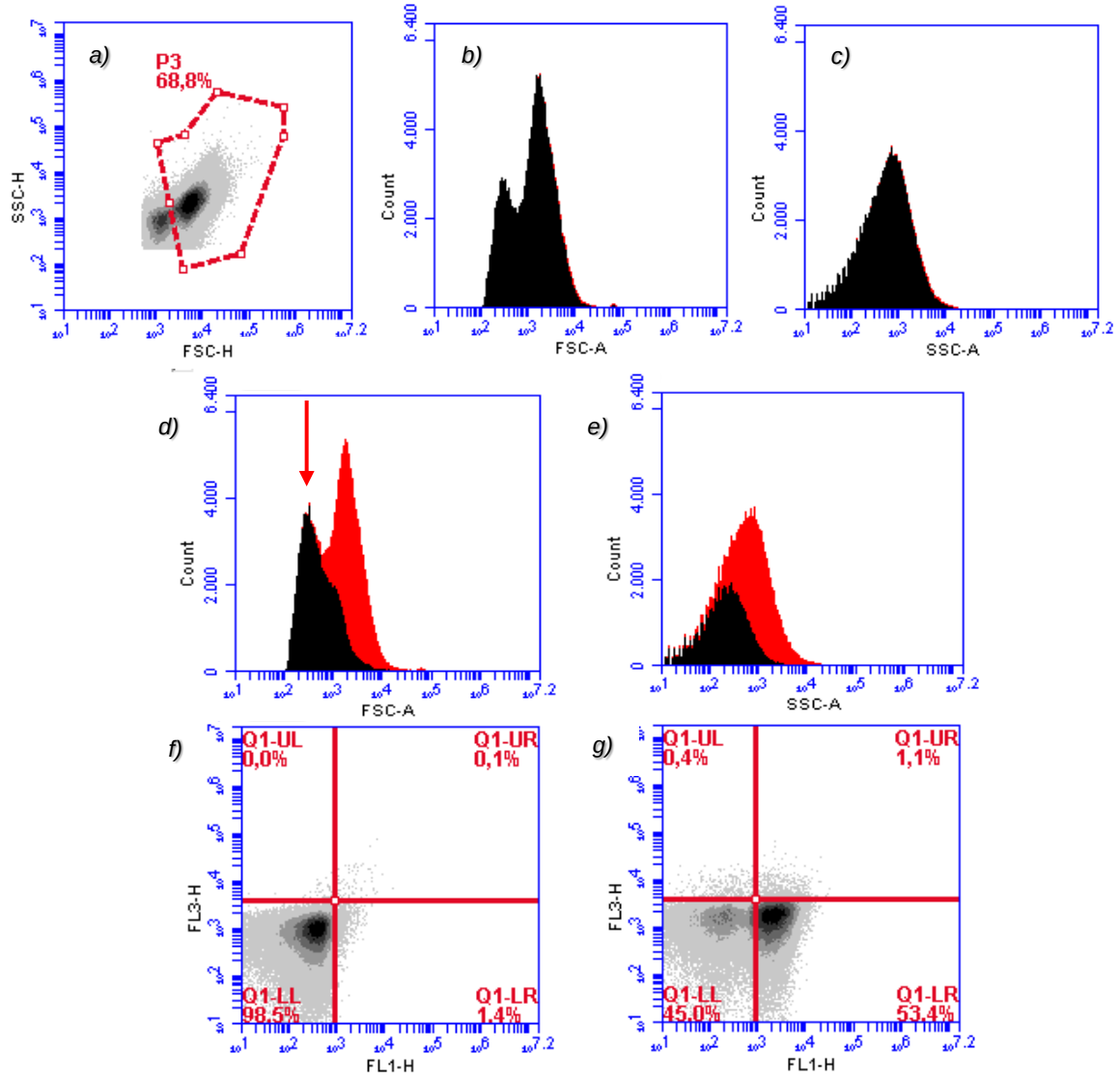

Figure 3.7 – Flow cytometric analysis of A. muciniphila DSM22959. Data acquisition was obtained with a single dye (TO). a) FSC vs SSC gated A. muciniphila population; b) uni-parametric FSC histogram of unstained A. muciniphila; c) uni-parametric SSC histogram of unstained A. muciniphila; d) uni-parametric FSC histogram of TO stained A. muciniphila; e) uni-parametric SSC histogram of TO stained A. muciniphila; f) FL1 vs FL3 dot plot of unstained A. muciniphila; g) FL1 vs FL3 dot plot of TO stained A. muciniphila;

This technique was tested during the oxygen tolerance assay (section 3.2) and compared with results from conventional plating methods. However, the expected increase in PI labelling, and consequent enumeration of dead cells during oxygen

exposure, didn't occur which was incongruent with data obtained by viable cell counts (Figure 3.4). The number of events remained constant but without increment of the dead population.

Despite the apparent success in detection *A. muciniphila* in the preliminary assay, the combination of TO and PI failed to accurately depict bacterial death during an oxygen survival kinetics assay. TO is used to evaluate total bacterial population present in the sample and PI, a DNA-binding dye, impermeable to intact membranes, to evaluate damaged/dead bacterial cells (Sibanda & Buys 2017). As mentioned previously, *A. muciniphila* Muc^T strain (=DSM22959) was found to be capable of aggregation via its capsular fibers, which could allow adhesion and colonization in the GI, and the fact that it was able to exclude Indian ink suggests that it is a capsule-possessing bacterium (Derrien et al. 2004). Additionally, a genetic screening discovered two capsule-building genes in *Akkermansia* genus, one which is a family 2 glycosyl transferase (gene Amuc_2081), a capsular polysaccharide biosynthesis pathway with the potential to protect against oxygen presence (Ouwerkerk et al. 2016). Although capsules are usually viewed as a pathogenic colonization strategy (Roberts 1996), we propose that in the case of *A. muciniphila* it may, not only protect against oxygen toxicity and other harsh conditions (Rendueles et al. 2017), but also could lead to the incapacity of PI binding and consequently loss of PI stain to the extracellular space, resulting in a decreased fluorescent signal. In a FCM yeast study, it seemed that capsular presence could be a cause for the ineffective nuclear DNA staining by PI (Medwid 1998). Recently, after ineffective yeast staining with classical PI, Zhang and collaborators were able to bypass this issue by pretreating cellular suspension with dimethyl sulfoxide (DMSO) (Zhang et al. 2018). DMSO widely known as a "universal solvent", is a dipolar aprotic solvent that has been used for a long time as a permeabilization enhancer for drug delivery (Williams & Barry 2004). The authors were able to demonstrate that DMSO treatment enabled PI penetration in recalcitrant yeasts, which translated into the successful detection of the fluorescence signal by FCM (Zhang et al. 2018). If the dye permeabilization problem was to be overcome, other dyes aimed at detecting different extrinsic parameters, such as intracellular enzymatic activity (using carboxy-fluorescein diacetate; cFDA), or membrane potential ($DiBAC_4$) could be assayed (Tracy et al. 2010).Since FCM analysis has to deal with both background and bacteria, the sensitivity of the bacterial measurement may be lower. Therefore, FCM can be combined with other cell detection/enumeration methods. The conjugation of rt-PCR, FCM and fluorescence *in situ* hybridization (FISH) - FLOW-FISH- consists of 16s RNA probes conjugated with i.e. PI where FISH probe labelled cell numbers against PI positive events determine the relative population FCM and Fluorescence *in situ*

hybridization (FISH) (Friedrich & Lenke 2006). Identically, peptide nucleic acid probes (PNA) used in a fluorescent in-situ hybridization FCM (FISH-FCM) assay (Gunasekera et al. 2003) could also be employed to distinguish *A. muciniphila* cells from background noise. The application of qPCR and FISH methodologies may also aid in the differentiation and enumeration of complex cultures and when incorporated in matrix formulations.

Based on the current literature, FCM is unarguably becoming a broad technique promising to circumvent the VBNC limitation, with a high degree of statistical agreement with the traditional plate count methods (Wilkinson 2018). Despite this, the danger of overestimation of bacterial viability might follow. Nonetheless, although it shouldn't be considered as a direct replacement for the traditional plating method, FCM can act as an analytic aid to accelerate assessment procedure.

3.2. Design and study of technological strategies

Upon definition of physiological traits, the next step was to define new technological strategies that could be employed to not only increase survival at ambient air but also enable an efficient delivery through the harsh high acidity and high bile conditions encountered during gastrointestinal passage. Such strategies depend on the adaptation capacity of these bacteria and their resistance to the different processing techniques. Protective formulations coupled to freeze-drying or an encapsulation technology are two of the most promising strategies. Formulation with vitamins, antioxidants and/or prebiotics among other compounds has been tentatively studied and several microencapsulation systems, including internal gelation, have been used successfully to protect probiotic strains.

Freeze-dried formulations incorporating *A. muciniphila*

To determine the best conditions to increase the survival rate of *A. muciniphila* to ambient air, different compounds with different properties were selected such as prebiotic compounds (inulin and FOS), antioxidants and/or redox compounds (cysteine, glutathione and riboflavin) and bulking agents, with possible growth enhancing/stabilizing action (starch).

3.2.1.1. Effect of prebiotics on growth of *A. muciniphila*

Due to their prebiotic properties in stimulating bacterial biomass growth and potentiating production of metabolic products of many probiotic bacteria, mainly

Bifidobacterium and *Lactobacillus* strains (Capela et al. 2006; Savini et al. 2010; Scott et al. 2014), the reference growth medium for *A. muciniphila* (PYGM broth) was supplemented with either inulin or FOS at 2.5% (w/v). Although slightly higher $OD_{600\ nm}$ values were observed in the PYGM broth containing inulin, in comparison to PYGM broth alone or PYGM broth with FOS (Figure 3.8a), no statistical differences were recorded ($p=0.121$). In terms of *A. muciniphila* viable cell numbers (Figure 3.8b), the incorporation of inulin or FOS in the growth media did not contribute positively to their increment in comparison to PYGM broth alone; in fact, viable cell numbers were found at statistically significant ($p<0.05$) lower values in PYGM broth containing inulin or FOS.

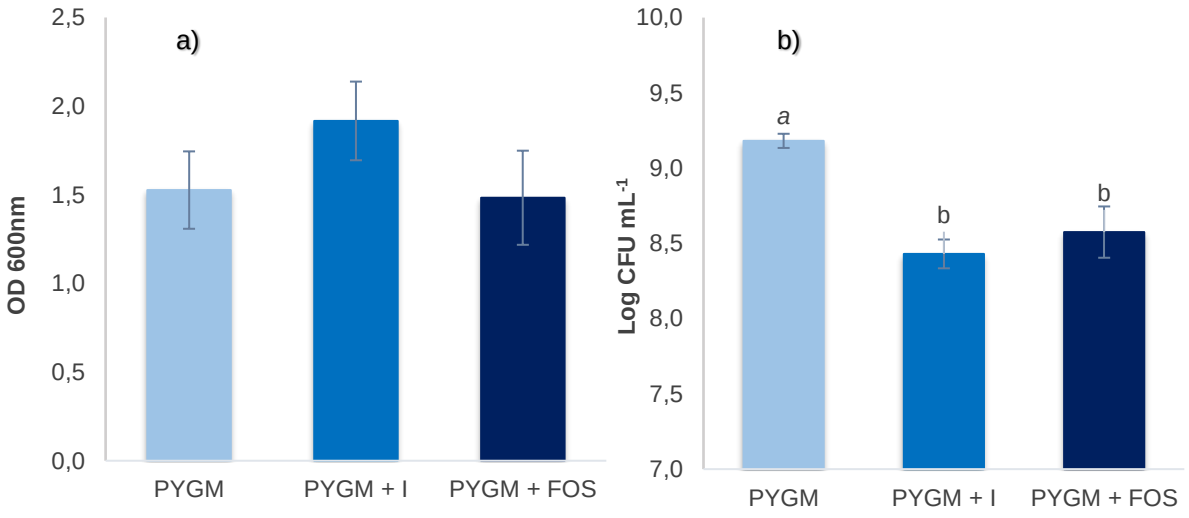

Figure 3.8 - Values of OD at 600 nm (a) and of viable cell numbers [Log (CFU mL-1)] (b) of A. muciniphila grown in PYGM broth with or without prebiotic agents [I: Inulin 2.5% (w/v); FOS: FOS 2.5% (w/v)]. Different letters represent statistically significant differences ($p < 0.05$) found between each growth medium.

These results are in alignment with previous findings suggesting that the prebiotic effect of inulin, and possibly of FOS, on *A. muciniphila* growth and subsequent colonization in GI tract, might be an indirect effect, since these compounds induce mucin secretion and aid in pH homeostasis, promoting the ideal conditions for *A. muciniphila* gut establishment (Van-den-Abbeele et al. 2011; Van Herreweghen et al. 2017). Therefore, by not being able to directly metabolize these sugars *in vitro*, *A. muciniphila* does not benefit from the presence of prebiotic compounds in growth media which, consequently, doesn't translate into a higher number of viable cell numbers. Despite these rationale, the reported decrease of approximately 1 logarithmic cycle in cell counts in PYGM containing 2.5 % (w/v) of inulin or FOS was not at all expected; this slightly negative effect warrants further study to understand why in future work.

3.2.1.2. Effect of antioxidant and/or redox agents in freeze-dried formulations incorporating *A. muciniphila*

Given their impact in protecting susceptible strains from the toxic effects of oxygen by mediating electron shuttle via redox reactions (Ross et al. 2005), the efficacy of antioxidant agents such as cysteine and glutathione or exogenous redox mediator such as riboflavin in the formulation matrix was explored. Therefore, it was studied the impact of inulin (10% w/v) and riboflavin (16.5 mM) with or without cysteine (0.2 % w/v) or glutathione (0.2 % w/v) in the formulation composition incorporating *A. muciniphila*. As it can be observed in Figure 3.9, a significant higher amount of freeze-dried powder containing *A. muciniphila* was obtained in the formulation containing inulin, riboflavin and glutathione ($p<0.05$); a direct effect caused by mass addition is excluded because the amount of cysteine was equal to that of glutathione, both at 0.2 % on a weight basis.

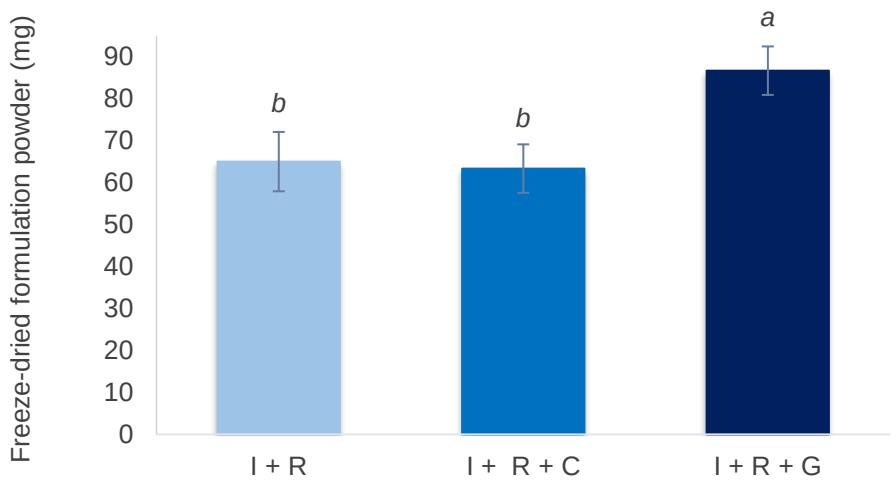

Figure 3.9 - Freeze-dried formulations incorporating A. muciniphila: I+R, formulation with 10 % (w/v) inulin and 16.5 mM riboflavin; I+R+C, formulation with 10 % (w/v) inulin, 16.5 mM riboflavin and 0.2 % (w/v) cysteine (; I+R+G, formulation with 10 % (w/v) inulin, 16.5 mM riboflavin and 0.2% (w/v) glutathione. Different letters represent the statistically significant differences ($p < 0.05$) found between each formulation composition.

Glutathione and its precursor cysteine are antioxidants widely used as a strategic measure to minimize bacteria oxidative stress via thiol-redox buffer reactions (Loi et al. 2015). On one hand cysteine is produced from the amino acid serine via the incorporation of thiosulfate and is usually used for thiolations in Gram positive bacteria (Fahey 2013; Loi et al. 2015). On the other hand, glutathione is a major thiol-redox

buffer in Gram-negative bacteria and is synthesized from its precursors cysteine, glutamate and glycine (Fahey 2013; Loi et al. 2015). During oxidative stress, thiols of cysteine or glutathione are oxidized forming reversible disulfide bonds that protect bacteria from overoxidation (Khan et al. 2012; Loi et al. 2015). Since *A. muciniphila* is a Gram-negative bacteria, it might benefit more from the presence of glutathione to shuttle electrons to oxygen as a detoxification strategy. For this reason, and owing to the results presented, glutathione was used as the antioxidant to be implemented in all further experiments.

Additionally, riboflavin was also included in the formulation to induce a reducing environment further favoring the survival of the anaerobic bacteria under oxygenized conditions, as previously employed for anaerobic *F. prausnitzii* (Khan et al. 2014). Riboflavin is a vitamin exploited as exogenous redox mediator that drives microbial extracellular electron transfer with oxygen as the terminal electron acceptor (M. T. Khan et al. 2012). However, the role of riboflavin in our formulation assays is questionable since recent work of Ouwerkerk and coworkers (2016), in a gas tube assay based on the turning point of a redox indicator (resazurin), observed no effect on the growth ring when in presence of riboflavin, suggesting that *A. muciniphila* cannot employ a riboflavin dependent extracellular electron shuttling system.

3.2.1.3. Impact of different formulations on *A. muciniphila* survival and resistance to GI

Taking into account the evidences supporting the use of glutathione as a major antioxidant by gram negative bacteria (Fahey 2013; Loi et al. 2015), and according to the better results obtained with the freeze-dried formulation comprised of inulin (10% w/v), glutathione (0.02% w/v) and riboflavin (16.5 mM), henceforth designated as “core-formulation”, this matrix was chosen for further assays to assess viability and stability promotion of incorporated *A. muciniphila* viable cells, under stress conditions over storage.

Akkermansia muciniphila incorporated in core-formulation after freeze-drying as well as cells in PYGM broth were evaluated for their ability to withstand simulated GI conditions. It is well established that in order for probiotic bacteria be able to confer positive health effects on the host, they must survive the transit through the stomach and small intestine to reach the colon in sufficient viable cell numbers (7 to 9 log CFU per serving to exert their impact (Hill et al. 2014; Hungin et al. 2018).

As can be observed in Figure 3.10, the number of viable cells of *A. muciniphila* in core-formulation suffered a substantial reduction (approximately 7 log decrease with the sharpest decrease of ca. 4 log cycles in the first hour of gastric phase) during simulated gastrointestinal conditions. In contrast, the number of viable cell numbers in PYGM broth counterpart only underwent very slight fluctuations along the simulated GI passage.

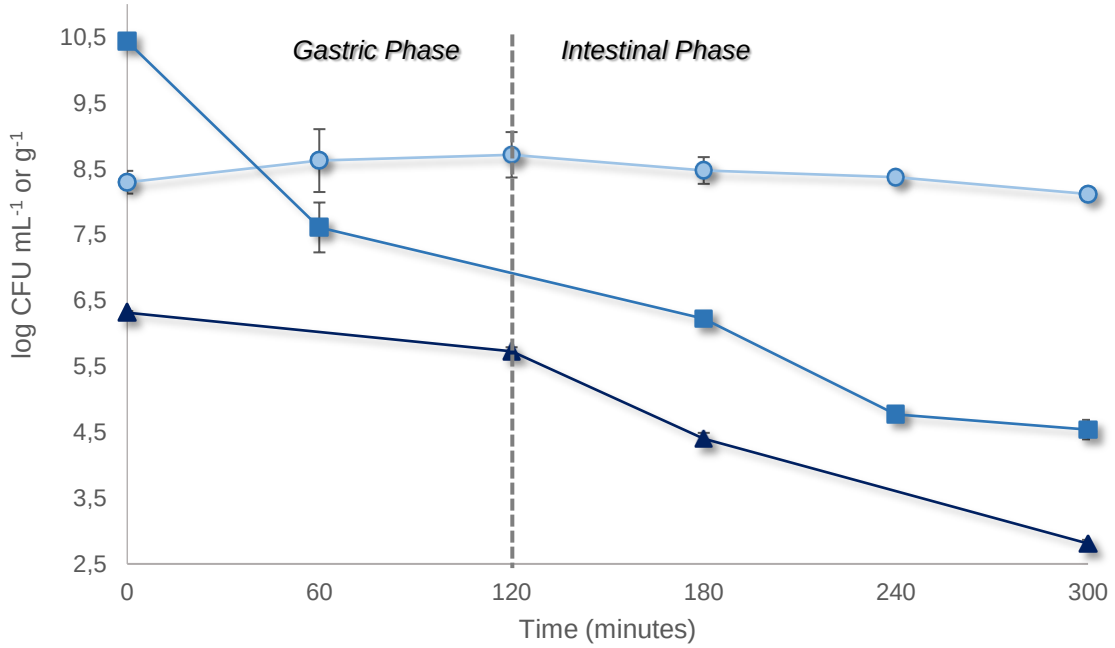

Figure 3.10 - Viable cell numbers of A. muciniphila in PYGM broth (●) [Log (CFU/mL)], incorporated in freeze-dried core formulation (■) [Log (CFU/g)] and incorporated in freeze-fried in core formulation with starch (▲) [Log (CFU/g)] during simulated gastrointestinal conditions

A plausible explanation for the apparent survival of non-formulated *A. muciniphila* cells to GI harsh conditions may lie on the active acid resistance system of *A. muciniphila* (Ottman 2015). In this context, it is important to recall that *A. muciniphila* has been detected in human breast milk (Collado et al. 2012; Ward et al. 2013), which eludes its capacity to survive GI passage in order to colonize nursing infants. Its presence in human milk coupled with acid resistance system has been proposed as a reason for early life colonization (Petschacher & Nidetzky 2016). With that said, during the formulation procedure, the removal of intracellular water is a mandatory step to reversibly inactivate microorganisms facilitating preservation during long-term storage (Prakash et al. 2013). One such method is freeze-drying, which usually provides stability of bacterial cultures. However, during this process, bacterial cells integrity can be affected by numerous factors, such as the formation of intracellular ice crystals

protein unfolding and denaturation (Arakawa et al. 2001). Therefore, to prevent any significant deterioration of viability, the inclusion of excipients and stabilizing agents before lyophilization is a proven strategy of bacterial protection (Fowler & Toner 2005).
Sugars are a conventional group of cryoprotectants that can prevent freeze-induced damage (Carpenter & Crowe 1989), mostly due to the formation of a glassy matrix that not only acts as a physical barrier, but it reduces diffusion and vitrification (molecular mobility), maintaining structural integrity of bacterial containing formulation both during drying and subsequent storage (Molina et al. 2004). Furthermore, high viability has been observed after freeze-drying when using sugars at 10 % concentrations (Zhao & Zhang 2005). In addition to its prebiotic properties, inulin has been linked to the stabilization of cell components by interacting with membrane lipids and enhancing surface pressure, being considered a potent cryoprotectant (Kolida & Gibson 2007; Mensink et al. 2015; Bircher, Geirnaert, et al. 2018). Therefore it was the selected protectant to be included in the formulation, as previously employed for anaerobic *F. prausnitzii* (Khan et al. 2014).

In light of the lower survival that core-formulated *A. muciniphila* cells displayed during GI passage, we are unable to assess if inulin is the best choice of cryoprotectant to ensure the proper stabilization of the formulation. Regardless, inulin protective effects have been shown to be significant, particularly when compounded with other agents such as glycerol, in preserving butyrate-producing bacteria (Bircher, Geirnaert, et al. 2018; Bircher, Schwab, et al. 2018), and in preserving LAB (Capela et al. 2006; Avila-Reyes et al. 2014). However, the efficacy of these strategies seems strain dependent, because the intrinsic sensitivity to drying methods fluctuates from strain to strain (Otero et al. 2007; Peiren et al. 2015) so, as best practice other alternatives must be explored for freeze-drying and the excipient with the best stability results must be chosen.
Coupling all the above information it can be concluded that the freeze-dried formulation contributed negatively (strains actually became more vulnerable) to *A. muciniphila* resistance to harsh conditions such as low pH in the gastric phase or bile in the intestinal phase.

Considering that the core formulation containing inulin (10% w/v), riboflavin (16.5 mM) and glutathione (0.2% w/v) provoked a significantly lower tolerance of incorporated *A. muciniphila* to simulated GI conditions, it was investigated if the addition of a bulking agent such as starch (0.5 g) to the formulation matrix could improve the efficacy in preserving *A. muciniphila* viability upon freeze-drying The inclusion of starch, a compound used to enhance probiotic strain survival in adverse environmental

conditions including pH shifts and oxygen exposure (Hoyos-Leyva et al. 2018), is an approach that has already been tested with the strictly anaerobic *F. prausnitzii* (Khan et al. 2014).

Therefore, *A. muciniphila* incorporated in core-formulation containing starch (0.5 g) after freeze-drying was also evaluated for its ability to withstand simulated GI conditions. As presented in Figure 3.10, starch did not offer enhanced protection during the freeze-drying process; in fact, the number of viable cell numbers in the formulation with starch upon freeze-drying (6.31 ± 0.03 log CFU g^{-1}) was 4-fold lower than those obtained in the freeze-dried core formulation under the same conditions (10.43 ± 0.09 log CFU g^{-1}). Due to exposure to simulated GI conditions, the viability of *A. muciniphila* decreased approximately 3.5 log CFU in the starch containing core formulation (Figure 3,10).

Despite the lower *A. muciniphila* cell counts upon the freeze-drying process, the declining trend was however much lower in the core formulation containing starch in comparison to that obtained with the non-starch containing core formulation. In particular, a 3-fold higher resistance to lower pH values in the gastric phase is observed (Figure 3.10) which probably is due to the bulking effect of starch enabling a protective matrix effect for *A. muciniphila,* even when submitted to the intestinal conditions.

Given the successful use of starch as a coating material in combination with other compounds, such as calcium alginate, in the encapsulation of *Bifidobacterium* and *Lactobacillus* strains (Sultana et al. 2000; Krasaekoopt et al. 2004), we theorized similar effects would be attained in the powdered core formulation. However, the results presented herein suggest that it promoted a viability reduction during freeze-drying compared to simple core-formulated *A. muciniphila* (Figure 3.10). One can only speculate on such phenomena, but the reason behind the poor viability might be related to the hydrophilic nature of starch that causes the absorption of bacterial intracellular fluid, leading to the decrease in survival, as previously suggested (Amiet-Charpentier et al. 1998). Since moisture content during freeze-drying is rapidly removed, it is plausible that the presence of a hydrophilic compound would cause an opposite effect on preservation. In fact, the application of starch as a co-encapsulation compound in conjunction with alginate results in a low decline in viable cell counts of the alginate-starch encapsulated bacteria (Krasaekoopt et al. 2004; Sultana et al. 2000). The protection offered with this mix can be attributed to the stabilizing effect of starch on the alginate microcapsules, by favoring the formation of polymeric structures (Krasaekoopt et al. 2004; Burgain et al. 2013). Thus, we hypothesize that in

microencapsulation by decreasing the contact of starch with bacterial cells, contrary to the occurrence in the present formulation, this interaction may prevent the absorption of bacterial fluid content promoting efficacy of the method. Taking all in the above consideration, starch seems to, on the one hand, impact negatively on the freeze-drying process, probably by promoting excessive intracellular water removal and on the other, to impact positively as a matrix protector to the harsh conditions of GI transit. Further studies are warranted to consolidate this function duality.

There is also, however, a further point to consider. Freeze-drying stress can induce bacterial cells to enter a “non-recoverable stage of existence” while still rendering them viable (Xu et al. 1982). As mentioned previously in the current thesis (see section 3.1.5), certain stressful conditions, such as freeze-drying can induce a VBNC state in cells. In these dormant stages bacteria are not detected by plating methods (Ramamurthy et al. 2014). Nonetheless, given sufficient conditions, they could revive, in other words, return to a culturable actively metabolizing state (Oliver 1995). Considering this, it was reasoned that the formulation could also evoke this sort of protective response on *A. muciniphila*, not allowing detection by conventional culture techniques, given their VBNC state.

Overall, optimization of formulation protocols requires a careful consideration of all the variables influencing the viability of the bacterial culture. However, information on preservation of this bacterial species remains scarce. Indeed, only one study, conducted by Ouwerkerk and colleagues (2017) demonstrated the optimization of a potentially scalable workflow, paralleled with the application of quality assessment and control practices, for the preparation and preservation of viable cells of *A. muciniphila*. Yet, this preservation method had as main limitation storage in anaerobiosis, which does not depict common household use.

These studies show that there is no single agent that efficiently protects all organisms, and the effect of a protective agent seems to be strain specific. Additionally, the combination of compounds with distinct protective mechanisms yields higher level of probiotic protection during cryopreservation compared to single component application (Hubálek 2003), and this needs to be considered for future assays.

3.2.1.4. Stability of the formulated *A. muciniphila* during storage

Since the probiotic term should only be applied to products that deliver live microorganisms within the appropriate viable count range of the defined strains (7- 9 log CFU/g or mL) to provide health benefits to the host (Hill et al. 2014; Hungin et al. 2018), a proof of delivery in viability of an efficacious dose at end of shelf-life must be

performed. In this manner, with the purpose of assessing the impact of the formulation, and subsequent freeze-drying on stability throughout storage, the viability of formulated *A. muciniphila* cells was compared with cells in PYGM submitted to different storage conditions [-20 ºC, 4 ºC, and room temperature (22 ºC)] up to 7 or 14 days in an aerobic environment. As expected, the mass of freeze-dried formulation incorporating *A. muciniphila* with starch was about ten-fold higher than without starch (Table 3.4).

Table 3.4 – Freeze-dried mass (mean ± SD; g) of formulation with or without starch incorporating A. muciniphila.

Formulation	Lyophilized weight (g)
With Starch	0,49 ± 0,02
Without Starch	0,046 ± 0,006

For both storage at 4ºC and -20ºC, both storage time and *A. muciniphila* form revealed to be statistical significant factors ($p<0.001$). After 7 days of refrigerated aerobic storage (4 ºC), cells in PYGM remained above 8.5 log CFU ml^{-1}, displaying a small viability reduction (0.4 log CFU ml^{-1}), and hence the cell viability was highly stable (Table 3.5). However, frozen aerobic storage lead to a 1.4 log CFU ml^{-1} decrease in cells viability. Concerning the formulated cells, core-formulation without starch revealed a higher initial number of viable cells in comparison to its counterpart with starch (10.43 log CFU $g^{-}1$ vs 6.32 log CFU g^{-1}). After 7 days of refrigerated and frozen storage, core-formulation with starch seemed to contribute toward stability of *A. muciniphila* cells. In fact, core-formulation with starch demonstrated a lower drop in viable cell numbers (below 1 log for both storage conditions), when compared with core-formulation without starch (higher than 1.5 log for both storage conditions). Interestingly, storage temperature had a more significant influence on core-formulated *A. muciniphila* cells viability, since at 4ºC *A. muciniphila* underwent a higher reduction than when stored at -20 ºC for 7 days, i.e. 3.94 log CFU g^{-1} against 1.83 log CFU g^{-1}, respectively.

Table 3.5 - Viability of A. muciniphila DSM2295 cells in PYGM (log CFU mL^{-1}) and formulated (with/without starch) (log CFU g^{-1}) stored aerobically at 4 ºC and -20 ºC for 7 days.

Storage Temperature (ºC)	*Akkermansia muciniphila*	Viable cells (log CFU mL^{-1} or g^{-1})	
		0 d	7 d
4ºC	*Cells in PYGM broth*	8.55 ± 0.21^{a}	8.51 ± 0.19^{a}
	Core formulation without starch	10.43 ± 0.09^{a}	6.49 ± 0.09^{b}
	Core formulation with Starch	6.32 ± 0.03^{a}	5.83 ± 0.17^{b}
-20ºC	*Cells in PYGM broth*	8.55 ± 0.21^{a}	7.12 ± 0.15^{b}
	Core formulation without Starch	10.43 ± 0.09^{a}	8.60 ± 0.04^{b}
	Core formulation with Starch	6.32 ± 0.03^{a}	5.51 ± 0.04^{b}

Different letters represent the statistically significant differences ($p < 0.05$) found between periods of time of storage for each form (A. muciniphila in PYGM broth or incorporated in formulation).

This could be explained by the fact that microbial metabolic activity is greater at temperatures closer to the optimum growth/stability conditions stability (Pedroso et al. 2013). Indeed, Pedroso and colleagues (2013) proposed that storage temperature stability is negatively correlated with bacterial viability and thus, a decrease in temperature is associated with an increased stability.

For storage at room temperature (22 ºC), both storage time and *A. muciniphila* form revealed to be statistical significant factors ($p<0.001$) and again the impact of starch was similar, i.e. negative impact in the freeze-drying process (10.21 log CFU g^{-}1 vs 5.94 log CFU g^{-1}) but positive in terms of lower decreasing trend over 4 d of storage even at 22 ºC.

Table 3.6 - Viability (log CFU g^{-1}) A. muciniphila DSM22959 cells, formulated with and without starch, stored aerobically at ambient air (22 ºC) for 4 days.

Akkermansia muciniphila	**Viable cells (log CFU g^{-1})**				
	0 d	1 d	2 d	3 d	4 d
Core formulation	10.21 ± 0.02^{a}	9.04 ± 0.28^{b}	7.55 ± 0.11^{bc}	7.16 ± 0.07^{bcd}	7.65 ± 0.03^{bc}
Core formulation with starch	5.94 ± 0.27^{a}	$5,92 \pm 0.20^{a}$	ø	5.18 ± 0.21^{b}	5.35 ± 0.12^{b}

Different letters represent the statistically significant differences ($p < 0.05$) found between periods of time of storage for each form (A. muciniphila in PYGM broth or incorporated in formulation).

According to these results, we can hypothesize that the freeze-drying process can induce stress in *A. muciniphila* formulated cells, resulting in a higher susceptibility to the subsequent conditions imposed, namely aerobic storage and simulated GI conditions.

3.2.2. Microencapsulation

Amongst the many encapsulation techniques, emulsification/internal gelation emerges as one of the most user friendly, since it is easy to perform and scale-up the production of microcapsules whilst providing good process efficiency and cell viability (Šipailienė & Petraitytė 2018). As stated previously, in the last years Na-alginate has been one of the most widely employed enteric coating material for microorganism encapsulation (Lopes et al. 2017). Indeed, in a comprehensive biocompatibility study, sodium alginate at 4 % (w/v) was found to be one of the most adequate vectors for immobilization on the tested *Lactobacillus* and *Bifidobacterium* strains, revealing the highest levels of noninhibition and biocompatibility (Rodrigues et al. 2011). Na-alginate is also recognized by its mucoadhesive properties due to its ability to form hydrogen bonds with mucin-type glycoproteins (Lee & Mooney 2012). Moreover, the structure of this natural hydrophilic polysaccharide allows the formation of a 3-dimensional hydrogel matrix by cross-linking with divalent cations such as Ca^{2+}, which can lead to a controlled probiotic release from the polymer matrix (Lee & Mooney 2012). For that, selection of an adequate Ca^{2+} source is crucial for the optimal efficacy of the method. With that in mind, Poncelet et al. (1995) examined potential Ca^{2+} sources to cross-link with alginate and found that microcapsules prepared with the insoluble salt $CaCO_3$ were more stable, sphere and uniform in size (Poncelet et al. 1995; Cai et al. 2014). Notwithstanding, considering that alginate microcapsules tend to be porous (Oddo et

al. 2010), co-encapsulation with other compounds has been shown to have a stabilizing effect on the resulting beads (Sultana et al. 2000; Martin et al. 2013; Wang et al. 2014). For this reason, whey protein isolate (WPI), in particular denatured whey protein isolate (DWPI), was considered as an alginate co-polymer (Rosenberg & Sheu 1996; Doherty et al. 2010). In short, denatured WPI is preferred since thermal denaturation induces structural changes that allow inter- and intramolecular bonding (Anandharamakrishnan et al. 2007; Nicolai et al. 2011), resulting in a firmer, stronger and more flexible matrix, which ultimately provides advantageous conditions for improved cell viability at harsh conditions (Rajam et al. 2012; Doherty et al. 2010; Rajam & Anandharamakrishnan 2015).

Considering all of the above, emulsification/internal gelation was selected to encapsulate *A. muciniphila* and this technique involves a microencapsulation protocol that can be briefly divided into 3 main steps: i) Emulsification, in which the discontinuous phase (Na-alginate solution, $CaCO_3$, DWPI and bacterial suspension) is added to the continuous phase (vegetable oil and surfactant), forming a water in oil (W/O) emulsion; ii) Acidification, in which Ca^{2+} ions are dissociated from $CaCO_3$ by the addition of glacial acetic acid, causing *in situ* gelation and thus forming alginate microcapsules; and iii) Harvesting, where the formed microcapsules are separated from the remainder materials, washed, through the addition of Tween 80 solution and collected.

3.2.2.1. Encapsulation Efficiency

In order to assess de protection efficiency of Na-alginate:$CaCO_3$:DWPI microcapsules for *A. muciniphila* DSM22959, the bacterial survival rate during the microencapsulation process (% EE) was determined. The procedure carried out assured an encapsulation yield of 95.8% ± 0.01.

The encapsulation efficiency in the present work was shown to be greater to those in other studies using Na-alginate microcapsules prepared by a method similar to emulsification/internal ionic gelation such as: Zou et al. (2011) with an EE of 43-50%, for *Bifidobacterium bifidum* F-35; Song et al. (2013) with an EE of 70% for Y235 yeast cells and, Holkem et al. (2016) with an EE of 90% for *Bifidobacterium* spp BB-12, respectively. One of the appointed explanations for the lower EE in some of these studies, was the addition of glacial acetic acid during capsule preparation, causing acid stress that eventually induces cell damage. The concentration and rate at which glacial acetic acid is added into the emulsion should be adequate to initiate gelation reaction, but at the same time decrease cell damage (Song et al. 2013). In fact, Qu et al. (2016)

demonstrated that the optimization of acid/Ca molar ratio and acidification time during microencapsulation by internal gelation can improve encapsulation efficiency by 90%. Conversely, Sánchez et al. (2017) reported one of the most successful studies on emulsification/internal gelation technique using Na-alginate described so far, indicating an EE of 100% for a *Lactobacillus* bacteria (undisclosed strain). The authors modified the original technique published by Poncelet et al. (1992) by replacing the aqueous content with MRS broth. Identically, the values of EE in the present work is similar to of those obtained by extrusion/external gelation, which are typically higher (80-98%) than those reported by other authors employing internal gelation (Krasaekoopt et al. 2003; Martín et al. 2015).

Concerning encapsulation of *A. muciniphila* and according to our best knowledge only two studies have been published (van der Ark et al. 2017; Marcial-Coba et al. 2018). Interestingly, Van der Ark and colleagues (2017) were the pioneers in exploring the encapsulation of this bacteria. They employed a double emulsion technique in which *A. muciniphila* was immobilized in the inner water droplets of double water-in-oil-in-water (W/O/W) emulsion. The obtained initial particle encapsulation efficiency was of 97.5%, which then decreased to 89.6% after 4 days of storage. More recently, Marcial-Coba et al. (2018) described a microencapsulation protocol of *A. muciniphila* DSM22959, in which they used a xanthan and gellan gum matrix, and subsequently freeze-dried the microencapsulated bacteria with different combination of cryoprotective agents. The sweetener agave syrup 10 % (w/v) conjugated with xanthan and gellan gum matrix using extrusion method, exhibited a higher EE (76.2 %) when compared with other cryoprotective agents tested. Even so, this EE value was much lower than reported in the present work (95.8% ± 0.01).

The distinct values obtained in this work and in those described in the literature are mainly due to the diverse immobilization materials and methods, and such, any comparison efforts can be limited. Nonetheless, these results indicate that *A. muciniphila* did not suffer marked damage during the microencapsulation process based on internal gelation, showing it to be an achievable and suitable technique to produce encapsulated *A. muciniphila*. However more encapsulation essays should be performed to consolidate the obtained EE values.

3.2.2.2. Microcapsule morphology and size

As it can be observed in Figure 3.11, the fresh Na-alginate:$CaCO_3$:DWPI microcapsules exhibited good sphericity and clear limits after its production (0 d). The spherical shape provides protection for encapsulated bacteria when implemented in

food products by maintaining textural quality of such products (Song et al. 2013). The particle size is a physical parameter of importance, since it has been suggested that microcapsule diameter should remain smaller than 100 µm in order to avoid a negative impact on the sensory characteristics in food products (Burgain et al. 2011). In the present work, *A. muciniphila* loaded microparticles presented mean diameters of 85 ± 13 µm, which is below the threshold value.

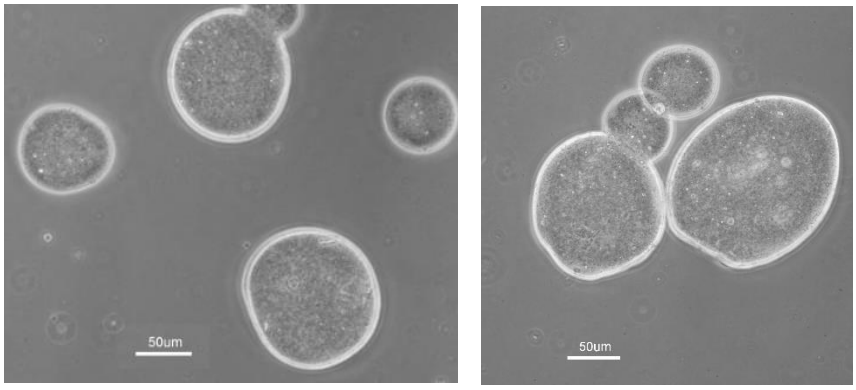

Figure 3.11. Morphology of wet microencapsulates, as assessed under inverted optical microscope post-microencapsulation (0 d). Scale bar = 50 µm.

Importantly, many factors may influence the shape and size of beads. In fact, it has been described that the acidification step in internal gelation greatly affects microcapsule physical properties. Namely Qu et al. (2016) demonstrated that increasing acetic acid/Ca molar ratio and acidification time can result in smaller microcapsules with higher mechanical strength. In similar fashion the presence of insoluble $CaCO_3$ suspended in Na-alginate solutions could prevent correct emulsification, leading to the production of droplets with a wide size distribution (Cai et al. 2014). Additionally, stirring rate has also been appointed as a significant parameter since the resulting shearing force leads to the production of spherical microcapsules (Song et al. 2013). Moreover, it seems that higher diffusion rates of Ca^{2+} through alginate matrix can be detrimental to encapsulation efficiency (Rosas-Flores et al. 2013). Furthermore, the relation between microcapsule size and cell protection is of utmost importance and should be considered (Anal & Singh 2007). While the production of smaller sized microcapsules is important for sensorial acceptance, cell viability must be assured in order to microencapsulation, by internal gelation, meet its designed purpose. The key lies on finding equilibrium between an acceptable viability loss and the production of adequate size microcapsules with low diameters.

3.2.2.3. Stability of the microencapsulated *A. muciniphila* during storage

The variation of viable cells of *A. muciniphila* during 14 days at 4 ºC under aerobic storage, is displayed in Figure 3.12. Microencapsulated *A. muciniphila* demonstrated good stability throughout the 14 d of storage, with a slight decrease in survival from 9.53 ± 0.10 log (CFU g^{-1}) to 9.10 ± 0.07 log (CFU g^{-1}) with a viability loss of only 0.43 log (CFU g^{-1}). For free cells, the survival ranged from 8.98 ± 0.06 log (CFU mL^{-1}) to 8.71 ± 0.12 log (CFU mL^{-1}) presenting a 0.27 log CFU decline, respectively. These variations over the 14 d of storage revealed to be not statistically significant ($p=0.05$); only differences for viable cells after 5 d of storage in comparison to 0 d revealed to be statistically significant ($p<0.05$).

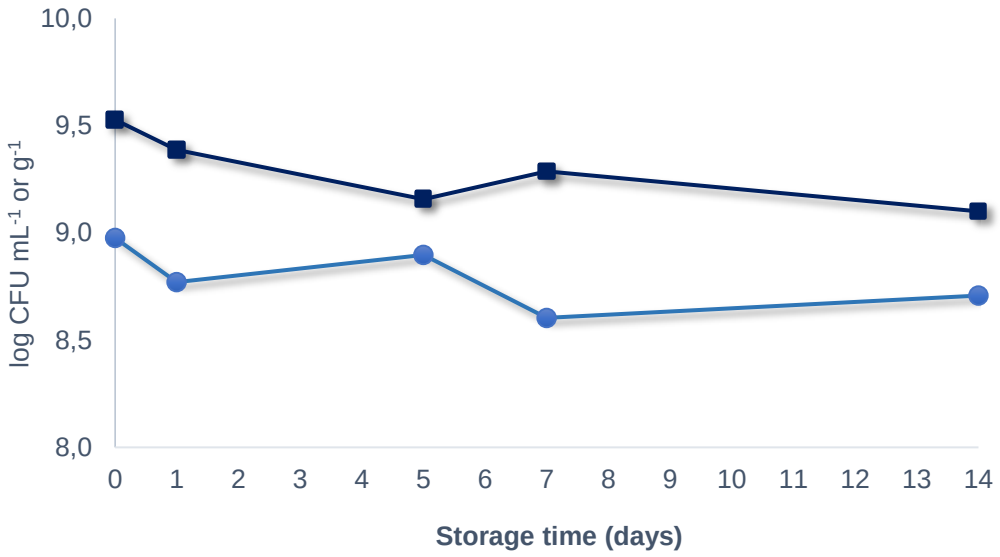

Figure 3.12. Viable cell numbers of free (●) [Log (CFU/mL)] and microencapsulated (■) [Log (CFU/g)] A. muciniphila during 14 days of aerobic storage at 4ºC.

The recommended number of viable cells per dose of probiotic, to ensure the positive health effects on the host, as already stated should range from 7 to 9 log CFU (Hill et al. 2014). For this reason, one of the most important aspects assessed in any formulation matrix is the ability to retain cell viability during storage.

The high values of stability of microencapsulated *A. muciniphila* found in the present work, contrasts with that of Van der Ark and colleagues (2017) work, who reported a 2 log CFU g^{-1} reduction of double emulsion *A. muciniphila* microcapsules after only 72 h of anaerobic storage, at 4ºC.

Another key parameter that may affect encapsulated cells survival seems to be the water content present at the time of storage. The most common strategy to remove residual water to maintain cell culture viability is through procedures such as freeze-drying. Sanchez et al. (2017), reported improvements in cell counts when microcapsule freeze-drying was implemented prior to storage. The same authors appointed that the 100% cell survival throughout long storage periods was also due to the replacement of the aqueous content, that acts as the pellet resuspension fluid, with the bacterial reference growth broth. Furthermore, prolonged storage generates increased stress on the encapsulated probiotics, which may lead to ruptures in the microparticles, increasing the probiotic cells susceptibility to harsh external conditions (Sousa et al. 2015). Hence, it would have been of interest to assess the impact of storage, at 4ºC, in the morphology and structure of the microcapsules. The information about the microcapsules surface topography would have permitted to understand the structure of the capsules, in particular surface porosity, since very porous capsules permit fast diffusion of fluids through the matrix, which can ultimately lead to cell damage (Anal & Singh 2007).

Studies of Sanchez et al. (2017) suggest that freeze-drying of encapsulated bacteria, prior to storage, seems to be a benefic strategy with potential to be implemented. Despite its benefits in preserving cell viability through the removal of water content, freeze-drying still presents deleterious effects to sensitive bacteria, as mentioned before (Arakawa et al. 2001). So, as to protect the bacterial physiological state from deterioration during freeze-drying, cryoprotectants addition would be mandatory if lyophilization was to be implemented during or after microencapsulation procedure.

3.2.2.4. Survival rate of free and microencapsulated *A. muciniphila* exposed to simulated gastrointestinal conditions over storage

As mentioned previously in this work, one of the key requirements of a good probiotic candidate is the ability to reach the large intestine with high viable bacteria load (Succi et al., 2014). Therefore, the resistance to gastrointestinal harsh conditions is paramount. Free cells and Na-alginate:$CaCO_3$:DWPI microcapsules, containing *A. muciniphila*, after 1 and 7 d storage at typical refrigeration conditions (aerobiosis, at 4 ºC), were exposed to simulated GI tract - due to constraints pertaining to the amounts of sample available, it was not possible to carry out GI assays of microcapsules for 14 days of storage. The resulting data is shown in Figure 3.12. Overall, there was observed a significant ($p<0.05$) reduction in viability of both free and

microencapsulated *A. muciniphila* with 1 d storage when exposed to GI. During simulated gastric phase, viability of both free and microencapsulated *A. muciniphila* cells remained relatively stable with a 0.16 log CFU and 0.29 log CFU drop, respectively. On the other hand, after a considerable 0.57 log CFU drop in the first hour of intestinal phase, microencapsulated *A. muciniphila* viability remained stable with no significant alterations ($p>0.05$) in viability, whilst its free counterpart maintained stability throughout the entire phase.

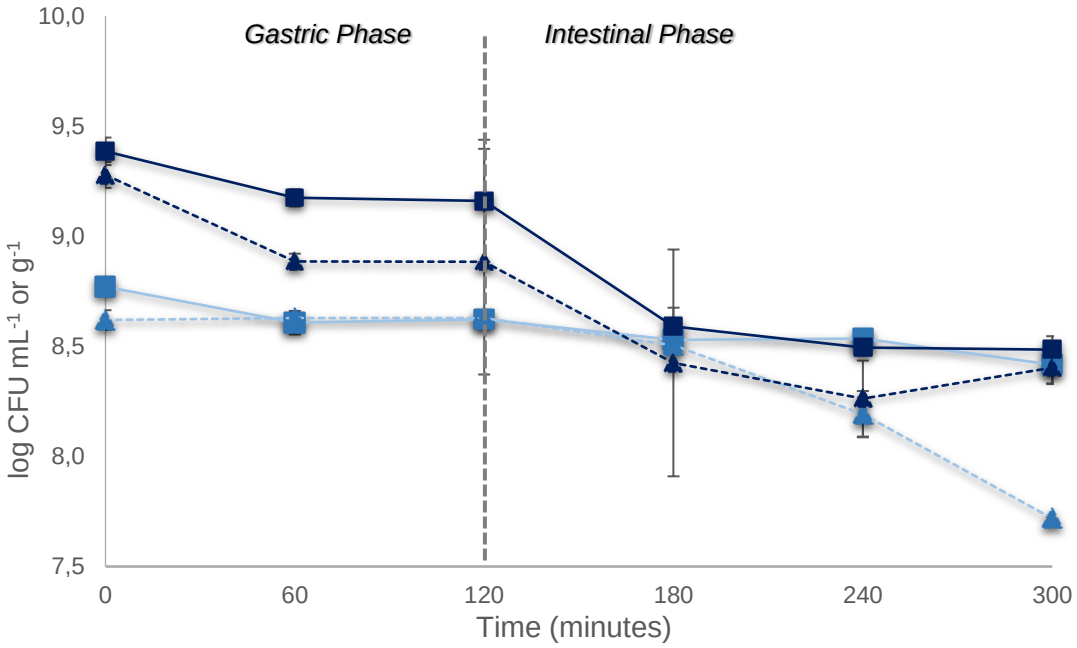

Figure 3.13 - Viable cell numbers of free [Log (CFU mL^{-1})] and microencapsulated [Log (CFU g^{-1})] A. muciniphila during simulated GI conditions, after aerobic storage, at 4 ºC. Caption: free (light blue) and microencapsulated A. muciniphila (dark blue); 1 d storage (■) and 7 d storage (▲ and dotted).

Despite some authors question alginate protective effects in the presence of chelating agents – low stability has been observed in pH conditions bellow 2.0 (Smidsrød & Skjåk-Braek 1990; Ding & Shah 2007) - the improvement in protection to acidic conditions in gastric phase by the alginate:$CaCO_3$ combination demonstrated in previous reports goes in line with our results (Poncelet et al. 1992; Cai et al. 2014; Qu et al. 2016). Furthermore, the implementation of DWPI as a co-encapsulant agent might overcome this limitation since it reduces microcapsule porosity offering higher stability to the end product (Doherty et al. 2010; Rajam et al. 2012). On the other hand, free cell stability to GI conditions might be explained by an acid resistance system (Ottman 2015) and the ability to colonize nursing infants, as aforementioned in the

current thesis (Petschacher & Nidetzky 2016). The resistance of *A. muciniphila* free cells against simulated intestinal conditions may be related with reduced activity of bile salts upon membrane of gram negative bacteria (Begley et al. 2005). In contrast, bile exposure is able to induce a swelling effect on alginate microcapsules with subsequent cell damage (Qu et al. 2016), which may explain the viability loss of alginate microcapsules in intestinal phase.

With respect to the performance to GI conditions after 7 d of aerobic refrigerated storage (Figure 3.12), microencapsulated *A. muciniphila* cells displayed a similar pattern to that of GI assay at the 1 d of storage with differences at the final stages of intestinal phase. After the first hour of the intestinal phase, free cells suffered a significant viability drop ($p<0.05$), contradicting the stable tendency displayed with 1 d of storage. With the prolonged storage time of 7 d, microcapsules showed higher stability when compared to free cells demonstrating the potential protecting effect of microencapsulation. Overall, emulsification/ionic internal gelation with Na-alginate:$CaCO_3$:DWPI matrix provides an additional protection against detrimental storage conditions. providing a gentle method, with limited physical stress on the cells. Nevertheless, microencapsulation did not bring on as much protection as needed to assure high viable cells levels in the intestinal phase, suggesting that coating reinforcement with other polymers, such as a lipidic component, is needed upon intestinal phase. Therefore, future works should contemplate studying the addition of compounds aimed at promoting a more robust capsule.

4. Concluding Remarks

Nowadays, *A. muciniphila* has been described as a promising candidate for NGP category with exciting potential to positively impact the fight against metabolic and inflammatory dysbiosis-derived diseases. However, a high sensitivity of this bacterium to oxygen and pH shifts reported in previous studies has so far precluded its application namely in the successful development of nutraceutical/therapeutical formulations. In this thesis, strategies to increase viability and stability of *A. muciniphila* throughout detrimental GI conditions and aerobic storage were investigated.

In the first phase, where an initial phenotypic characterization of *A. muciniphila* DSM 22959 was performed due to scarcity of information regarding physiological features of this novel probiotic microorganism results confirmed that *A. muciniphila* cells are Gram-negative, coccobacillary-shaped, and demonstrated myristoleic and pentadecanoic acids to be the major fatty acids present in the bacterial membrane. Morphologically, colonies were shown to be small, circular and translucent.

An important conclusion to be highlighted is the fact that it is shown for the first time that *Akkermansia muciniphila* has a higher oxygen tolerance than that reported in literature; data reported herein demonstrated that *A. muciniphila* was able to survive up to 60 hours after exposure to aerobic environment, contradicting previously classification as being an extreme oxygen sensitive bacteria. Furthermore, results also showed that *A. muciniphila* adheres avidly to colon epithelial cell lines (Caco-2 and HT29-MTX), confirming their ability to colonize gut epithelium. In the second phase, freeze-dried formulations and encapsulation were explored as technological strategies to enhance viability and stability of *A. muciniphila* to GI transit and aerobic storage. Overall, the freeze-dried formulation containing inulin (10 % w/v), riboflavin (16.5 mM) and glutathione (0.2 % w/v) enabled the achievement of viable cell numbers above the 10^{10} CFU g^{-1} threshold. However, this freeze-dried formulation, even when supplemented with starch (0.5 g), did not offer any additional protection to *A. muciniphila* throughout simulated GI conditions and aerobic storage. In fact, the number of *A. muciniphila* viable cells when present in formulations (with or without starch) dropped more significantly under *in vitro* GI transit than did non-formulated cells submitted to the same conditions. Likewise, higher stability during refrigerated and frozen storage was reported for non-formulated *A. muciniphila* cells, followed by formulation with starch.

In contrast, microencapsulation by internal gelation using a Na-alginate:$CaCO_3$:DWPI matrix was demonstrated to be an efficient method to immobilize a high *A. muciniphila* cells load (%EE = 95.8% ± 0.01). Morphologically, these microcapsules displayed a

spherical shape with diameter size smaller than 100 μm that are pivotal features in sensory analysis. Furthermore, the Na-alginate:$CaCO_3$:DWPI microcapsules were able to protect *A. muciniphila* against detrimental aerobic refrigerated storage as well as against simulated harsh GI conditions. An almost 10-fold higher survival of the encapsulated cells was observed by 7 d of storage. Hence, microencapsulation with Na-alginate:$CaCO_3$:DWPI matrix seems to be a better strategy to enhance the viability of *A. muciniphila* throughout GI passage and to promote high stability in aerobic storage.

Overall, this thesis takes the first steps to modulate *A. muciniphila* growth, stability and viability at ambient air toward their manipulation and incorporation in new delivery vectors (food or supplement) and facilitating its mechanistic characterization.

5. Future Work

The results presented in this thesis provided valuable information on some phenotypic features of *A. muciniphila* and presented the first insights regarding freeze-dried formulations and microencapsulation as technological strategies to increase *A. muciniphila* viability during simulated GI conditions and stability during storage. Nevertheless, it has also raised some questions that should be addressed in future research work.

Since the phenotypic characterization of *A. muciniphila* DSM22959 in terms of membrane fatty acids composition and gut epithelium adhesion was performed when bacterium was grown according to optimal nutritional and environmental conditions, further studies involving a more comprehensive phenotypic characterization of this bacterium when exposed to stressful conditions, such as O_2 exposure, should be conducted. In this case, monitorization of organic acids including specific short chain fatty acids is also important to study in order to assess impact of stress conditions on metabolic modulation. Still in the context of adhesion to gut epithelium, it would be interesting to perform a structural analysis in order to identify the adhesins or bond type involved, since such knowledge would allow the development of better delivery tactics of this NGP. Also, the effect of selected prebiotic compounds such as inulin or FOS upon *A. muciniphila* adhesion to human intestinal epithelium should also be evaluated, which could reveal the need to develop synbiotic strategies. Also, further studies evaluating other probiotic properties of *A. muciniphila* such as production of antimicrobial substances, biofilm formation and ability to compete, displace or exclude adhesion of enteric pathogens, should be performed.

Notwithstanding the preliminary results with FCM analysis, this method remains as a promising complementary tool for the enumeration of *A. muciniphila* viable cells and its metabolic evaluation. The optimization of FCM analysis including strategies aimed at removing capsular involucre to enable the correct analysis shall be employed in future analyses. Also, coupling with molecular techniques such as RT PCR and FISH, could circumvent the issues inherent to FCM analysis.

It could also be said, that the present formulation efforts also showcased that there is no single agent that efficiently protects all organisms, and so the effect of a protective agent seems to be strain specific and mainly dependent on the formulation method. For this reason, other cryoprotective agents and their combinations (distinct protective mechanisms might yield higher level of probiotic protection during cryopreservation compared to single component application) need to be considered for future assays.

The most relevant advance proposed by this thesis is the microencapsulation by emulsification/internal gelation, using Na-alginate:$CaCO_3$:DWPI. Nevertheless, due to the sensitivity displayed at the intestinal phase, the incorporation of lipidic compounds into microencapsulation formula could futher improve *A. muciniphila* survival during GI conditions. In that regard, the assesment of storage and GI conditions impact on microcapsule structure could provide a more comprehensive understanding of the necessary method adjustments.

Lastly, both formulation and microencapsulation efforts could benefit with the application of sub-lethal stress treatments which allow the activation of metabolic pathways that permit the re-adaptation to harsh conditions, such as freeze-drying and storage, impacting the survival rates during industrial formulation and ultimately in the gut.

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