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Effect of emulsification/internal gelation-based microencapsulation on the viability of *Akkermansia muciniphila* upon prolonged storage and simulated gastrointestinal passage

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ABSTRACT

Akkermansia muciniphila is a common human intestinal commensal with a mucin-degrading nature. Its immunomodulatory characteristics and regulatory role of mucus layer and gut barrier integrity highlight the potential benefits of using this bacterium as an interventional player against inflammatory/cardio-metabolic disorders. In this work, we evaluate the effect of microencapsulation by the emulsification/internal gelation method on *A. muciniphila* survival during aerobic storage (0, 15, 30 and 95 days) and subsequent exposure to simulated gastrointestinal passage, in comparison with that of free cells. The present results show that microencapsulation by internal gelation promotes a 64.4 % entrapment efficacy of *A. muciniphila* cells (maintaining a 10^8 order of magnitude for cell viability). Moreover, physical characterization showed that microparticles mean size was 53,5 ± 12,1 μm and, as observed by electron scanning microscopy, microcapsules were spherical in shape. More importantly, as storage time increased, encapsulated *A. muciniphila* demonstrated higher stability in GI conditions, when compared to its free counterpart. In conclusion, microencapsulation by internal gelation seems to be an appropriate strategy in protecting *A. muciniphila* against the detrimental gastrointestinal transit after long periods of aerobic refrigerated storage.

1. Introduction

The ability that specific microbial strains possess in altering physical phenotypes in humans has been well documented. Specifically, when live microorganisms intervene beneficially in host health, whilst administered in sufficient quantities, they are to be considered as probiotics (Hill et al., 2014). Controlled studies clarify the mechanistic contexts through which these microorganisms operate on the host. Among these, *Lactobacillus* and *Bifidobacterium* genera are extensively investigated and, thus, the most widely employed as marketed probiotics (Almeida et al., 2020). The primary probiotic-mediated benefits are linked to pathogen colonization inhibition – via competitive exclusion –, enhancement of gut barrier function and neuroendocrine-modulatory capacities (Cani, 2018). With the advent of high throughput technologies promoting the understanding on gut microbial composition and its influence on human health (Papadimitriou et al., 2015), human-derived *Akkermansia muciniphila* emerged as a next-generation probiotic (NGP) (Andrade et al., 2020). This commensal is a mucin-degrading aerotolerant Gram-negative strain (Derrien, Vaughan, Plugge & de Vos, 2004) that resides in the mucus layer, representing 3 to 5 % of the total human intestinal microbiota (Derrien, Collado, Ben-Amor, Salminen, & De Vos, 2008). Growing scientific evidence has been associating *A. muciniphila* reduction or depletion to several inflammatory/cardio-metabolic disorders including obesity, type 2 diabetes, and inflammatory disorders (Everard et al., 2013; Li, Lin, Vanhoutte, Woo & Xu, 2016; Schneeberger et al., 2015), reflecting the central importance of this NGP in the kinetics of cardio-metabolic regulation and immune responses (Cani & de Vos, 2017; Ottman, Geerlings, Aalvink, de Vos & Belzer, 2017). The putative mechanisms by which *A. muciniphila* supports the enhancement of immune function, improvement of glucose metabolism and low-grade inflammation, are through the supplementation of energetic sources (such as SCFAs) and the maintenance of the mucus layer

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thickness (Belzer et al., 2017; Reunanen et al., 2015). After data collection regarding the qualified presumptions of safety, genomic sequence, antibiotic resistance, and toxicological profiles – required for the evaluation of any new potential probiotic strain (Brodmann et al., 2017) –, it is important to investigate optimal production and stability parameters for the possible probiotic incorporation into commercialized products. Assuring a strain effectiveness on the host comprehends essentially the assessment of survival during product shelf-life and, after consumption, during gastrointestinal tract (GI) passage (Andrade et al., 2020). Regarding the latter, the resilience of *A. muciniphila*, formerly regarded as strictly anaerobic, to GI conditions and different temperatures was demonstrated in a recent study (Machado et al., 2020). Notwithstanding the importance of the high survivability to GI passage, probiotic strains must also succeed in maintaining viability during long periods of aerobic storage (room temperature or refrigeration are usually recommended) until utilization (Andrade et al., 2020). In this manner, devising an approach that guarantees protection of *A. muciniphila* during the exposure to environmental effects is required to develop a probiotic supplement characterized by its effectiveness and stability.

Among the various strategies used to improve probiotic viability, microencapsulation has grown to be one of the most explored by providing a physical barrier. In particular, emulsification/internal gelation of biopolymers emerges as a user-friendly methodology, since it is easy to perform and scale-up, whilst providing good process efficiency and cell viability (Šipailienė & Petraitytė, 2018).

In the last years, sodium-alginate (SA) has been widely employed as an enteric coating material for microorganism encapsulation (Lopes et al., 2017; Zhang et al., 2021; Ramos et al., 2018). In a comprehensive biocompatibility study, the seaweed polysaccharide was found to be one of the most adequate vectors for immobilization at 4 % (m/v) on the tested *Lactobacillus* and *Bifidobacterium* strains, revealing the highest levels of non-inhibition and biocompatibility (Rodrigues et al., 2011). Sodium-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 (Lee & Mooney, 2012). The typically Ca^{2+}-mediated crosslinking events form a polymeric matrix, allowing an increased storage stability and a controlled release of the microencapsulated bioactive compounds. To achieve successful microencapsulation with crosslinked alginate, selection of an adequate Ca^{2+} source is crucial. Poncelet et al. (1995) examined potential Ca^{2+} sources and found that microcapsules prepared with the insoluble salt $CaCO_3$ were more stable, spheric in shape and uniform in size (Cai et al., 2014). These reversible meshes can be accomplished by either internal or external gelation. Encapsulation by extrusion/external gelation is a simpler method that, despite the high cell viability conservation, produces capsules larger than 500 μm, which can ultimately negatively impact sensorial analyses, impairing successful incorporation into food products (Prisco, Maresca, Ongeng & Mauriello, 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. This makes it as one the most promising encapsulation techniques for future applications (Šipailienė & Petraitytė, 2018). Notwithstanding, considering that crosslinked alginate microcapsules tend to be porous - an unwanted factor that could lead to burst effects and facilitate the release of active low molecular weight compounds (Oddo et al., 2010) -, co-encapsulation with other compounds has been shown to have a stabilizing effect on the resulting beads (Martin, Lara-Villoslada, Ruiz & Morales, 2013; Sultana et al., 2000; Wang, Korber, Low & Nickerson, 2014). Whey proteins are used as gelling agents in the food industry and produce robust, pH sensitive hydrogels, with controlled permeability, thereby improving cell viability. For example, when added as co-polymer, whey protein isolate (WPI), in particular in its denatured form (DWPI), was shown to enhance the efficacy of the microcapsules by filling the pores of alginate capsules, which contributes to a firmer immobilization structure (Chen & Subirade, 2006; Doherty et al., 2010; Rosenberg & Sheu, 1996).

Based on the above rationale, the aim of this study was to produce *A. muciniphila* DSM 22,959 microcapsules by emulsification/internal gelation and assess survivability throughout simulated *in vitro* GI passage for a period up to 95 days of storage, presenting a good foundation for the commercial application of this strain.

2. Materials and methods

2.1. Materials

All solutions were prepared in previously sterile water H_2O. Sodium-alginate (SA) (Sigma-Aldrich, USA) was prepared to a concentration of 4 % m/v, homogenized at room temperature overnight with the aid of a magnetic stirrer, and stored at 4 °C. Denatured whey protein isolate (DWPI; Pure Whey Isolate™ 97, Bulk Powders, UK) at 8 % (m/v) was prepared following the procedure described by Hébrard et al. (2010), except for denaturation time, which was 30 min. Commercial vegetable oil (edible grade) was used as an oil phase to prepare the microcapsules without further purification. All other chemicals were of reagent or analytical grade.

2.2. Inoculum preparation

Freeze-dried culture of *Akkermansia muciniphila* strain DSM 22,959 was purchased from DSMZ collection (Leibniz Institute DSMZ - German Collection of Microorganisms and Cell Cultures, Germany) and stocked at −80 ºC in PYGM broth (DSMZ reference growth medium: 104 PYG modified, supplemented with 0.05 % (m/v) of mucin (Sigma-Aldrich, USA)) with 20 % (v/v) glycerol (Fisher Chemical, UK). Bacteria were propagated in PYGM broth in an anaerobic incubator (Whitley DG250 Anaerobic Workstation, Don Whitley Scientific Limited, UK) under an atmosphere of 85 % N_2, 5 % H_2 and 10 % CO_2. At least two subsequent culturing steps using the same growth conditions were performed, with a final incubation volume of 4 L of PYGM broth with 10 % (v/v) of cell inoculation, to obtain a higher *A. muciniphila* biomass yield. Cell biomass was harvested by centrifugation (Sorvall LYNX 4000, Thermo Scientific) at 12 000 x g/ 30 min, at 4ºC, and washed once with the same volume of sterile phosphate buffer saline (PBS x1; VWR, USA). After centrifugation, the pelleted biomass was resuspended in isotonic saline (NaCl; 0.9 % m/v) solution, to a final concentration of 1.3×10^9 colony forming units (CFU/mL). Five different batches were prepared and pooled for sample variability harmonization. The resulting bacterial suspension was either used for microencapsulation (10 mL per microencapsulation batch) or used directly for free cell control.

2.3. Microencapsulation by emulsification/internal gelation method

The emulsification/internal gelation technique used herein to encapsulate *A. muciniphila* (Figure S1), followed the procedure proposed by Poncelet et al. (1992) with some modifications suggested by Zou et al. (2011) and Holkem et al. (2016). Briefly, suitable volumes of DWPI and SA solutions were mixed with 10 mL of concentrated cell suspension to obtain a final concentration in the internal phase of 2 % SA and DWPI, respectively. Microcrystalline $CaCO_3$ (Omya, Switzerland) was added to the mixture (1.4 mmol), and after homogenization, the mixture was dispersed into vegetable oil (40 % internal phase ratio, v/v) containing 1 % (v/v) Span 80 (Sigma-Aldrich, USA) by stirring at 600 rpm using an electronic overhead stirrer (DLS, Velp Scientifica, Italy). After 15 min emulsification, 8.7 mmol of glacial acetic acid (Fisher Scientific, UK) was added to the w/o emulsion using a 1 mL syringe (Thermo®) and a syringe driver (Multi-Phase™ NE-1000; Pump Systems Inc, USA) set at a 0.05 mL min^{-1} flow. Stirring continued for 15 min to permit $CaCO_3$ solubilization. Microparticles were recovered

from lipid phase by using $CaCl_2$ 0.05 M (Honeywell Fluka™, USA). Once microspheres were settled down, the top layer lipid phase was removed by aspiration using a vacuum pump (KNF Laboport®, USA) and the microparticles were successively washed with 1 % Tween 80 (Sigma, Germany) in $CaCl_2$ 0.05 M and 0.9 % NaCl solution to remove remnant oil. The microparticles were harvested upon centrifugation for 5 min at 4470 xg (Universal 320|320 R; Hettich, Germany). The wet microparticles obtained this way were used in the subsequent experiments.

2.4. Enumeration of free and microencapsulated A. muciniphila cells

For the enumeration of cultivable free *A. muciniphila* cells decimal dilutions were performed in PBS and spotted, in triplicate, on PYGM agar plates (PYGM broth supplemented with 1.5 % (m/v) agar (Liofilchem, Italy)). Plates were incubated for 5–7 days at 37 ºC under anaerobic conditions, and results were expressed in colony forming units (CFU/mL). Concerning the encapsulated bacteria, the microparticles were disintegrated using a cordless pellet pestle (max speed 3000 rpm, Kimble® Chase Pellet Pestle®, USA) for 30–60 s in phosphate buffer (0.1 M, pH 7.0). This treatment was previously tested and it was shown not to affect the viability of *A. muciniphila* cells (unpublished results). The resulting suspension was then serial diluted, as described for the free cells, and the results were expressed as CFU/g.

2.5. Encapsulation efficiency (% EE)

The encapsulation efficiency (% EE) is a combined measurement of the efficacy of entrapment and the survival of viable cells during the microencapsulation process, and it was calculated according to the following Eq. (1) (Martin et al., 2013):

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

where N is the number of CFU released from the total obtained microcapsules mass (g) and N_0 is the CFU in the total cell concentrate (mL) used for the microencapsulation process.

2.6. Characterization of Alg:DWPI microparticles

2.6.1. Microcapsule size and morphology

Microcapsule characterization was achieved by size and morphology 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 acquire digital images. Microcapsule size was measured using ImageJ (1.52a; https://imagej.nih.gov/ij/), resulting from average of 650 individual measurements. To assess microcapsule morphology by scanning electron microscopy (SEM), microparticles were initially recovered using a glass filter funnel (porosity 1; Schott Duran®), by gravity so that the microparticles would not aggregate and, afterwards, fixation, dehydrating, and drying steps were performed. After recovery, freshly produced microcapsules were chemically stabilized by immersion fixation in a 2.5 % (v/v) glutaraldehyde (Sigma-Aldrich, USA) solution for 1 h. Then, microcapsules underwent a graded ethanolic series of dehydration ethanol (30, 50, 70, 80, 90, 100, 100 % for 10 min each), at room temperature, with a final drying step with hexamethyldisilazane (HMDS; Sigma-Aldrich, USA), which was immediately evaporated under a gentle stream of N2. Dried samples were placed on top of double-sided adhesive carbon tape (NEM tape; Nisshin, Japan) mounted on SEM stubs and coated with gold/palladium in a sputter coater (Polaron, Germany), and viewed under a JEOL JSM-5600LV (JEOL, Japan) scanning electron microscope at an accelerating voltage of 30 kV..

2.6.2. Determination of moisture

The moisture was gravimetrically determined by loss on drying. Wet *A. muciniphila* loaded microparticles were weighed and then submitted to freeze-drying: samples were placed at −80 ºC for 24 h and then freeze-dried in Edwards Modulyo 4 K Freeze Dryer at a condenser temperature of - 60 ºC and a chamber pressure of 10^{-1} torr for 46 h. The residual moisture was determined according to Eq. (2):

$$\% \text{ Water} = \frac{\text{Initial mass (g)} - \text{Final mass (g)}}{\text{Initial mass (g)}} \times 100 \quad (2)$$

2.7. Survival of free and microencapsulated A. muciniphila cells under storage

The impact of microencapsulation on *A. muciniphila* cells viability and stability was evaluated, in comparison to free cells, upon either aerobic or anaerobic storage, at 4ºC. Aerobic atmosphere was reached by incubating the storage containers at ambient air, allowing the permeation of oxygen, whereas anaerobiosis was achieved using AnaeroGen™ system (ThermoFisher Scientific, USA), in an anaerobic chamber. Total viable cell numbers of stored *A. muciniphila* samples were determined at 1, 15, 30 and 95 days, following the procedures described in 2.4. Cell viability of each condition was expressed in CFU/g, when resulting from the microcapsules, or CFU/mL from free cells, at the evaluated storage timepoint.

2.8. Survival of A. muciniphila cells through in vitro simulated GI passage

The viability of free and encapsulated *A. muciniphila* in simulated GI conditions was determined 1 day upon microencapsulation procedure and after 30 and 95 days of aerobic refrigerated storage, using a standardized procedure described by Minekus et al., 2014, with some modifications. Briefly, either 0.5 mL of free cells in 0.9 % NaCl (m/v) or 0.5 g of *A. muciniphila* loaded microcapsules, were distributed into independent tubes (two replicas per timepoint, per condition). To replicate the temperature and mixing conditions that prevail during human GI transit an orbital shaker incubator (Wiggen Hauser, Germany) was used at 37 ºC, at 150 rpm, and the tubes were placed at an acute-angled fashion. For each experiment, all enzymatic solutions were freshly prepared. For the esophagus-stomach step (gastric phase), samples were exposed to 2 mL of simulated gastric fluid (pH 3), with pepsin (2000 U/mL – from porcine gastric mucosa; Sigma Aldrich, USA) for 120 min. Intestinal conditions were simulated for an additional 180 min by adding 4 mL of simulated intestinal fluid, containing pancreatin (based on the trypsin activity at 100 U/mL in the final mixture; Sigma Aldrich, USA) and bile salts (10 mM; Sigma-Aldrich, USA); pH was adjusted to 7 with 6 M HCl. Sampling points were selected to follow the simulated gastric and intestinal effects and those were: upon 60 and 120 min in the gastric phase and upon 60, 120 and 180 min in the intestinal phase (Figure S2). For each pre-defined sampling point, cell viability of each condition was determined, according to procedures described in 2.4, and normalized to the value at $t = 0$ (V_0 = cell viability at 0 h) hours and plotted *versus* time. The GI protocol was performed under aerobic conditions, whilst the PYGM plates were incubated anaerobically.

3. Results and discussion

3.1. Microcapsule morphology characterization

To further understand the microcapsule morphology, freshly prepared *A. muciniphila* loaded microcapsules were observed under optical microscopy and scanning electron microscopy (Figs. 1 and 2, respectively).

As shown in Fig. 1, the optical micrographs of wet Alg:DWPI microcapsules presented mostly spherical structures, with no apparent formation of aggregates.

Interestingly, homogeneously distributed inner granules can be observed within wet Alg:DWPI microcapsules. Although not common

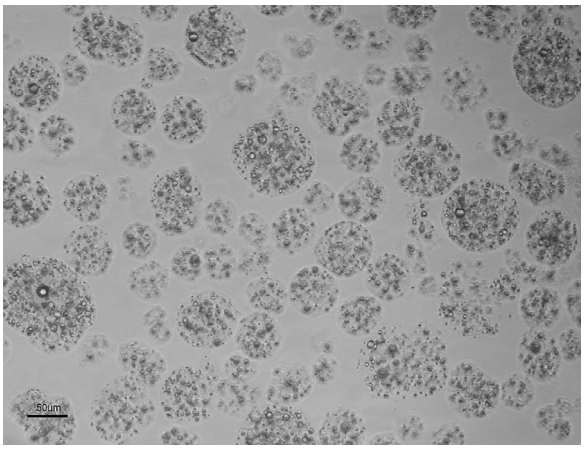

Fig. 1. Representative optical micrograph of *A. muciniphila* DSM 22,959 Alg:DWPI microcapsules with various diameters. Magnification: 100x. Samples were freshly prepared.

for single emulsions, these structures were previously observed by Chen and Subirade (2006), attributing their formation to the emulsion formed when whey protein and alginate solutions are mixed. The photomicrographs obtained by SEM (Fig. 2) illustrated microcapsules exhibiting a shriveled spherical "blackberry-like" structure (Fig. 2a-b). SEM results also showed the presence of entrapped *A. muciniphila* throughout the wall matrix surface of the fresh microcapsules (Fig. 2d).

The observed surface topography roughness has been previously detected in formulations containing heat-treated WPI (Rajam & Anandharamakrishnan, 2015; Rajam, Karthik, Parthasarathi, Joseph & Anandharamakrishnan, 2012), in which the induced structural changes provide inter- and intramolecular bonding that could explain these morphological features (Anandharamakrishnan, Rielly & Stapley, 2007; Nicolai, Britten & Schmitt, 2011). Comparatively, when DPWI was omitted from the formulation, microcapsules surface was smooth but also revealed slight surface depressions (results not shown). Particle size is also a physical parameter of importance, since it has been suggested that microcapsule diameter should remain smaller than 100 μm, to avoid a negative impact on the sensory characteristics in food products (Burgain, Gaiani, Linder & Scher, 2011). The present results demonstrate the production of microcapsules with a mean diameter of 53,5 ± 12,1 μm, which is below the abovementioned threshold value. As stated, adequate adjustments were previously conducted, focusing on the methodological aspects that are known to influence microcapsule physical properties (data not shown), such as acidification time and acetic acid volume, and $CaCO_3$ concentration, which has enabled the production of small sized microcapsules. For instance, Song, Yu, Gao, Liu and Ma (2013) indicated the emulsion stirring rate as a significant parameter, since the resulting shearing force leads to the production of spherical microcapsules; however, it seems that higher diffusion rates of Ca^{2+} through alginate matrix can be detrimental to encapsulation efficiency (Rosas-Flores, Ramos-Ramírez & Salazar-Montoya, 2013). Another important factor is acidification, in which an increased acetic acid/Ca molar ratio and acidification time can result in smaller microcapsules, with higher mechanical strength (Qu et al., 2016). Additionally, presence of residual insoluble $CaCO_3$ in SA solutions could prevent complete emulsification, which can result in an increase in microcapsule size, and thus high concentration of $CaCO_3$ should be avoided (Cai et al., 2014; Silva, Ribeiro, Figueiredo, Gonçalves & Veiga, 2006). Furthermore, fresh microcapsules were found to have an average moisture of 92,2 % (± 0.006), which is a typical property of alginate hydrogels due to the retention of large volumes of water – or biological fluids - in the intermolecular space as result of the presence of hydrophilic functional groups in the three-dimensional network (Lee & Mooney, 2012).

3.2. Encapsulation Efficiency

The encapsulation efficiency (% EE), based on the total *A. muciniphila* cell concentrate used for microencapsulation (6.5×10^{10} CFU) and the resulting microcapsule mass (4.2×10^{10} CFU), was of 64.4 % ± 6,28 %. These values indicate that this approach is adequate for *A. muciniphila* immobilization since the order of magnitude of cell density was maintained. A very wide range of % EE has been reported by several authors for different probiotic strains, using similar emulsification/internal ionic gelation methods, such as Zou et al. (2011) with an EE of 43–50 %, for *B. bifidum* F-35; Song et al. (2013) with an EE of 70 % for Y235 yeast cells, Holkem et al. (2016) with an EE of 90 % for *B. animalis* spp BB-12, and Ji et al. (2019) 95 % for *B. longum* DD98. It has been shown that several factors affect emulsification/internal gelation technique yield, one of which is 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 to 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 could improve encapsulation efficiency by 90 %. On the other hand, the replacement of the aqueous content with the appropriate media broth could also benefit EE, as Sánchez, Ruiz, Lasserrot, Hormigo and Morales (2017) reported for an undisclosed *Lactobacillus* strain, in which they also modified the original technique published by Poncelet et al. (1992) achieving an 100 % EE. Regarding *A. muciniphila* live cells immobilization, van der Ark et al. (2017) reported a 97.5 % EE with a water-in-oil-in water double emulsion, although viability suffered a sharp reduction after only 72 h of refrigerated anaerobic storage. Later, another research group described two microencapsulation protocols in which *A. muciniphila* strain DSM22959 was trapped using a conventional extrusion method (Marcial-Coba et al., 2018; Marcial-Coba, Saaby, Knøchel & Nielsen, 2019). According to the authors, the application of a xanthan/gellan gum polymeric matrix for cell immobilization, with a subsequent freeze-drying step, led to an EE of 76.2 %. It is worth noting that, in the referenced works, the % EE was determined using log-transformed data, which harmonizes raw data by conforming it to normality (Feng et al., 2014). Indeed, if applied to our data, the logarithmic transformation would render the present work an EE of 98.2 %. While the diversity of immobilization materials and methods can explain the distinct values obtained in this work and in those described in the literature, our results indicate that *A. muciniphila* microencapsulation process, based on emulsification/internal gelation, appears to be a suitable technique for production of *A. muciniphila* loaded microparticles.

3.3. Stability of the microencapsulated A. muciniphila during storage

The recommended number of viable cells per serving of probiotic, to ensure positive health effects on the host, should range from 10^7 to 10^9 CFU per dose (Hill et al., 2014). For this reason, one of the most important aspects in any formulation matrix is the ability to support cell viability during storage. In a previous study, regarding the culturability of free *A. muciniphila* cells, our group found that refrigeration and room temperature stood as the storage modalities with the least impact on survival (Machado et al., 2020). Furthermore, studies of Marcial-Coba et al. (2018); Marcial-Coba et al., 2019 reported better results at 4 ºC (refrigeration), when determining cell stability using various encapsulation formulations. Additionally, it is known that refrigeration promotes higher shelf-life of probiotic formulations, since it generates physiological responses in the bacteria which enables the adaptation to a colder environment and, ultimately, in a few instances, induce an increased resistance to gastric acid conditions (Nguyen et al., 2016). Taking this into account, free and microencapsulated *A. muciniphila* were

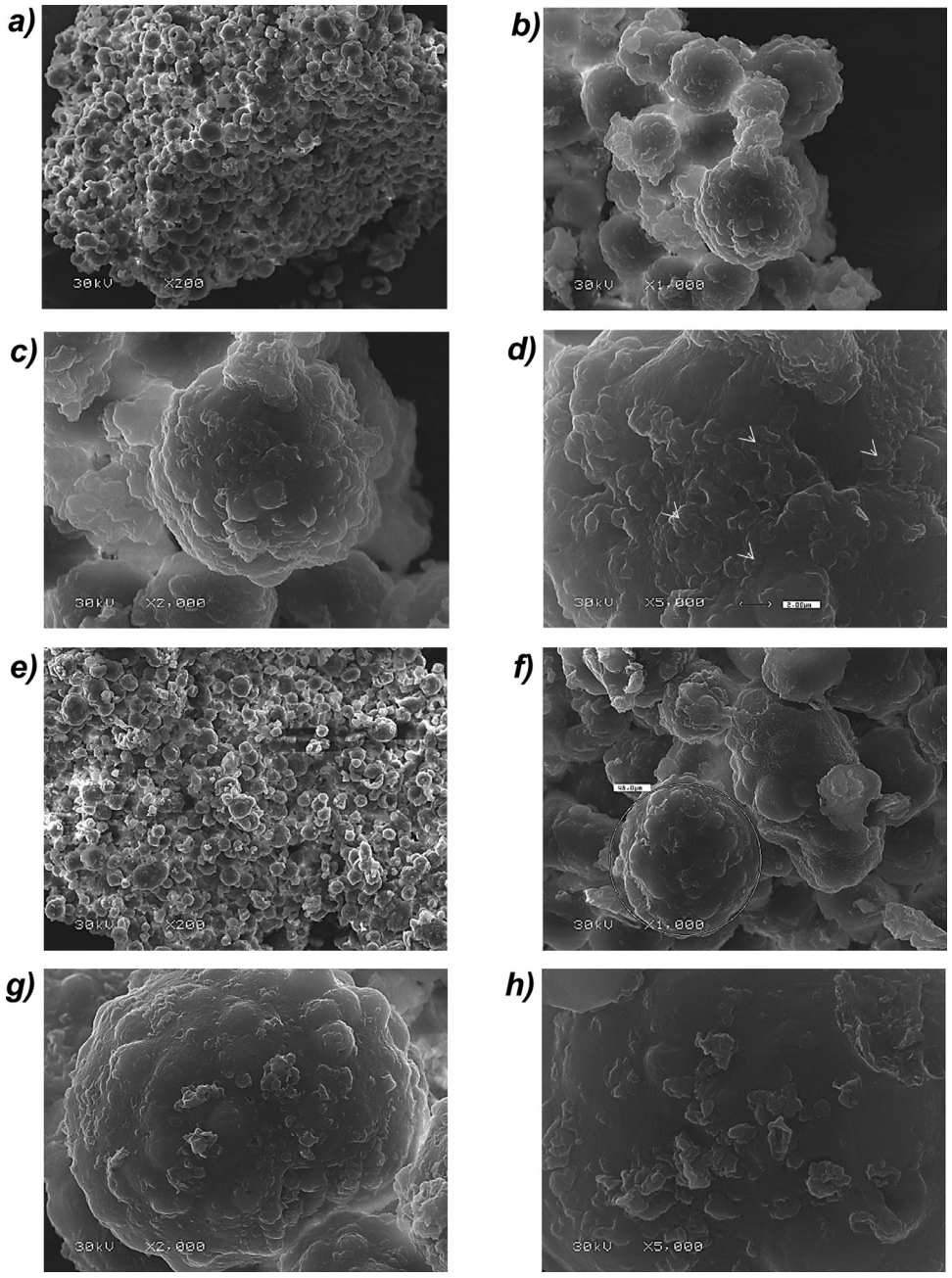

Fig. 2. SEM micrographs of *A. muciniphila* DSM 22,959 (a-d) loaded and (e-h) unloaded microcapsules, at different magnifications (x200, x1000, x2000 and x5000, respectively). Arrows indicate possible *A. muciniphila* cells. Samples were freshly prepared.

Table 1
Viability (CFU/g for microencapsulated cells and CFU/mL for free cells) of *A. muciniphila* DSM 22,959 throughout 95 days refrigerated storage at 4 ºC, in aerobic and anaerobic conditions. Data is shown as the mean ± standard deviation (n = 3).

Akkermansia muciniphila	Storage Conditions	Initial Cell Viability	1 day	15 days	30 days	95 days
Free	Anaerobic	$(1.30 \pm 0.27) \times 10^9$	$(1.23 \pm 0.12) \times 10^9$	$(2.70 \pm 0.55) \times 10^9$	$(2.70 \pm 0.32) \times 10^9$	$(5.95 \pm 0.42) \times 10^8$
	Aerobic	$(1.30 \pm 0.26) \times 10^9$	$(1.63 \pm 0.25) \times 10^9$	$(2.60 \pm 0.62) \times 10^9$	$(2.63 \pm 0.06) \times 10^9$	$(6.27 \pm 0.15) \times 10^8$
Microencapsulated	Anaerobic	$(4.70 \pm 0.46) \times 10^8$	$(1.60 \pm 0.36) \times 10^9$	$(4.13 \pm 0.15) \times 10^9$	$(1.64 \pm 0.01) \times 10^8$	$(6.33 \pm 1.00) \times 10^6$
	Aerobic	$(4.70 \pm 0.46) \times 10^8$	$(1.20 \pm 0.17) \times 10^9$	$(2.70 \pm 0.53) \times 10^9$	$(2.77 \pm 0.32) \times 10^8$	$(6.63 \pm 0.45) \times 10^5$

stored at 4ºC, in both aerobic and anaerobic conditions, and their viability was determined, at specific timepoints, for 95 days (Table 1).

Up until 30 days of refrigerated storage, free *A. muciniphila* cell counts in suspension remained constant, only with a slight decrease of *ca.* 1 log CFU/mL after 95 days stored either in anaerobic or aerobic conditions. Comparatively, an equal reduction in viability was observed for microencapsulated cells after 30 days, maintaining a 10^8 CFU/g magnitude for viable cells. However, after 95 days, encapsulated *A. muciniphila* viability suffered a sharper reduction of *ca.* 3 and 4 log cycles, in both anaerobic and aerobic conditions, respectively. As expected, the prolonged storage periods led to a decrease in viable cells counts; such reductions were curiously more evident for the encapsulated cells.

On the one hand, this might indicate that the physiological conditions generated within the Alg:DWPI microcapsules' hydrocolloid

Table 2
Viability of free (CFU/mL) and microencapsulated (CFU/g) *A. muciniphila* DSM 22,959 stored aerobically for 95 days at 4 ºC, throughout the simulated gastrointestinal conditions. Data is shown as the mean ± standard deviation ($n = 6$).

Gastrointestinal Phase [1]	Sampling Time (hours)	Condition					
		Free *A. muciniphila*(CFU/mL)			Microencapsulated *A. muciniphila*(CFU/g)		
		1d	**30d**	**95d**	**1d**	**30d**	**95d**
	0	$(1.63 \pm 0.25) \times 10^9$	$(2.63 \pm 0.06) \times 10^9$	$(6.27 \pm 0.15) \times 10^8$	$(1.20 \pm 0.17) \times 10^9$	$(2.77 \pm 0.32) \times 10^8$	$(6.63 \pm 0.45) \times 10^5$
Oral-Gastric	1	$(1.48 \pm 0.13) \times 10^9$	$(1.99 \pm 0.42) \times 10^9$	$(1.49 \pm 0.18) \times 10^7$	$(9.60 \pm 1.01) \times 10^8$	$(1.85 \pm 0.72) \times 10^8$	$(1.20 \pm 0.10) \times 10^5$
(Δ pH 6 to 3,							
37 ºC, 140 rpm)	2	$(4.27 \pm 2.23) \times 10^8$	$(4.35 \pm 3.03) \times 10^8$	$(6.27 \pm 2.15) \times 10^4$	$(6.27 \pm 1.09) \times 10^8$	$(1.07 \pm 0.37) \times 10^8$	$(8.80 \pm 2.37) \times 10^4$
Intestinal	3	$(5.37 \pm 0.58) \times 10^8$	$(1.53 \pm 6.49) \times 10^8$	N.D.	$(3.45 \pm 0.92) \times 10^8$	$(9.87 \pm 1.09) \times 10^7$	$(4.87 \pm 0.69) \times 10^4$
(Δ pH 3 to 7,	4	$(1.71 \pm 0.53) \times 10^8$	$(4.28 \pm 0.47) \times 10^7$	N.D.	$(2.65 \pm 0.36) \times 10^8$	$(9.52 \pm 2.27) \times 10^7$	N.D.
37 ºC, 140 rpm)	5	$(1.61 \pm 0.37) \times 10^8$	$(3.24 \pm 0.96) \times 10^7$	N.D.	$(4.90 \pm 1.40) \times 10^8$	$(1.52 \pm 0.48) \times 10^8$	N.D.

[1] Typical conditions prevailing in each gastrointestinal compartment: pH or variation of pH (Δ pH), temperature and agitation values (rpm) used in each compartment are indicated[2] N.D. no viable cells detected.

matrix were less optimal for the probiotic cells over storage. Indeed, prolonged storage generates an increased stress on the encapsulated probiotics, which may lead to loss of structural integrity causing ruptures in the microparticles, increasing the probiotic cells susceptibility to external conditions (Sousa et al., 2015). Furthermore, water activity measurements were taken to confirm how much water was available in the matrix, to participate in structural degradation reactions. For three weeks post-microcapsule production, water activity (a_w) ranged 1.00–0.99, as expected from the measured total water content (92.6 %). Ying et al. (2010) observed that when exposed to incremented percentages of relative humidity, spray-dried and freeze-dried samples of *Lactobacillus rhamnosus* GG encapsulated in an emulsion-based formulation, also demonstrated an increase in a_w, which was accompanied by a negative influence on the probiotic viability.

On the other hand, it is known that water possesses a small oxygen diffusion coefficient, which translates into a minimal depth of oxygen penetration in the culture media (Fenchel & Finlay, 2008). In this case, since free *A. muciniphila* cells were stored in static isotonic saline suspension without headspace volume (10 mL total volume), one can conjecture that it unintentionally created an oxygen-free environment that in conjunction with the low temperatures resulted in a lower loss of cell viability in comparison to the encapsulated cells. Another possible explanation for the viability decrease of microencapsulated *A. muciniphila* is the presence of residual Tween 80 that could also inhibit the bacterial growth (Nielsen, Kjems, Mygind, Snabe & Meyer, 2016).

Notwithstanding such limitations, the present microencapsulated *A. muciniphila* stability values contrast positively with those depicted in literature up to the present moment. For example, van der Ark et al. (2017) reported a 2 log CFU/mL reduction of double emulsion *A. muciniphila* microcapsules after only 72 h of anaerobic storage, at 4ºC. In a different study, Marcial-Coba et al. (2018) indicated that the best storage viability outcome was found after freeze-drying, and after 30 days of refrigerated anaerobic storage. In our present study, it is important to highlight that cell viability was still preserved to a magnitude of 10^8 CFU/g of wet microcapsules (above the required minimum threshold) after 30 days of refrigerated aerobic storage, a storage approach which represents the conventional preservation method of food products with less energy costs associated (no drying process was applied) and consequently of higher industrial relevance. The stability of freeze-dried probiotics is mainly observed when stored in inert atmospheres (nitrogen or vacuum), which increases the costs of an already expensive procedure (Manojlović, Nedović, Kailasapathy & Zuidam, 2010). Additionally, this requires the inclusion of cryoprotectant agents to help retain probiotic activity upon storage, otherwise it could evoke harmful effects to more vulnerable microorganisms (Arakawa, Prestrelski, Kenney & Carpenter, 2001).

3.4. *Survival rate of free and microencapsulated* A. muciniphila *exposed to* in vitro *simulated GI conditions over long storage*

To exert its beneficial effects, a probiotic has to be able to reach the large intestine with high viable bacteria load (Hill et al., 2014), and thus, resistance to Gl harsh conditions is paramount in what concerns its delivery. Since exposure to oxygen is expected to present greater challenge for *A. muciniphila* viability, the stability to simulated GI tract conditions was assessed following 1, 30 and 95 d of refrigerated aerobic storage. The resulting data is shown in Table 2 and Fig. 3 (normalized data to allow the comparison between the relative viability of microencapsulated and free forms of *A. muciniphila*).

Overall, the observed reduction in viability for both free and microencapsulated *A. muciniphila* after simulated GI passage, was not noteworthy, except for 95d of storage for both conditions. Nevertheless, the second hour of gastric phase ($t = 2$ h) seems to be the step responsible for the highest reduction in cell counts for both conditions. Interestingly, the relative viability also indicates that as storage time elapsed (Fig. 3b-c) the survival of free *A. muciniphila* to GI conditions decreases. A decrease in resistance to GI passage for free *A. muciniphila* was noticed after 30 days of aerobic storage, depicted by a faster decline after 1 h of intestinal conditions ($t = 3$ h). After 95 days of storage, *A muciniphila* in the free form could not survive past the gastric phase while the encapsulated cells were still viable up to the first hour of the intestinal phase. Free cells had a detected viability of $6{,}3 \times 10^4$ CFU/mL of at the end of gastric phase ($t = 2$ h), which comprehended a *ca.* 4-log reduction in viability. Comparatively, microencapsulated *A. muciniphila* presented a viability of 8.8×10^4 CFU/g at the end of the gastric phase (corresponding to *ca.* 1-log cycle reduction) which was roughly maintained during the first hour of the intestinal phase (4.96×10^4 CFU/g). Unfortunately, no viable cells were detected at the end of the intestinal phase. Thus, although microencapsulation by the emulsification/internal gelation within an Alg:DWPI matrix can considerably mitigate the effects of prolonged aerobic storage on cell viability during the GI transit, it was not sufficient to ensure enough viability after 95 days of refrigerated aerobic storage conditions. According to Ottman (2015), the early observed free cell stability to GI conditions might be explained by an acid resistance system, which enables nursing infants' colonization (Petschacher & Nidetzky, 2016). Moreover, the reduced activity of bile salts upon Gram-negative bacteria membrane can also be a factor explaining *A. muciniphila* natural resiliency (Begley, Gahan & Hill, 2005). Indeed, this resilience regarding oxygen and low pH exposure has been empirically confirmed previously (Machado et al., 2020). Microcapsule carrier materials must have the capacity to not only immobilize, but also form a physical barrier between the active agent and the external environment (Šipailienė & Petraitytė, 2018). Alginate protective effects in the presence of chelating agents have been questioned by

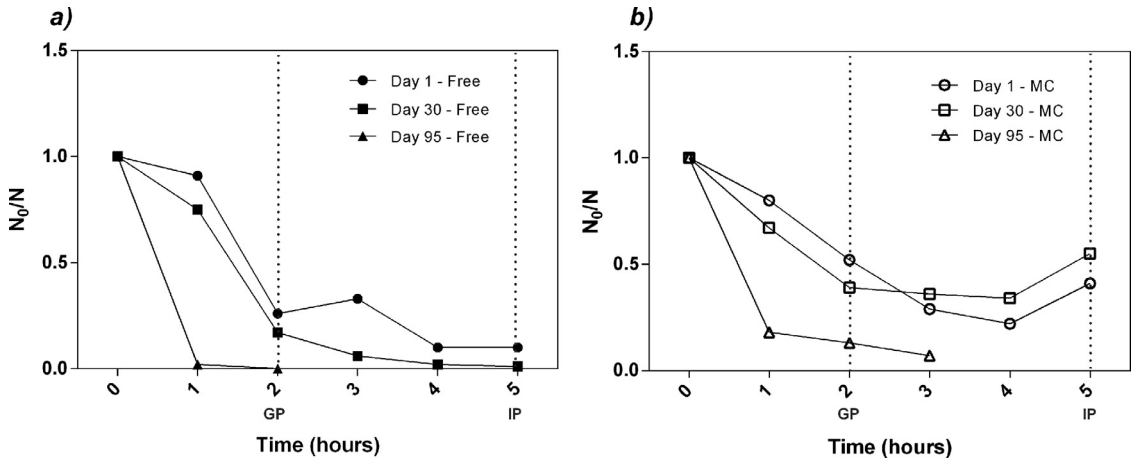

Fig. 3. Viability of free and microencapsulated (MC) *A. muciniphila* DSM 22,959 when exposed to simulated GIT conditions for 5 h. The assessment was performed at defined timepoints after aerobic, refrigerated storage: 1; 30 and 95 days. GP = end of the gastric phase; IP = end of the intestinal phase. Data was treated as CFU/mL or CFU/g ± standard deviation (SD), for free (a) and microencapsulated (b) *A. muciniphila* cells, respectively. At the initial timepoint (t = 0) the values were then normalized to 1, and the following timepoints were calculated with respect to survival at t = 0 for each condition and storage period and plotted *versus* time.

some authors, since a low stability has been observed in pH conditions below 2.0 (Ding & Shah, 2007; Smidsrød & Skjåk-Braek, 1990). However, the improvement in protection to acidic conditions in gastric phase was demonstrated in previous reports (Cai et al., 2014; Poncelet et al., 1992; Qu et al., 2016). Recently, Chang et al. (2020) evaluated the effect of a modified SA containing polyphenol on the improvement of *A. muciniphila* viability during spray-drying. The authors found that this improved its storability and stability towards GI fluid. However, viability of microencapsulated cells, maintained in anaerobic buffer, during 12 days, was measured by optical density, which does not always reflect the true cultivable cell numbers available (Chang et al., 2020). Similarly, the presence of other compounds, specifically, proteinaceous constituents in matrices, has been proved to grant a protective effect, mainly due to its buffering capacity, in particular, under low pH gastric conditions (Heidebach, Först & Kulozik, 2009). Herein, the implementation of DWPI as a co-encapsulant agent can reduce microcapsule porosity, offering higher stability to the final product (Doherty et al., 2010; Rajam et al., 2012). Additionally, the inclusion of colloidal antiacids, such as $CaCO_3$ have been shown to provide a neutral internal pH when microcapsules are exposed to acidic conditions - as experienced under simulated stomach conditions - mainly due to the neutralizing effects of the released hydroxyl ions (Gu et al., 2019; Zhang et al., 2021).

Alginate microcapsules are also prone to undergo a swelling effect, due to bile exposure, which can result in subsequent cell damage (Qu et al., 2016). This may explain the viability loss of *A. muciniphila* in Alg:DWPI microcapsules in the intestinal phase. Despite that, microencapsulated *A. muciniphila* displayed higher stability when compared to free cells during intestinal phase. Data depicting the relative cell viability from the simulated GI assays (Fig. 3a-c) shows that until 30 days of aerobic refrigerated storage, encapsulated cells behaved positively in the intestinal phase, considering that survivability was maintained at levels corresponding to 10^8 CFU/g, whilst its free counterpart dropped to levels corresponding to 10^7 CFU/g. Although the number of live *A. muciniphila* cells required to exert beneficial effects on humans is yet to be determined, it has been shown that the oral administration of 10^8 CFU of live *A. muciniphila* cells in murine models was sufficient to reverse high-fat diet-induced metabolic disorders (Everard et al., 2013). According to these criteria, these results confirmed the presence of *A. muciniphila*, until 30 days of aerobic refrigerated storage, at levels that can putatively guarantee the delivery of the recommended therapeutic dose in functional products.

4. Conclusion

The results show that the adapted emulsification/internal gelation technology using the dual hydrocolloid matrix Alg:DWPI matrix is an interesting and suitable immobilization strategy for *A. muciniphila*. This technology ensured large production yields of high-loaded microcapsules (maintained a cell density of 10^8 CFU/g even in aerobic storage conditions), with uniform morphology, maintaining a small average size and narrow capsule size distributions. Furthermore, such technology enables an improved control over each stage of particle production process. Despite the considerable effect of the extended storage period, *A. muciniphila* cells capitulated the microcapsules' protective features, since the survival rates under *in vitro* simulated GI conditions increase as storage time elapses, when compared to that of free cells. Thus, although this protective effect could not be immediately observed when analyzing storage and GI simulation independently, it becomes evident when looking for the cumulative effect on *A. muciniphila* survival rates following the sequential exposure to storage and simulated GI stages. This first-time methodology could be used to enhance *A. muciniphila* survival during long shelf-life to achieve the intestinal delivery of the recommended therapeutic level of probiotics (10^7–10^8 CFU/g) in functional products. Given the present data additional *in vivo* studies are required to ascertain the efficacy of the microencapsulated *A. muciniphila* on gut microbiota composition modulation and other host health-related parameters, and also to possibly define therapeutic dosages for specific disease models.

Ethical statement - Studies in humans and animals

The authors declare that the study presented herein did not involve studies in humans or animals.

Declaration of competing interest

The authors declare no conflicts of interest.

CRediT authorship contribution statement

Diana Almeida: Conceptualization, Formal analysis, Investigation, Methodology, Writing – original draft, Writing – review & editing. **Daniela Machado:** Formal analysis, Writing – review & editing. **Sérgio Sousa:** Methodology, Formal analysis, Writing – review & editing.

Catarina Leal Seabra: Methodology. **Joana Cristina Barbosa:** Formal analysis, Writing – review & editing. **José Carlos Andrade:** Conceptualization, Resources, Supervision, Writing – review & editing. **Ana Maria Gomes:** Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Writing – review & editing. **Ana Cristina Freitas:** Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Writing – review & editing.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.fhfh.2022.100084.

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