Commensal Gut Bacterium Akkermansia Muciniphila Secretome Induces Mitochondrial Calcium Overload and α-Synuclein Aggregation in Enteroendocrine Cells

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Abstract

**Background:** The notion that the gut microbiota plays a role in neurodevelopment, behavior and outcome of neurodegenerative disorders is recently taking place. A number of studies have consistently reported a greater abundance of *Akkermansia muciniphila* in Parkinson's disease (PD) fecal samples. Nevertheless, a functional link between *A. muciniphila* and sporadic PD remained unexplored. Here, we investigated whether *A. muciniphila* conditioned medium could initiate the misfolding process of α-synuclein (αSyn) in enteroendocrine cells (EECs), which are part of the gut epithelium and possess many neuron-like properties.

**Results:** We found that *A. muciniphila* conditioned medium is directly modulated by mucin, induces intracellular calcium (Ca$^{2+}$) release, and causes increased mitochondrial Ca$^{2+}$ uptake in EECs, which in turn leads to production of reactive oxygen species (ROS) and αSyn aggregation. Indeed, oral administration of *A. muciniphila* cultivated in the absence of mucin to aged mice also led to αSyn aggregation in cholecystokinin (CCK)-positive enteroendocrine cells. Noteworthy, buffering mitochondrial Ca$^{2+}$ reverted all the damaging effects observed.

**Conclusion:** Thereby, these molecular insights provided here offer evidence that bacterial proteins are capable of inducing αSyn aggregation in enteroendocrine cells.

Background

Traditionally, Parkinson's disease (PD) has been characterized as a progressive neurodegenerative disorder caused by loss of dopaminergic neurons in the substantia nigra pars compacta of the midbrain [1]. Neuronal loss leads to Parkinsonism, an array of motor symptoms comprehending muscle rigidity, slowness, tremors and difficulty in controlling movement [2]. However, recent studies have shown that drug-naïve PD patients frequently report gastrointestinal complaints such as constipation, nausea and prolonged intestinal transit time even years before the disease is diagnosed [3–5]. Therefore, this pathology is now considered as a multisystemic disease gathering a plethora of non-motor symptoms [6, 7].

Currently, PD is the second most common neurodegenerative disease and the interest of the scientific community to unveil the cellular and molecular mechanisms of this complex pathology has grown substantially, triggered especially by the discovery of a number of causative monogenic mutations [8]. Nevertheless, these mutations only explain a small percentage of all PD cases since about 90% are sporadic [9].

The key dogma of PD consists in the aggregation of the protein alpha-synuclein (αSyn) within neurons [10]. This presynaptic protein is linked genetically and neuropathologically to PD. It is accepted that αSyn aberrant soluble oligomeric conformations (protofibrils) are the toxic species that disrupt cellular homeostasis and lead to neuronal death through effects on several intracellular targets, including synaptic function [11]. This aggregation process can be caused by genetic or sporadic factors due to
mitochondrial dysfunction, oxidative stress and altered proteostasis [12]. Although this toxic aggregation occurs more widely throughout the central system, abundant clinical and pathological evidence shows that misfolded αSyn is found in enteric nerves before it appears in the brain [13–15]. It was recently reported that enteroendocrine cells (EECs), which are part of the gut epithelium and are directly exposed to the gut lumen and its microbiome, possess many neuron-like properties, such as αSyn expression, and connect to enteric nerves [16]. This leads to the hypothesis that PD might originate in the gut and then spread to the central nervous system via cell-to-cell prion-like propagation [16]. Such a concept has gathered significant momentum in recent years and great attention has been given to the brain-gut connection. Therefore, the gut microbiome raises as a promising target to be investigated in the outcome of sporadic PD. Several reports have shown that individuals with PD display an imbalanced gut microbiome (dysbiosis) [17–19] where commensal bacteria (e.g., phylum Firmicutes) are reduced, while pathogenic Gram-negative bacteria (Proteobacteria sp, Enterobacteriaceae sp, Escherichia sp.) and mucin-degrading Verrucomicrobiaceae, such as Akkermansia muciniphila are increased [17, 18, 20–22].

The mucin-degrading microorganism A. muciniphila (Derrien, 2004) comprises about 1–4% of the fecal microbiome in humans (Naito et al. 2018). While numerous diseases have been associated with a decrease in A. muciniphila abundance [23, 24], an increase of this microorganism has been consistently reported in PD patients [25]. In addition, it was shown that A. muciniphila abundance had the largest contribution to the significantly altered metabolite secretion profiles of sporadic PD patients [25].

The microbial surface and secreted proteins contain many proteins that interact with other microbes, host and/or environment [26]. These proteins (e.g., receptors, transporters, adhesins, secreted enzymes, toxins) not only allow bacteria to interact with and adapt to their environment, but also modulate the host cells activities [27]. Identifying the effects of A. muciniphila secreted proteins on the physiology of enteroendocrine cells could therefore increase our current understanding on the cell mechanisms that could lead to one of the possible outcomes of sporadic PD.

Based on the common occurrence of gastrointestinal symptoms in PD, dysbiosis among PD patients, and strong evidence that the microbiota influences central nervous system (CNS) function, in this work we investigated whether and how A. muciniphila conditioned medium alters enteroendocrine cells homeostasis leading to αSyn aggregation. Herein, we found that the protein fraction of A. muciniphila mucin-free conditioned medium induces inositol 1,4,5-trisphosphate (IP$_3$)-independent endoplasmic reticulum (ER)-calcium (Ca$^{2+}$) release by directly modulating Ryanodine Receptors (RYR), leading to increased mitochondrial Ca$^{2+}$ uptake. Increasing levels of A. muciniphila by oral administration in aged mice, which present thinner mucus barrier when compared to younger mice, was not sufficient to cause any motor deficit, however, it was shown to increase aSyn aggregation in enteroendocrine cells.

In addition, mitochondrial Ca$^{2+}$ overload leads to ROS generation culminating with αSyn aggregation and these events were efficiently inhibited once we buffered mitochondrial Ca$^{2+}$. These molecular insights provided here will push further the understanding of the pathogenesis of PD by offering mechanistic
evidence that bacterial secreted proteins are capable of inducing αSyn aggregation in enteroendocrine cells.

Materials And Methods

Animals

Old (18–20 months) SPF male FVB mice were maintained under specific pathogen-free conditions. After 1–2 weeks of acclimation, mice were randomly assigned to two groups. Group AKK (A. muciniphila) mice were administrated $2 \times 10^8$ A. muciniphila cells determined by OD 600nm\[28\] suspended in 0.15 ml sterile anaerobic PBS by oral gavage per day, while mice in Group Control (PBS) were given an equivalent volume of sterile anaerobic PBS instead \[29\]. Treatment was continued for 28 days. All mice were maintained in a temperature- and humidity-controlled environment under a 12-h light–dark cycle and had ad libitum access to food and water. All experiments were approved by the Institutional Committee of Animal Use & Protection (CEUA 92-B).

Fecal Microbial Community Analysis

To confirm the effectiveness of A. muciniphila oral administration, fecal samples were collected before and after the last day of treatment, immediately frozen and stored at -80°C freezer after collection. Total bacterial DNA was extracted from fecal samples (approximately 200 mg) using a QIAamp FastDNA Stool Mini Kit (Qiagen, Hilden, Germany) following the manufacturer’s procedures. Specific primers for detection of the variable regions of the 16S rRNA gene sequence of A. muciniphila were used (AM forward- CAGCACGTGAAGGTGGGGAC; AM reverse CCTTGCGGTTGGCTTCAGAT) \[30\]. All reactions were carried out on an Applied Biosystems® 7900HT Fast Real-Time PCR machine (Applied Biosystems® Foster City, CA, USA). Each reaction was performed in duplicate and consisted of 1X Syber®Green PCR Master Mix (Applied Biosystems), forward and reverse primers at final concentration of 200 nM and 4 µl of template. Standard cycling conditions and melt curve analysis were employed. The cycle threshold (CT) value of each sample was then compared with a standard curve made by diluting genomic DNA of A. muciniphila. Log10 of A. muciniphila number per gram of fecal content was used to indicate the abundance of A. muciniphila \[24, 31\].

Behavioral tests

To determine the possible effects of A. muciniphila of sensory motor function in mice, wire-hang and the cylinder test were performed. To acclimatize to the behavioural testing condition, the mice were trained on the last 2 days before the last gavage. Tests were performed as per the protocol described with slight modification\[32\]. For the cylinder test, a transparent glass cylinder with a 14-cm diameter and 19-cm height was used. A video recording device was set up and mice were then placed inside the cylinder one-by-one for a 3-min recording period to estimate the number of rearing. The number of rearing was defined as the number of times the mouse stood with the support of its hind limbs solely. Thorough cleaning of the cylinder was performed between experiments. The wire-hang test is a useful tool to evaluate motor
function and motor strength in rodents, and the experiments were performed according to Sango et al. (1996) and Prado et al. (2006) [33, 34]. Mice were habituated at the behavioral room 2 h before the test. We placed each animal individually in the top of a wire cage lid (22 × 22 cm) and then the lid was gently turned upside down by the investigator. The latency of mice to lose their grip and fall off the lid was visually evaluated in three trials with a cut-off time of 60s.

**Cell lines**

STC-1 (CRL-3254) cell line was obtained from the America Type Culture Collection (ATCC). STC-1 cell was cultured in DMEM (Gibco) supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin antibiotics (PSA) and incubated at 37ºC with 5% CO₂: 95% air.

Lyophilized *A. muciniphila* (DSM-22959) and *E. coli* (ATCC 25922) were purchased from DSMZ and America Type Culture Collection respectively. Strains were grown individually in pure Brain & Heart Infusion Broth (BHI) (BD, Heidelberg, Germany) or in BHI broth supplemented with 0.4% mucin (Sigma-Aldrich, MO, USA) [35–37].

Conditioned media collection.

Briefly, lyophilized *A. muciniphila* (DSM-22959) and *E. coli* (ATCC 25922) were grown individually in pure Brain & Heart Infusion Broth (BHI) (BD, Heidelberg, Germany) or in BHI broth supplemented with 0.4% mucin (Sigma-Aldrich, MO, USA) [35–37].

After being supplemented and sterilized, the vials containing the media were gassed with N₂ injection system for 30 minutes, and then placed into an anaerobic chamber (Whitley DG250 Anaerobic Workstation, Don Whitley Scientific) kindly provided by the Brazilian Bio renewables National Laboratory (LNBR, CNPEM). The bacterial inoculum was incubated for up to 72 hs at 37°C. After 12, 24, 36, 48, 60 and 72 hs of incubation, an aliquot of each of the vials was collected and evaluated under light microscopy and spectrometry for optical density (OD) measurement, using a spectrometer (Evolution™ 60S UV-Visible Spectrophotometer, Thermo Fisher Scientific Inc.) in order to monitor bacterial growth.

After 36–40 hs of incubation, conditioned (CM) and unconditioned media were collected and concentrated. Briefly, media were centrifuged for 4000 rpm at 4°C to pellet cells and the supernatant was concentrated at 4000 rpm at 4°C for 20min using Centricon® 3kD Plus-70 Centrifugal Filter Units. These conditioned (BHI CM) or unconditioned media (BHI) containing were then filtered (0.22 µm) and stored at -80°C until used. For some experiments, conditioned media as heat inactivated by boiling (100°C, 2 hs) [38]. Subsequently, conditioned medium was cooled to 37°C and employed in calcium signaling experiments.

**Mass Spectrometry**

*A. muciniphila* CM and CM + 0.4% mucin were submitted to protein electrophoresis technique using 10% acrylamide-SDS page separation. After the electrophoretic run, the lanes were cut into small fragments,
micro-purified, enriched, and digested using trypsin[39, 40]. Then, samples were directed to mass spectrometry (LTQ Orbitrap Velos, Thermo-Fischer). The peptides were separated with a 2–30% acetonitrile gradient in 0.1% formic acid using a PicoFrit analytical column (20 cm x 75 nm, particle size from 5 µm, New Objective) at a flow rate of 300 nL/min over 173 min. The nanoelectrospray voltage was adjusted to 2.2 kV and the source temperature was set at 275°C. All instrument methods were set in data dependent acquisition mode. The full scan MS spectra (m/z 300-1600) were acquired in the Orbitrap analyzer after accumulation to a target value of 1x10^6. The resolution in the Orbitrap was set at 60,000 and the 20 ions of the most intense peptides with states of charge ≥ 2 were sequentially isolated to a target value of 5,000 and fragmented into linear ion traps using low energy CID (35% normalized collision energy). The signal limit for triggering the MS/MS event was set to 1,000 counts. Dynamic exclusion was activated with an exclusion size list of 500, exclusion duration of 60 s and a repeat count of 1. An activation q = 0.25 and an activation time of 10 ms were used.

The data obtained by mass spectrometry were processed using the MaxQuant 1.3 software based on the A. muciniphila protein database.

**Plasmids and transfection**

cDNA for the Ca^{2+} binding protein parvalbumin (PV) fused to mitochondrial targeting sequence (PV-MTS-GFP) and respective control (MTS-GFP) were kindly donated by Dr. Mateus Guerra (Yale University, USA) [41]. cDNA for the human αSyn (αSyn) was amplified and cloned between the HindIII and SmaI restriction sites of pEGFP-N2 vector. For αSyn mCherry-tagged version, the human αSyn sequence was inserted between BamH1 and SalI of the pCDNA5-mCherry vector.

**Immunofluorescence**

Cells were cultured onto glass-slides and fixed with 4% PFA for 20 min. Samples were blocked in PBS 1X containing 5% Normal Horse Serum and 5% bovine albumin (Sigma-Aldrich) for 1h. After washing in PBS 1X, cells were incubated with primary antibodies anti-αSyn (1:250, Abcam); anti-pser129 αSyn, (1:100; Abcam) for 2 h at room temperature, followed by PBS washes and incubation with secondary antibody (anti-mouse Alexa-488, 1:500; Thermo-Fischer) for 1 h at room temperature. Fluorescence intensity of αSyn and αSyn-p-serine-129 was quantified on at least 25 cells from 3 different experiments. Data are expressed as percentage relative to the untreated group (control). The images were obtained using a Leica SP8 confocal microscope, using a ×63 objective lens, 1.4 NA.

**Cell viability**

Cellular viability was measured using the CellTiter 96® AQueous One Solution Cell Proliferation Assay (MTS) (Promega) according to the manufacturer's protocol. STC-1 cells were treated with 1 or 10% BHI and BHI CM for 48 hs. Following this period, cells were incubated in the presence of MTS Tetrazolium Compound for 2 hr at 37°C. Absorbance measurements (490nm) were performed using a plate reader (PerkinElmer; Waltham, MA).

**Immunoblotting**
Cell proteins were extracted with RIPA buffer supplemented with inhibitors of proteases and phosphatases followed by centrifugation at 10,000 rpm for 10 min at 4°C. Proteins were separated by SDS-PAGE in 12% Bis-Tris gels and transferred onto 0.45 µm nitrocellulose membranes (BioRad). For dot blot quantification of αSyn fibrils, 1 µg of tissue homogenate from the specified region was spotted in 1 µL volume aliquots onto 0.45 mm nitrocellulose membranes. The blots were incubated overnight at 4°C with anti-αSyn (1:1000, Abcam), GATA-2 (1:1000, R and D Systems), anti-pser129 αSyn, (1:1000; Abcam) and anti-aggregated αSyn (1:2000, Abcam) antibodies followed by incubation with horseradish peroxidase (HRP)-conjugated secondary IgGs (anti-rabbit, 1:5000, Thermo-Fischer; anti-mouse, 1:5000, Thermo Fischer). β-actin (1:5000, Santa Cruz) or GAPDH (1:5000, Santa Cruz) were used as loading controls. Membranes were developed using the BioRad chemiluminescence detection system (Clarity Western ECL, BioRad). Chemiluminescence signals were quantified using Image J software.

Cytosolic and mitochondrial calcium measurements.

For cytosolic calcium measurements, cells were loaded with the Ca\(^{2+}\) indicator Fluo-4/AM (for intracellular Ca\(^{2+}\)) or Rhod-2/AM (for mitochondrial Ca\(^{2+}\)) (Thermo Fisher Scientific) for 15 min at 37°C, placed onto the stage of a Leica SP8 Confocal System and continuously perfused with HEPES buffer solution (142.2 mM NaCl, 5.4 mM KCl, 1.0 mM NaH\(_2\)PO\(_4\), 10 mM HEPES, 5.6 mM dextrose, 0.8 mM MgSO\(_4\) and 1 mM CaCl\(_2\)), unless otherwise noted. 1 or 10% BHI or BHI CM (v/v) medium with or without 0.4% mucin was used to trigger Ca\(^{2+}\) release. 40 µM Adenosine triphosphate (ATP) was used to trigger InsP3-dependent Ca\(^{2+}\) release and to evaluate mitochondrial-calcium response after 48h-treatment with the conditioned medium. To investigate whether intracellular calcium signaling was from endoplasmic reticulum stores, cells were incubated for 30 min in HEPES Ca\(^{2+}\)-free buffer containing 10µM thapsigargin prior to stimulation with the conditioned medium. To investigate IP\(_3\) dependence for the A. muciniphila conditioned media-triggered calcium response, cells were incubated with Fluo-4/AM and 2.5 µM xestospongin C for 30 min before stimulation with the conditioned medium. Dantrolene sodium salt (75 µM) was used as RYR blocker. For mitochondrial calcium measurements in PV-MTS-GFP or MTS-GFP transfected cells, cells were transfected 48 hs before the experiment using FUGENE HD (Promega) according to the manufacturer’s instructions. Data are expressed as fluorescence/baseline fluorescence × 100% of the average values of samples from 3–6 biological replicates (>20 cells/replicate). The images were obtained using a Leica SP8 confocal microscope, using a ×63 objective lens, 1.4 NA, excitation at 488 nm and emission at 505–525 nm for both dyes.

Detection of reactive oxygen species (ROS)

To evaluate the formation of reactive oxygen species (ROS), STC-1 cells were previously seeded on glass slides were treated with 5 µM DHE (Thermo-Fischer) for 30 min in HEPES buffer solution (142.2 mM NaCl, 5.4 mM KCl, 1.0 mM NaH\(_2\)PO\(_4\), 10 mM HEPES, 5.6 mM dextrose, 0.8 mM MgSO\(_4\) and 1 mM CaCl\(_2\)). The glass slides were transferred to a perfusion chamber attached to the confocal microscope. The cells were
stimulated with 1 or 10% (v/v) *A. muciniphila* conditioned or unconditioned media. As a positive control of ROS formation, 100 µM H$_2$O$_2$ was used.

For each stimulus, the emission of fluorescence in response to the general indicator of oxidative stress was monitored in individualized cells during stimulation with the conditioned and unconditioned media. Data are expressed as fluorescence/baseline fluorescence × 100% of the average values of samples from 3–6 biological replicates (> 20 cells/replicate). The images were obtained using a Leica SP8 confocal microscope, using a ×63 objective lens, 1.4 NA, excitation at 488–518 nm and emission at 606 nm.

**Evaluation of mitochondrial membrane potential**

STC-1 cells were treated for 48 hs with 1 or 10% (v/v) *A. muciniphila* conditioned or unconditioned media and then incubated 500 nM of MitoTracker Red CMXRos (Thermo-Fischer) for 30 min. Cells were washed 2x with PBS1x and then fixed in 4% paraformaldehyde at room temperature, for 20 min, washed with PBS and in sequence, were mounted in Vectashield. At least 25 cells from 3 different experiments were analyzed. Data are expressed as percentage relative to the untreated group (control). The images were obtained using a Leica SP8 confocal microscope, using a ×63 objective lens, 1.4 NA, excitation at 579 nm and emission at 599 nm. Finally, the intensity of fluorescence of the dye was quantified.

**Immunohistofluorescence.**

Mice were euthanized and perfused with 4% paraformaldehyde. Ileum was harvested, embedded in 4% low melting agarose and sections (100µm) were collected with a Vibratome VT1000S (Leica). All sections were rinsed with 1X PBS, permeabilized with 0.5% Triton X-1000 solution and blocked in 10% normal horse serum in 10 mM Tris, pH 7.4, 0.9% NaCl, 0.1% Triton X-100 (TBST) for 60 minutes at room temperature to limit nonspecific antibody association. After washing in PBS 1X, cells were incubated with primary antibodies anti-αSyn conformation specific (1:250, Abcam); and anti-CCK (1:100, Abcam) overnight at 4ºC, followed by PBS washes and incubation with secondary antibody and rhodamine-phalloidin (Thermo Fisher Scientific) for 1 h at room temperature. Slices were mounted in Vectashield solution containing DAPI.

**Statistical analysis**

Data analysis for PCR assays performed on the Applied Biosystems platform was performed using SDS 2.4 software (Applied Biosystems®). Target copy number in each sample was determined based on the fold change ($2^{-\Delta Ct}$) relative to the $10^7$ DNA standard. Copy numbers were normalized for dilution volume, elution volume, DNA concentration and sample weight. The statistical relevance of the bar graphs was obtained by calculating the P-value using the unpaired two-tailed Student's t-test. The bar graphs showed in the Figures are presented as mean ± SEM. For the imaging experiments, at least 25 independent cells for each condition were analyzed. Comparison of multiple groups was performed by one-way analysis of variance with Bonferroni post-tests. All column graphs, plots and statistical analyses were done using GraphPad Prism version 6 software. Detailed information regarding statistic test and number of experiments are embedded in figure legends.
Results

Akkermansia muciniphila growth curve pattern and conditioned medium composition are modulated by mucin

*A. muciniphila* is a mucin-degrading Gram-negative bacterium of the phylum Verrucomicrobia [36]. However, the intestinal mucus layer is thought to be inversely correlated with *A. muciniphila* abundance in the gut [42]. Prolonged lack of dietary fibers induces damage to the mucus barrier and is directly associated with increased abundance of *A. muciniphila*. This would bring gut bacteria closer to the intestinal epithelium, which could trigger deleterious effects or other host compensatory responses [43]. To test whether mucin could interfere with *A. muciniphila* conditioned medium composition, the strain DSM-22959 was harvested and monitored for 72 hs in both BHI culture medium and BHI supplemented with 0.4% mucin (from porcine stomach, Type II) (Fig. 1a). It is clearly observed that the addition of mucin maintains the growth of *A. muciniphila* in BHI medium (Fig. 1a). When mucin was provided, *A. muciniphila* grew faster at log phase and maintained a plateau for a longer time than when cultivated in mucin-free BHI medium. In addition, when analyzing the conditioned medium (CM) by MS-MS mass spectrometry, we identified 285 differentially expressed proteins in the conditioned medium obtained from *A. muciniphila* when cultivated for 36–40 hs (peak of growth for both conditions) in 0.4% mucin-supplemented BHI medium as opposed to 30 in mucin-free medium (Fig. 1b, Table S1). In both cases, many of these proteins have not yet been characterized by the scientific community. In summary, *A. muciniphila* conditioned medium is directly modulated by the presence of mucin.

Intracellular calcium signaling is elicited by Akkermansia muciniphila mucin-free conditioned medium in a model of enteroendocrine cells

Enteroendocrine cells (EECs) are chemosensory cells distributed throughout all the mucosal lining of the intestine and with their apical surface exposed to the lumen of the organ. In addition, it was recently described that EECs also connect to enteric neurons [16, 44, 45]. Due to their location at the interface between gut contents and the nervous system, EECs provide a direct route for substances in the gut to affect neural function. The STC-1 cell line is widely accepted as a model of native EECs [46] due to the expression of several gastrointestinal hormones, including cholecystokinin (CCK) and peptide YY (PYY), whose secretion pattern is compared to that of native EECs [47–49]. In addition, these cells present many neuronal-like features, including the expression of α-synuclein (αSyn) [16]. Since native EECs are hard to culture or to be collected from intestinal tissue in a sufficient number for *in vitro* assays, STC-1 are considered an attractive cell model for evaluating properties of EECs.

Calcium (Ca$^{2+}$) is known to regulate several important cell functions, such as secretion, proliferation, apoptosis, protein biosynthesis and folding [50–52]. In order to study the effects of *A. muciniphila* conditioned media in the fluctuations of intracellular Ca$^{2+}$ signaling in STC-1 cells, we first stimulated Fluo-4/AM-loaded cells with 1 or 10% conditioned BHI medium (BHI CM) or unconditioned BHI medium (BHI). We observed that *A. muciniphila* mucin-free BHI CM induces a strong increase in Ca$^{2+}$ transient in a
concentration-dependent manner (Fig. 2a-c). On the other hand, 0.4% mucin-supplemented BHI CM induced weaker Ca^{2+} signals when compared to the mucin-free condition (Fig. 2, S1a-c). In order to observe whether this Ca^{2+} fluctuation was due to bacterial secreted elements and not to the unconditioned culture medium, STC-1 cells were also stimulated with mucin-supplemented and mucin-free unconditioned media (BHI) and no fluctuation on intracellular Ca^{2+} signals was observed (Figure S2).

Therefore, secreted elements found in *A. muciniphila* conditioned media are key to elicit intracellular Ca^{2+} response in STC-1 cells.

**Mucin-free A. muciniphila conditioned media increases expression of endogenous α-synuclein in STC-1 cells**

Induced transient increase in free intracellular Ca^{2+} concentration by thapsigargin or Ca^{2+} ionophore chemical treatments lead to a significant increase in the number of cells presenting microscopically-visible αSyn aggregates [53]. Also, it is already reported that increased expression or decreased degradation of αSyn can initiate the formation of amyloid aggregates that can assemble to form Lewy bodies and Lewy neurites over the course of a lifetime [54].

In addition, misfolded αSyn is found in enteric nerves before it appears in the brain [13–15]. However, it is yet to be demonstrated whether the secreted proteins of a gut bacterium could initiate this pathologic sequence of events.

Therefore, we next analyzed whether *A. muciniphila* CM could modulate αSyn homeostasis in STC-1 cells. MTS assay confirmed that 48h-incubation of cells with 1 or 10% BHI CM or BHI did not decrease cell viability (Figure S3). When STC-1 cells were incubated with 1 or 10% mucin-free BHI CM for 48 hs, but not with the unconditioned one (BHI), we detected a significant clear overexpression of αSyn analyzed by immunofluorescence and Western blotting (Fig. 2d-f). However, this was not observed when the cells were incubated with 0.4% mucin-containing BHI CM (Figure S1d-f).

The SNCA gene expression in neurons, which encodes for αSyn, is known to be controlled by the GATA-2 transcription factor [55], which also plays a crucial role in central nervous system development, and erythroid cells differentiation [56]. In addition, GATA-2 has a critical role in neuronal development, particularly in cell fate specification of catecholaminergic sympathetic neurons [57, 58]. We observed that STC-1 cells not only express GATA-2 transcription factor but also exhibit increased expression of this factor when incubated with either 1% or 10% mucin-free *A. muciniphila* BHI CM. This supports the idea that *A. muciniphila* conditioned medium upregulates GATA-2 which in turn induces SNCA overexpression (Fig. 2g). To go further into the effects of *A. muciniphila* mucin-free conditioned media and to understand whether these phenomena were specifically due to the protein fraction of *A. muciniphila* conditioned medium, we stimulated STC-1 cells with 1 and 10% of heat-inactivated BHI CM. No fluctuation on Ca^{2+} signaling neither alteration on αSyn homeostasis evaluated by western blotting for GATA-2, αSyn and pser-129 αSyn was observed (Figure S4).
In order to confirm if these observed effects were specifically due to *A. muciniphila* conditioned medium, we conducted the same set of above experiments employing *Escherichia coli (E. coli)* conditioned medium. *E. coli* was chosen because it is an abundant Gram-negative microorganism from the gut. This strain was also cultivated in BHI medium under anaerobic condition as for *A. muciniphila*. Although we also observed a transient increase in free intracellular Ca\(^{2+}\) in STC-1 cells stimulated with 1 or 10\% *E. coli* BHI CM, the amplitude of the signal was smaller than the one elicited by *A. muciniphila*, (Figure S5a-e). In addition, we did not detect alteration on αSyn expression levels when STC-1 cells were incubated for 48 hs with *E. coli* CM (Figure S5f-h).

In summary, the protein fraction of *A. muciniphila* mucin-free CM leads to a transient increase in free intracellular Ca\(^{2+}\) and induces GATA2-regulated-overexpression of αSyn in the STC-1 enteroendocrine cell model.

*A. muciniphila* conditioned medium induces calcium release from stores in the endoplasmic reticulum in an IP\(_3\) -independent manner

Several maneuvers were performed to define the mechanism by which *A. muciniphila* mucin-free CM increases free cytoplasmic Ca\(^{2+}\) in STC-1 cells. To determine the source of the Ca\(^{2+}\), cells were stimulated in Ca\(^{2+}\)-free medium. We observed that *A. muciniphila* CM induced cytoplasmic Ca\(^{2+}\) oscillations in a concentration-dependent manner even in Ca\(^{2+}\)-free medium (Fig. 3a,b). Additionally, induced-Ca\(^{2+}\) signals initiate/predominate in the cytoplasm (Figure S6) and were elicited in a similar fraction of STC-1 cells regardless of the presence of extracellular Ca\(^{2+}\). On the other hand, selective depletion of stored calcium by 10µM thapsigargin significantly blocked Ca\(^{2+}\) oscillations-induced by *A. muciniphila* CM (Fig. 3c,d). Thereby, these findings demonstrate that *A. muciniphila* CM sample increases cytoplasmic Ca\(^{2+}\) levels by mobilizing intracellular Ca\(^{2+}\) stores.

A classic manner by which extracellular factors initiate an intracellular Ca\(^{2+}\) mobilization is by generating InsP3 to bind and release Ca\(^{2+}\) from InsP3 receptors in the endoplasmic reticulum [59]. In order to investigate whether the cytoplasmic Ca\(^{2+}\) increase was triggered by InsP3 generation, we stimulated STC-1 cells in the presence of the InsP3 receptor inhibitor xestospongin C [60]. Incubation of cells for 30 min and continuous perfusion with 2.5 µM xestospongin C did not impair *A. muciniphila* CM -induced Ca\(^{2+}\) mobilization, suggesting an InsP3-independent release of intracellular Ca\(^{2+}\) stores (Fig. 3e,f). To go further into the mechanism by how *A. muciniphila* CM evokes Ca\(^{2+}\) release from intracellular stores, we incubated cells for 30 min with dantrolene (75 µM), an inhibitor of Ca\(^{2+}\) release through ryanodine receptor (Ryr) channels [61–63]. In the presence of 75 µM dantrolene, only a very small Ca\(^{2+}\) increase was observed following stimulation with 1 or 10% *A. muciniphila* CM (Fig. 3g,h).

Thus, dantrolene eliminated *A. muciniphila* CM Ca\(^{2+}\) response in enteroendocrine cells. Taken together, these results show that proteins contained in *A. muciniphila* conditioned medium works as a
physiological RYR gating agent, eliciting intracellular Ca\(^{2+}\) signals by directly modulating RYR in the cytoplasm.

**Mitochondrial calcium overload and impaired membrane potential (ΔΨ\(_m\)) is elicited by A. muciniphila conditioned medium**

Global changes in Ca\(^{2+}\) homeostasis accompanied by the alteration in cellular bioenergetics status and thereby imposing oxidative stress in cells are reported in PD [64, 65]. The cytosolic Ca\(^{2+}\) concentration in unstimulated cells is maintained at low levels (∼100 nM) by several enzymes that translocate Ca\(^{2+}\) ions into intracellular stores or across the plasma membrane. Moreover, Ca\(^{2+}\) uptake into the mitochondria is not limited to the control of organelle function, but also has a direct impact on the intracellular Ca\(^{2+}\) signals evoked by agonist stimulation in the cytosol through modulation of their kinetics and spatial dimensions [66]. Enhanced cytosolic Ca\(^{2+}\) concentration, on the other hand, affects the bioenergetics of the cells by promoting increased ATP demand [67]. Furthermore, this alteration in cytosolic Ca\(^{2+}\) hampers the normal Ca\(^{2+}\) handling by various intracellular organelles, including mitochondria, and threatens neuronal survival. Although well established for neuronal cells, there is still a gap regarding changes in mitochondrial Ca\(^{2+}\) dynamics in enteroendocrine cells due to gut microbiome stimulation and how this event might be related to αSyn homeostasis.

Thereby, we aimed at evaluating mitochondrial Ca\(^{2+}\) under stimulation with *A. muciniphila* conditioned medium. When STC-1 cells loaded with the mitochondrial Ca\(^{2+}\) indicator Rhod-2/AM dye were stimulated with 10% of *A. muciniphila* CM, we observed a significant increase in mitochondrial Ca\(^{2+}\) uptake when compared to unconditioned BHI medium (Fig. 4a,b). In addition, when we incubated the cells for 48 hs with 1 or 10% CM and stimulated with ATP (10 µM), mitochondrial fluorescence was dramatically increased in the group incubated with 10% BHI CM when compared to 1% BHI CM or unconditioned BHI medium (1 and 10%) suggesting that long exposure to *A. muciniphila* conditioned medium induces increased uptake of Ca\(^{2+}\) by the mitochondria (Fig. 4c,d).

As previously mentioned, enhanced, or sustained Ca\(^{2+}\) stress results in mitochondrial injury due to Ca\(^{2+}\) overload. Excessive mitochondrial Ca\(^{2+}\) uptake or impaired Ca\(^{2+}\) efflux influences mitochondrial membrane potential (ΔΨ\(_m\)) leading to depolarization of mitochondrial inner membrane, swelling of the organelle, and ultimately cell death [68–70]. In order to observe whether mitochondrial Ca\(^{2+}\) uptake induced by *A. muciniphila* CM could lead to mitochondrial damage, we monitored ΔΨ\(_m\) in STC-1 cells under *A. muciniphila* CM incubation for 48 hs. After treatment, cells were stained with the mitochondrial-targeted probe Mitotracker Red CMXRos, which accumulates in mitochondria depending on its membrane potential and has been widely used as an indicator of reduced ΔΨ\(_m\) [71, 72]. As can be observed on Fig. 4e,f, cells incubated with 10% CM presented a reduced fluorescent signal of the probe what suggests impaired membrane potential.
Altogether, the results described so far demonstrate that *A. muciniphila* mucin-free conditioned medium induces exacerbated mitochondrial Ca$^{2+}$ uptake, which in turn is the driven force that causes mitochondrial damage, reflected by a loss of membrane $\Delta \Psi_m$.

Increased intracellular ROS level, $\alpha$-synuclein phosphorylation and aggregation as a consequent event of *A. muciniphila* conditioned medium stimulation of enteroendocrine cells

It is suggested that endogenous ROS mainly modulate cell signaling locally and stimuli that promote ROS formation or mitochondrial alterations highly correlate with mutant $\alpha$Syn phosphorylation at Serine 129 (Ser129), a promoter of $\alpha$Syn aggregation propensity and toxicity in PD [73–75]. Therefore, we next measured intracellular levels of ROS under stimulation with 1 or 10% *A. muciniphila* CM by live cell imaging. STC-1 cells were incubated for 30 min with DHE and continuously perfused with buffer containing 1 or 10% CM. Buffer/unconditioned media and $\text{H}_2\text{O}_2$ (100 µM) perfusion were used as negative and positive controls, respectively. The real-time fluorescence measurement indicates that the surge of ROS level after $\text{H}_2\text{O}_2$ or 1–10% CM stimulation was significantly higher than stimulation with buffer or 1–10% unconditioned BHI media for 5 min (Fig. 5a,b). In addition, cells stimulated with 1 or 10% CM presented increased DHE fluorescence in a similar manner.

As mentioned, stimuli that promote intracellular ROS formation and mitochondrial damage highly correlate with $\alpha$Syn phosphorylation at Ser129, an event that may precede cell degeneration in PD [73]. Previous observations have shown that both nigral and dorsal motor nucleus of the vagus nerve neurons present a high vulnerability to oxidative challenges [76]. Since the nigro-vagal pathway that controls gastric tone and motility connect these brain regions, it raises the possibility that an oxidative injury may be relayed and possibly amplified through this anatomical and functional connection.

In order to evaluate whether increases ROS levels induced by *A. muciniphila* CM could promote $\alpha$Syn phosphorylation and aggregation, we incubated the cells for 48 hs in the presence of CM or unconditioned BHI media and directed them to immunofluorescence and Western blotting. Confocal microscopy images showed strong deposits of pSer129-$\alpha$Syn in STC-1 cells incubated with 1 and 10% CM (Fig. 5c). In addition, quantification by Western blotting showed a 2-3-fold increase of p-Ser129-$\alpha$Syn in cells treated with the conditioned medium when normalized against total $\alpha$Syn (Fig. 5d). To establish whether *A. muciniphila* CM-induced p-Ser129 $\alpha$Syn might play a role on $\alpha$Syn aggregation in our STC-1 cell model, we transfected cells with full-length human $\alpha$Syn-GFP-tagged and incubated them with unconditioned or CM for 48hs. Unconditioned BHI media (1 or 10%) did not cause $\alpha$Syn to form cellular inclusions. However, 1 and 10% CM led to the formation of small to large $\alpha$Syn granules within the cytoplasm (Fig. 5e). When we quantified the number of GFP-positive cells containing intracellular aggregates, we observed that over 50% of the cells stimulated with *A. muciniphila* CM contained $\alpha$Syn granules (Fig. 5f). Thereby, conditioned medium of *A. muciniphila* grown in the absence of mucin induces intracellular $\alpha$Syn aggregation in enteroendocrine cell model.
Oral administration of Akkermansia muciniphila to aged mice leads to αSyn aggregation in CCK-positive enteroendocrine cells

So far, our results showed that the protein fraction of *A. muciniphila* conditioned medium grown in the absence of mucin induces mitochondrial stress and ROS generation which in turn led to αSyn aggregation. In addition, previous works have shown that aged mice have impaired mucus barrier in the colon and ileum and this thinner mucus layer was associated with increased bacterial penetrability and contact with the epithelium[42, 77]. Therefore, we wondered whether the increased levels of *A. muciniphila* in aged mice could be a trigger to αSyn pathology in the gut. To assess if *A. muciniphila* could cause motor deficits, we treated aged mice with bacterial cells (AKK group) for 28 continuous days (Figure S7a). After 28 days of oral administration, AKK group did not exhibit alteration in body weight but presented significantly higher number of *A. muciniphila* 16S rRNA copies in stool (Figure S7b,c).

We used three measures of gross motor function: time to cross a challenging beam, the cylinder test and wire hanging. In none of the test we observed differences between control and AKK group (Figure S7d-f). Utilizing an antibody that recognizes only conformation-specific αSyn aggregates and fibrils, we performed dot blot analysis for aggregated αSyn in total protein extract from ileum of control and *A. muciniphila*-treated animals and observe similarly low levels of αSyn aggregation in both groups (Fig. 6a,b). Interestingly, by immunofluorescence, we observe αSyn aggregation in cholecystokinin (CCK) - positive enteroendocrine cells (Fig. 6c,d). In addition, the number of CCK-positive cells containing αSyn aggregates in AKK group was ~ 4 times higher when compared to control animals which barely presented αSyn-aggregate-containing cells (Fig. 6e). These data suggest that *A. muciniphila*, when exposed to a mucin-deprived environment, regulates pathways that promote αSyn aggregation and/or prevent the clearance of insoluble protein aggregates in enteroendocrine cells, suggesting that αSyn pathology can indeed start in the gut.

**Mitochondrial calcium buffering reverts the damaging effects to mitochondria and prevents α-synuclein aggregation**

Inhibition of mitochondrial Ca\(^{2+}\) uptake was shown to diminish the oxidative stress in substantia nigra pars compacta dopaminergic neurons (SNpc DNs) suggesting that mitochondrial oxidative stress could also be due to mitochondrial Ca\(^{2+}\) overload [78]. Several lines of investigation point out to mitochondrial Ca\(^{2+}\) imbalance as key factor to be modulated in order to control the progression of PD. In order to observe whether modulating mitochondrial Ca\(^{2+}\) in enteroendocrine cells could reverse intracellular ROS generation and αSyn aggregation, we transfected the cells with parvalbumin (PV) fused to a mitochondrial targeting sequence (MTS) and GFP [41]. Parvalbumin (PV) is a cytosolic Ca\(^{2+}\)-binding protein of the large EF-hand protein family, involved in intracellular Ca\(^{2+}\) regulation and buffering. GFP targeted to the mitochondrial matrix was used as a control (MTS-GFP) (Fig. 7a).
One or 10% BHI CM elicited a robust increase in mitochondrial Ca\(^{2+}\) in cells expressing MTS-GFP alone, but this was reduced by approximately 90% in cells expressing PV in mitochondria (Fig. 7b,c). These results demonstrated that PV-MTS-GFP was correctly targeted to the mitochondrial matrix and efficiently buffered mitochondrial Ca\(^{2+}\) overload driven by stimulation with \textit{A. muciniphila} conditioned medium.

Once mitochondrial Ca\(^{2+}\) was buffered, the next set of experiments aimed to observe whether the damaging effects caused by \textit{A. muciniphila} conditioned medium could be prevented. When we stimulated the cells expressing PV-MTS construct with 1 and 10% BHI CM, the increase in intracellular ROS was significantly suppressed (Fig. 7d,e) indicating that mitochondrial Ca\(^{2+}\) buffering prevents intracellular oxidative stress.

To test the effect of mitochondrial Ca\(^{2+}\) on Ser129-phosphorylation of αSyn induced by \textit{A. muciniphila} conditioned medium, we incubated the transfected cells with 1 or 10% CM for 48hrs. Total cell lysate evaluated by Western blotting showed that levels of Ser129-phosphorylated αSyn significantly decreased in PV-MTS expressing cells when compared with control cells (MTS-GFP) (Fig. 8a,b). However, no effect was observed in the total expression level of αSyn, which remained higher when compared to untreated cells (Fig. 8a,c).

We then extended our observation that mitochondrial Ca\(^{2+}\) can suppress intracellular ROS generation and αSyn phosphorylation to the formation of αSyn aggregates. Hence, we double-transfected cells with the PV-MTS-GFP construct and human αSyn mCherry-tagged. Large number of αSyn aggregates were observed in cells expressing the control construct (MTS-GFP) after 48 hs of treatment with 1 or 10% conditioned medium. However, the number of αSyn aggregates in cells expressing the PV-MTS-GFP constructed was markedly reduced (Fig. 8d,e).

Taking together, these findings provide evidence on the mechanism by which \textit{A. muciniphila} conditioned media induces αSyn aggregation in enteroendocrine cells (Fig. 9).

**Discussion**

Parkinson’s disease is a growing health concern for an ever-aging population. Although genetic risks have been identified, environmental influences and gene-environment interactions are so far considered responsible for most PD cases\[79\]. Besides the plethora of neurological and motor symptoms, PD patients present prominent gut manifestations \[4, 6, 80–83\].

Aggregates of the protein αSyn is a hallmark of PD. Interestingly, αSyn pathology in PD is not limited to the brain. It was also observed in the peripheral nervous system, including the enteric nervous system \[84\]. Therefore, the interaction between the gut microbiota, EECs and αSyn aggregation in PD is receiving increasing attention. The idea that αSyn aggregation process is initiated in the gut following continuous gastrointestinal symptoms aggravation and spreading to the nervous system in a prion-like manner has gathered significant force in recent years. Some pathophysiological evidence helps to support this notion:
αSyn inclusions appear earlier in the enteric nervous system and the glossopharyngeal and vagal nerves[13, 85]; and vagotomized individuals are at reduced risk for PD [86]. In addition, injection of αSyn fibrils into the gut tissue of healthy rodents seemed sufficient to induce disease within the vagus nerve and brainstem [87].

The finding that EECs connect to nerves raises an array of possibilities for how nutrients, bacteria, toxins, and potential pathogens gain access to and communicate with the nervous system. The discovery of αSyn in EECs, which are directly exposed to A. muciniphila secreted proteins in the gut lumen and connected to enteric nerves, provides a location in which misassemble and spread of αSyn could initiate [16]. However, the knowledge of how αSyn aggregation initiates in the gut and spreads to the central nervous system via retrograde transmission and whether the gut microbiome could directly trigger this process remains controversial [86].

The specific mechanisms by which gut bacteria promote αSyn-mediated pathophysiology are likely diverse, complex, and poorly explored. Nonetheless, in this work we have identified that mitochondrial Ca²⁺ overload in EECs led by the conditioned medium of a commensal gut bacterium A. muciniphila is a molecular pathway by which αSyn homeostasis is disturbed in EECs, providing experimental support for a gut-microbial connection to PD.

Since its discovery in 2004, Akkermansia muciniphila [36] has gathered a great amount of scientific attention. It has been shown that intestinal Akkermansia abundance is significantly reduced in a many metabolic disorders, including type 2 diabetes, obesity, dyslipidemia [88]. Therefore, this has stimulated several studies in order to investigate Akkermansia supplementation. Evidence shows Akkermansia supplementation restores epithelial mucosal integrity, reduces weight gain and fat accumulation in the liver, improves glucose tolerance, and reduces inflammation and metabolic endotoxemia in animal models of diabetes and obesity [29, 36].

However, we cannot rule out recent reports showing that alteration in gut microbiota is associated with PD. Several lines of evidence suggest that PD patients present strong gut dysbiosis with remarkable abundance of A. muciniphila which is consistently high in PD stool samples [18, 19, 21, 22, 25, 80, 89–92]. Additionally, emerging studies have described increased abundance of A. muciniphila in multiple sclerosis (MS), one of which enrolled monozygotic twins discordant for MS diagnosis, therefore excluding genetic confounders [93, 94].

In this work, we showed that the proteins obtained from A. muciniphila conditioned medium cultivated in mucin-free medium induces RYR-dependent ER Ca²⁺ release. This persistent Ca²⁺-mediated signals is followed by increased mitochondrial Ca²⁺ uptake in STC-1 cells, which in turn culminates with aSyn phosphorylation and aggregation. On the other hand, the conditioned media collected of A.muciniphila grown in mucin-supplemented medium cause no intracellular alteration in the enteroendocrine cell line studied. This phenomenon leads us to the fact that in order to maintain the mammalian intestinal homeostasis with the microbiota, a key element is to minimize and regulate contact between luminal
microorganisms and the intestinal epithelial cell surface. In the gut, physical separation of bacteria and the epithelium is greatly accomplished by secretion of mucus, antimicrobial proteins, and IgA into the lumen [95, 96]. Interestingly, it was previously shown that aged mice (15–19 months old) have an impaired mucus barrier in the colon and ileum accompanied by major changes in the fecal microbiota composition, a fact that has also been observed in humans [42, 77]. Therefore, besides changing the microbiome pattern of secretion (Fig. 1), a decrease on mucus barrier thickness leads to increased contact of gut bacteria and their secreted components with the intestinal epithelium that could therefore modulate gut cells homeostasis, especially misbalancing intracellular Ca\(^{2+}\) dynamics.

An emerging, key pathological feature in neurons affected by PD is the global dysregulation of Ca\(^{2+}\) homeostasis [97]. Ca\(^{2+}\) handling through contact sites between the endoplasmic reticulum and mitochondria (mitochondria-associated endoplasmic reticulum membranes – MAMs) have attracted great attention in the study of cell homeostasis and dysfunction, especially in the context of neurodegenerative disorders. Emerging evidence suggests that the abnormality and dysfunction of MAMs have been involved in a number of neurodegenerative disorders including Alzheimer’s disease, amyotrophic lateral sclerosis, and Parkinson’s disease [98–100]. Also, increased intracellular Ca\(^{2+}\) levels alter Ca\(^{2+}\) handling in intracellular organelles such as the endoplasmic reticulum and mitochondria which may potentiate pathological effects [64]. Indeed, Ca\(^{2+}\) uptake into the mitochondria is a key mechanism by which cells maintain intracellular Ca\(^{2+}\) homeostasis [101, 102]. However, excessive mitochondrial Ca\(^{2+}\) uptake or impaired Ca\(^{2+}\) efflux results in ROS production [103, 104] and disruption of membrane potential inducing neuronal cell death, an important indicator of several different neurological disorders including Alzheimer’s Disease (AD) and PD. Moreover, it has been suggested that impaired mitochondrial biogenesis, Ca\(^{2+}\) buffering and oxidative stress may precede the development of PD and AD pathology [105–108]. Recent studies indicate that increased intracellular free Ca\(^{2+}\) and oxidative stress synergistically augment the number of cytoplasmic αSyn-enriched aggregates \textit{in vitro} and \textit{in vivo} [109]. In addition, it is also known that αSyn aggregates trigger increased mitochondrial Ca\(^{2+}\) transient and then leads to oxidative stress [110, 111]. Thus, an increase in oxidative stress can cause αSyn aggregation which can also induce further oxidative stress within the cell creating a positive feedback loop.

Although very clear for neuronal cells, we show here that this cascade of events is also triggered in EECs stimulated by \textit{A. muciniphila} secreted proteins. The outcome of this persistent Ca\(^{2+}\) mishandling is very similar to what has already been reported for neurons in PD. Additionally, since αSyn-containing EECs directly connect to enteric nerve terminals forming a neural circuit between the gut and the nervous system, influences in the gut lumen affect αSyn folding in the EECs which can then propagate to the nervous system. Hence, we offer some mechanistic insights of how misfolded αSyn could be generated in EECs and then be propagated from the gut epithelium to the brain, being a possible outcome of Sporadic PD, as once hypothesized by Braak [85].

Conclusions
In summary, this work shows that the protein fraction of *A. muciniphila* secretome is directly modulated by mucin and induces an IP$_3$-independent ER-calcium release in enteroendocrine cells. This Ca$^{2+}$ release is triggered by direct activation of RYR leading to increased mitochondrial Ca$^{2+}$ uptake. Mitochondrial Ca$^{2+}$ overload leads to ROS generation culminating with αSyn aggregation. In addition, oral administration of *A. muciniphila* to old mice led αSyn to aggregate in CCK-positive enteroendocrine cells. Buffering mitochondrial Ca$^{2+}$ efficiently inhibits *A. muciniphila*-induced αSyn aggregation in enteroendocrine cells. Finally, the data presented here provide further information to be considered in the design of microbial interventions for therapeutic use and in the importance of maintaining the integrity of the mucus-barrier. In addition, this data set the stage for dissection of the biochemical mechanisms underlying gut microbiome-induced- αSyn aggregation.

**Abbreviations**

ΔΨm  
mitochondrial membrane potential  
AD  
Alzheimer's Disease  
AKK  
*Akkermansia muciniphila* or *A. muciniphila*  
ATP  
denosine triphosphate  
BHI  
Brain & Heart Infusion Broth  
Ca$^{2+}$  
calcium  
CCK  
cholecystokinin  
CM  
conditioned medium  
CNS  
central nervous system  
EECs  
enteroendocrine cells  
ER  
endoplasmic reticulum  
FBS  
fetal bovine serum  
IP$_3$  
inositol 1,2,4 triphosphate  
MAMs
mitochondria-associated endoplasmic reticulum membranes

**MS**
multiple sclerosis

**MS-MS**
mass spectrometry

**MTS**
mitochondrial targeting sequence

**PBS**
phosphate-buffered saline

**PD**
Parkinson's disease

**PFA**
Paraformaldehyde

**PSA**
penicillin/streptomycin antibiotics

**PV**
protein parvalbumin

**PYY**
peptide YY

**ROS**
reactive oxygen species

**RYR**
ryanodine receptors

**SNpc DNs**
substantia nigra pars compacta dopaminergic neurons

**αSyn**
α-synuclein or alpha-synuclein

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**Declarations**

**Ethics approval**

The experiments were conducted according to the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals (NIH publications No. 8023, revised 1978). All experiments with animals were approved by the Institutional Committee of Animal Use & Protection (CEUA 92-B).

**Consent for publication**

Not applicable

**Availability of data and materials**
No obtained data required submission to a public repository. Raw data or further details related to the conducted experiments can be obtained upon request from the corresponding author.

Competing interest

The authors declare that they have no competing interests.

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Author’s contribution

Conceptualisation, MCF and DPAN; Formal Analysis, DPAN, BPG, and MCF; Investigation DPAN, BPB, JVPG, KT, PV, CCCT; Resources, CCCT and MCF; Writing, DPAN, CGB and MCF; Funding Acquisition, MCF.

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Figures
Figure 1

Growth curve of A. muciniphila and conditioned media protein composition. a Growth curve as a function of culture media supplementation. (Error bars indicate the media ± SEM of 6 individual bacterial culture for each condition; unpaired Student's t-test, *p<0.05). b Qualitative Venn diagram showing the common and unique expressed proteins between the 0.4% mucin and mucin-free culture condition identified by mass spectrometry. Mass spectrometry data were obtained from at least 6 vials for each culture condition.
Akkermansia muciniphila conditioned medium induces intracellular calcium signals and increased levels of α-synuclein in STC-1 cells. a Confocal microscopy imaging of STC-1 cells incubated with Fluo-4/AM (6μM) and stimulated with 1 or 10% A. muciniphila conditioned media (BHI CM) (scale bar: 10 μm). b Representative time-course of total Ca2+ signal. Arrow indicates the moment when culture medium was applied. c Quantification of the peak fluorescence following stimulation with 1 or 10% conditioned (BHI
CM) and unconditioned (BHI) media. (Error bars indicate the media ± SEM; n= at least 25 cells for each group, * p< 0.05 by unpaired Student’s t-test). d αSyn staining (green) in STC-1 cells after 48 hs incubation with 1-10% conditioned (BHI CM) or unconditioned media (BHI) demonstrating increased expression of the protein. Nuclei were stained with DAPI (blue) and immunofluorescence control is shown as NSB (non-specific binding control) (scale bar: 10 µm). e Quantification of αSyn fluorescence intensity in images shown in d. (Error bars indicate the media ± SEM; n= at least 25 cells for each group from 3 individual experiments, * p< 0.05 by unpaired Student’s t-test). f Immunoblots (upper image) of total cell lysates showing the increased expression of αSyn after 48 hours-incubation with 1-10% conditioned (BHI CM) or unconditioned media (BHI). Densitometric analysis shows increased expression of αSyn in 1-10% BHI CM condition when compared to 1-10% BHI. (Error bars indicate the media ± SEM; n= 4 individual experiments, *p< 0.05 by two-way Student’s t-test). g Immunoblots (upper image) of total cell lysates showing the increased expression of GATA-2 after 48 hours-incubation with 1-10% conditioned (BHI CM) or unconditioned media (BHI). Densitometric analysis shows increased expression of GATA-2 in 1-10% BHI CM condition when compared to 1-10% BHI. (Error bars indicate the media ± SEM; n=3 individual experiments; *p < 0.05 by unpaired Student’s t-test).
Akkermansia muciniphila conditioned medium induces InsP3-independent intracellular calcium signals by acting directly on ryanodine receptors. 

a STC-1 cells were stimulated with 1 or 10% A. muciniphila conditioned media (BHI CM) in the presence of Ca2+-free buffer. Graph shows a representative time-course of total Ca2+ signal in STC-1 cells. The arrow indicates the time when culture medium was applied. 

b Quantification of the peak fluorescence following cells stimulation with 1 or 10% conditioned media.
(BHI CM) and unconditioned (BHI) media in the presence of 1 mM Ca2+ buffer or Ca2+ free buffer. c STC-1 cells were incubated with 10 µM thapsigargin for 30 min and stimulated with 1 or 10% A. muciniphila conditioned media (BHI CM) in the presence of Ca2+-free buffer containing 10 µM thapsigargin. Graph shows a representative time-course of total Ca2+ signal in STC-1 cells. Arrow indicates the time when culture medium was applied. d Quantification of the peak fluorescence following cells stimulation with 1 or 10% conditioned (BHI CM) and unconditioned (BHI) media shows that the Ca2+ signal induced by BHI CM is blocked by thapsigargin 10 µM. e STC-1 cells were incubated with 2.5 µM xestospongin C for 30 min and stimulated with 1 or 10% A. muciniphila conditioned media (BHI CM) in the presence of Ca2+-free buffer containing 2.5 µM xestospongin C. Graph shows a representative time-course of total Ca2+ signal in STC-1 cells. The arrow indicates the time when culture medium was applied. f Quantification of the peak fluorescence following cells stimulation with 1 or 10% conditioned (BHI CM) and unconditioned (BHI) media shows that the Ca2+ signal induced by BHI CM is not blocked by the InsP3 receptor inhibitor xestospongin C (2.5 µM). g STC-1 cells were incubated with 75 µM dantrolene for 30 min and stimulated with 1 or 10% A. muciniphila conditioned media (BHI CM) in the presence of Ca2+-free buffer containing 75µM dantrolene. Graph shows a representative time-course of total Ca2+ signal in STC-1 cells. The arrow indicates the time when culture medium was applied. h Quantification of the peak fluorescence following cells stimulation with 1 or 10% conditioned (BHI CM) and unconditioned (BHI) media shows that the Ca2+ signal induced by BHI CM is completely blocked by the RYR receptor inhibitor, dantrolene (75 µM). (Data in a, c, e and g represent a representative tracing recorded from one individual STC-1 cell of each group. Data in (b, d, f and h) represent the mean ± SEM of three independent experiments in which at least 25 individual cells were analyzed for calcium transient. * p< 0.05 by one-way Student’s t-test).
Figure 4

Increased mitochondrial Ca2+ uptake elicited by Akkermansia muciniphila conditioned media leads to mitochondrial stress and reduced $\Delta\Psi_m$. a Representative time-course of mitochondrial Ca2+ signal. Cells were incubated with the mitochondrial Ca2+ indicator Rhod-2/AM and stimulated with 10% A. muciniphila conditioned medium (BHI CM) in the presence of Ca2+-free buffer. The arrow indicates the time when culture medium was applied. b Graphs show quantification of the peak of fluorescence
following stimulation with 10% BHI CM. c Representative time-course of mitochondrial Ca2+ signal of cells incubated for 48 hs with 1-10% A. muciniphila conditioned medium (BHI CM) and stimulated with 10 µM ATP in the presence of Ca2+-free buffer. The arrow indicates the moment when culture medium was applied. d Graphs show quantification of the peak of fluorescence following stimulation with ATP. e Confocal images of STC-1 cells incubated for 48 hs with 10% BHI CM and then stained with MitoTracker Red CMXRos (red). Nuclei were stained with DAPI (blue). f Quantification of fluorescent signal in untreated and treated cells. Data in (a and c) represent a representative tracing recorded from one individual STC-1 cell of each group. Data in (b, d and f) represent the mean ± SEM of three independent experiments in which at least 25 individual cells were analyzed. * p< 0.05 by unpaired Student’s t-test.
α-synuclein phosphorylation and aggregation as a result of increased intracellular levels of ROS due to Akkermansia muciniphila conditioned media treatment of enteroendocrine cells. 

**Figure 5**

a) Time lapse of ROS production in STC-1 cells measured by DHE fluorescence intensity under confocal live imaging. 1 and 10% BHI or BHI CM was used as stimuli. 100 μM H2O2 was used as positive control. 

b) Quantitative summary of the effects of A. muciniphila conditioned and unconditioned media on ROS production. * p<
0.001 by unpaired Student's t-test. c Confocal images of pSer129 αSyn staining (green) in STC-1 cells after 48 hs incubation with 1-10% conditioned (BHI CM) or unconditioned media (BHI) demonstrating increased phosphorylation of the protein. Nuclei were stained with DAPI (blue) and immunofluorescence control is shown as NSB (non-specific binding control). Arrows point to aberrant fibrillary-like structures. (Scale bar: 10 µm). d Immunoblots (upper image) of total cell lysates showing the increased phosphorylation of αSyn on Ser129 (normalized against total αSyn) after 48 hours-incubation with 1/10% conditioned (BHI CM) or unconditioned media (BHI). Densitometric analysis shows phosphorylation of αSyn (pSer129) in 1-10% BHI CM condition when compared to 1/10% BHI. * p< 0.05 by unpaired Student’s t-test. e Confocal images of hαSyn GFP-tagged plasmid transfected into STC-1 cells exhibits diffuse distribution in untreated or BHI-treated cells. Cells treated with 1-10% BHI CM medium forms inclusions of different sizes (bottom images). (Scale bar: 10 µm). f Graph shows the number of GFP-positive cells containing inclusions in each condition. *p< 0.05 by unpaired Student’s t-test. Data in (A) represent a representative tracing recorded from one individual STC-1 cell of each group. Data in (B and F) represent the mean ± SEM of independent experiments in which at least 25 individual cells were analyzed in B and 6 slides for F. Densitometric analysis of western blot (D) are derived from triplicates of three different experiments.
Commensal gut Akkermansia muciniphila induces α-synuclein aggregation in enteroendocrine cells. 

**Figure 6**

a) Aggregate-specific aSyn dot blots derived from total ileum homogenate of aged mice. Each dot is representative of a piece of tissue from one animal. 

b) Densitometry quantification of dot blots in (A).

c) 3D Z-stack image of a 150µm section of the ileum of aged mice treated with PBS or with mucin-free cultivated A. muciniphila (AKK) for 28 days. CCK-positive enteroendocrine cells are stained for anti-CCK.
(white), specific antibody for αSyn-aggregates (green), actin cytoskeleton (phalloidin, red) and DAPI. d Quantiﬁcation of αSyn aggregates and ﬁbrils ﬂuorescence (% of control). e Quantiﬁcation of the number of CCK-positive cells containing αSyn aggregates and ﬁbrils in control and AKK-treated mice. Error bars indicate the media ± SEM. Each dot represents the mean ﬂuorescence of at least 15 cells per slice (at least 3 slices of 5 individual animals). Scale bar = 10µm.

Figure 7
Mitochondrial Ca2+ buffering reduces intracellular ROS levels elicited by Akkermansia muciniphila conditioned medium. a Confocal images of STC-1 cells transfected with mitochondrial parvalbumin (PV) expression and control vectors showing the expression and mitochondrial localization of targeted PV-MTS-GFP and MTS-GFP fusion proteins. Scale bar= 10 µM. b Representative changes in mitochondrial Ca2+ signals over time are shown. Cells transfected with the indicated vectors were loaded with Rhod-2/AM and induced by 1-10% unconditioned (BHI) or conditioned medium (BHI-CM) (arrow). Ca2+ signals were attenuated in cells expressing PV in mitochondria. c Peak Ca2+ signals were observed in three separate experiments for STC- cells transfected with MTS-GFP, and cells transfected with PV-MTS-GFP. *p< 0.05 by unpaired Student’s t-test. d Representative changes in intracellular ROS levels over time are shown. Cells transfected with the indicated vectors were loaded with DHE and induced by 1/10% unconditioned (BHI) or conditioned medium (BHI-CM) (arrow). DHE fluorescence intensity was significantly reduced in cells expressing PV-MTS-GFP fusion protein. e Peak ROS signals were observed in three separate experiments for STC- cells transfected with MTS-GFP, and cells transfected with PV-MTS-GFP stimulated with each represented condition. * p< 0.05 by unpaired Student’s t-test. Data in (b and d) represent a representative tracing recorded from one individual STC-1 cell of each group. Data in (c and e) represent the mean ± SEM of three independent experiments in which at least 25 individual cells were analyzed.
α-synuclein phosphorylation and aggregation induced by Akkermansia muciniphila conditioned medium are prevented due to mitochondrial Ca2+ buffering. a Immunoblots (upper image) of total cell lysates from PV-MTS-GFP or MTS-GFP transfected cells showing a decrease in αSyn phosphorylation on Ser129 after 48 hours-incubation with 1-10% conditioned (BHI CM) or unconditioned media (BHI). b Densitometric analysis shows that mitochondrial Ca2+ buffering reduced αSyn phosphorylation in 1-10%
BHI CM condition when compared to cells expressing MTS-GFP fusion protein. * p< 0.05 by two-way Student’s t-test. c Densitometric analysis shows that mitochondrial Ca2+ buffering did not reduce αSyn expression induced by 1/10% BHI CM condition when compared to cells expressing MTS-GFP fusion protein. * p< 0.05 by two-way Student’s t-test. d Confocal images of STC-1 cells co-transfected with PV-MTS-GFP and αSyn-mCherry tagged construct show reduced number of intracellular αSyn aggregates after 48 hs incubation with 1-10% BHI CM. (scale bar: 10µM). e Quantification of the number of GFP-positive cells containing mCherry-tagged αSyn aggregates. Data are expressed as percentage of total GFP-positive cells per image. * p< 0.05; Ns, not significant by unpaired Student’s t-test. Densitometric analysis of western blot (b and c) are derived from triplicates of three different experiments. Data in (e) represent the mean ± SEM of at least three independent experiments in which at least 6 individual slides were analyzed.
Synopsis figure. The protein fraction of the conditioned media isolated from the commensal gut bacterium Akkermansia muciniphila triggers intracellular Ca2+ signaling in enteroendocrine cells, leading to increased mitochondrial Ca2+ uptake. Mitochondrial Ca2+ overload leads to ROS generation culminating with αSyn phosphorylation and aggregation (left panel). All these events were inhibited once mitochondrial Ca2+ is buffered (right panel).
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- TableS1.xlsx