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Supplemental information

Akkermansia muciniphila induces mitochondrial calcium overload and α -synuclein aggregation in an enteroendocrine cell line

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Supplemental Figures and Data

Figure S1

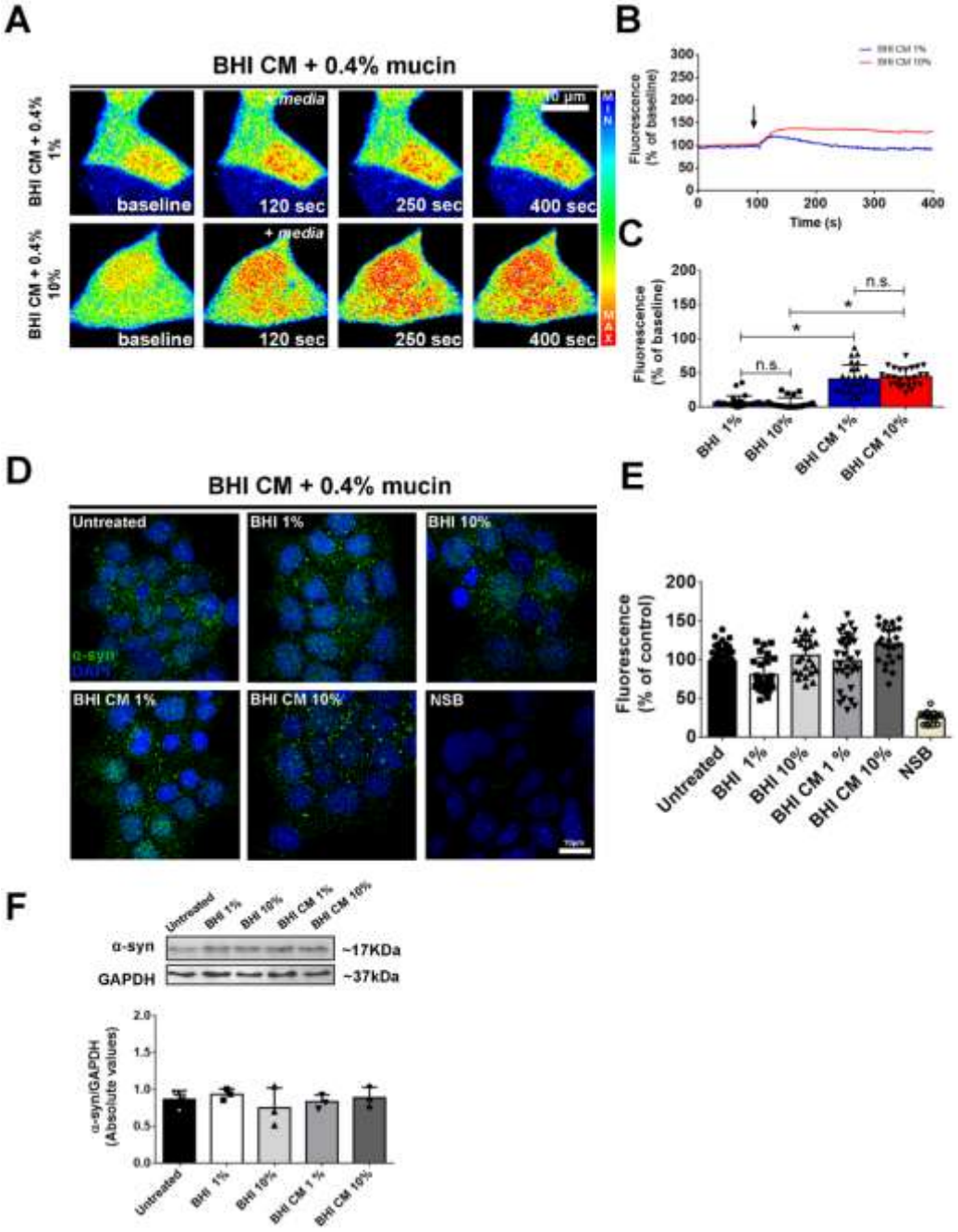

Figure S1. 0.4% mucin-supplemented *Akkermansia muciniphila* conditioned medium induces discrete intracellular calcium signals and does not influence total levels of α-synuclein in STC-1 cells, related to Figure 2. (A) Confocal microscopy imaging of STC-1 cells incubated with Fluo-4/AM (6µM) and stimulated with 1 or 10% >3kDa fraction of mucin-supplemented *A. muciniphila* conditioned media (BHI CM + 0.4% mucin) (scale bar: 10 µm). **(B)** Representative time-course of total Ca^{2+} signal. The arrow indicates the time 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 mucin-supplemented media (BHI). 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 expression of αSyn after 48 hours-incubation with 1-10% conditioned (BHI CM) or unconditioned mucin-supplemented media (BHI). Graphic representation of densitometric analysis showing expression of αSyn in 1-10% BHI and BHI CM mucin-supplemented condition (Data are represented as mean +/- SEM; n=3 individual experiments; *p < 0.05 by unpaired Student's *t-test*).

Figure S2

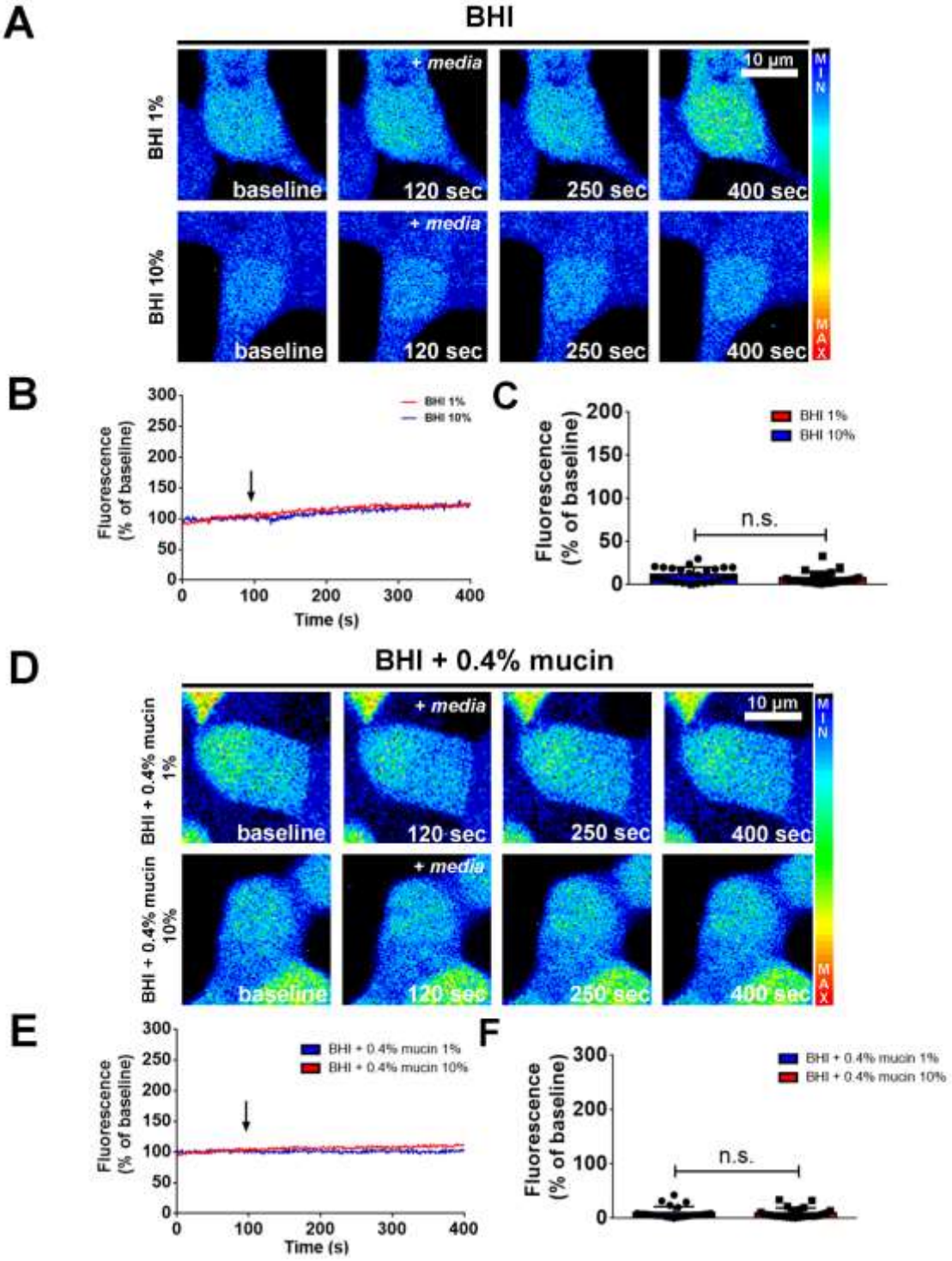

Figure S2. Mucin-free or mucin-supplemented unconditioned media do not induce intracellular calcium signals in STC-1 cells, related to Figure 2. (A) Confocal microscopy imaging of STC-1 cells incubated with Fluo-4/AM (6 µM) and stimulated with 1 or 10% >3kDa fraction of unconditioned mucin-free media (BHI) (scale bar: 10 µm). **(B)** Representative time-course of total Ca^{2+} signal. Arrow indicates the moment when culture medium was applied. **(C)** Quantification of the peak fluorescence following stimulation with 1 or 10% unconditioned mucin-free media (BHI) (Error bars indicate the media ± SEM; n= at least 35 cells for each group from 3 individual experiments, n.s.= not significant by unpaired Student's *t-test*). **(D)** Confocal microscopy imaging of STC-1 cells incubated with Fluo-4/AM (6µM) and stimulated with 1 or 10% unconditioned 0.4% mucin-supplemented media (BHI + 0.4% mucin) (scale bar: 10 µm). **(E)** Representative time-course of total Ca^{2+} signal. The arrow indicates the time when culture medium was applied. **(F)** Quantification of the peak fluorescence following stimulation with 1 or 10% unconditioned 0.4% mucin-supplemented media (BHI + 0.4% mucin).) (Data are represented as mean +/- SEM; n= at least 25 cells for each group from 3 individual experiments, n.s.=not significant by unpaired Student's *t-test*).

Figure S3

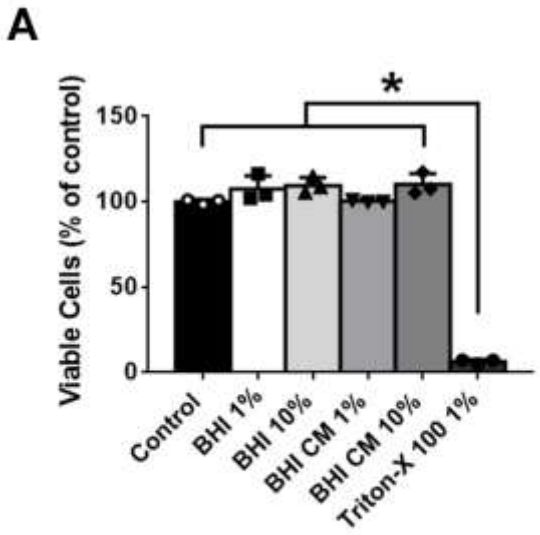

Figure S3. Incubation of STC-1 cells with *Akkermansia muciniphila* conditioned or unconditioned media does not alter cell viability, related to Figure 2. (A) MTS assay expressed as percentage of control showing no reduction of STC-1 cell viability after treatment. 1% Triton-X 100 was used as positive control. (Data are represented as mean +/- SEM; n=3 individual experiments; *$p < 0.001$ by unpaired Student's *t-test*).

Figure S4

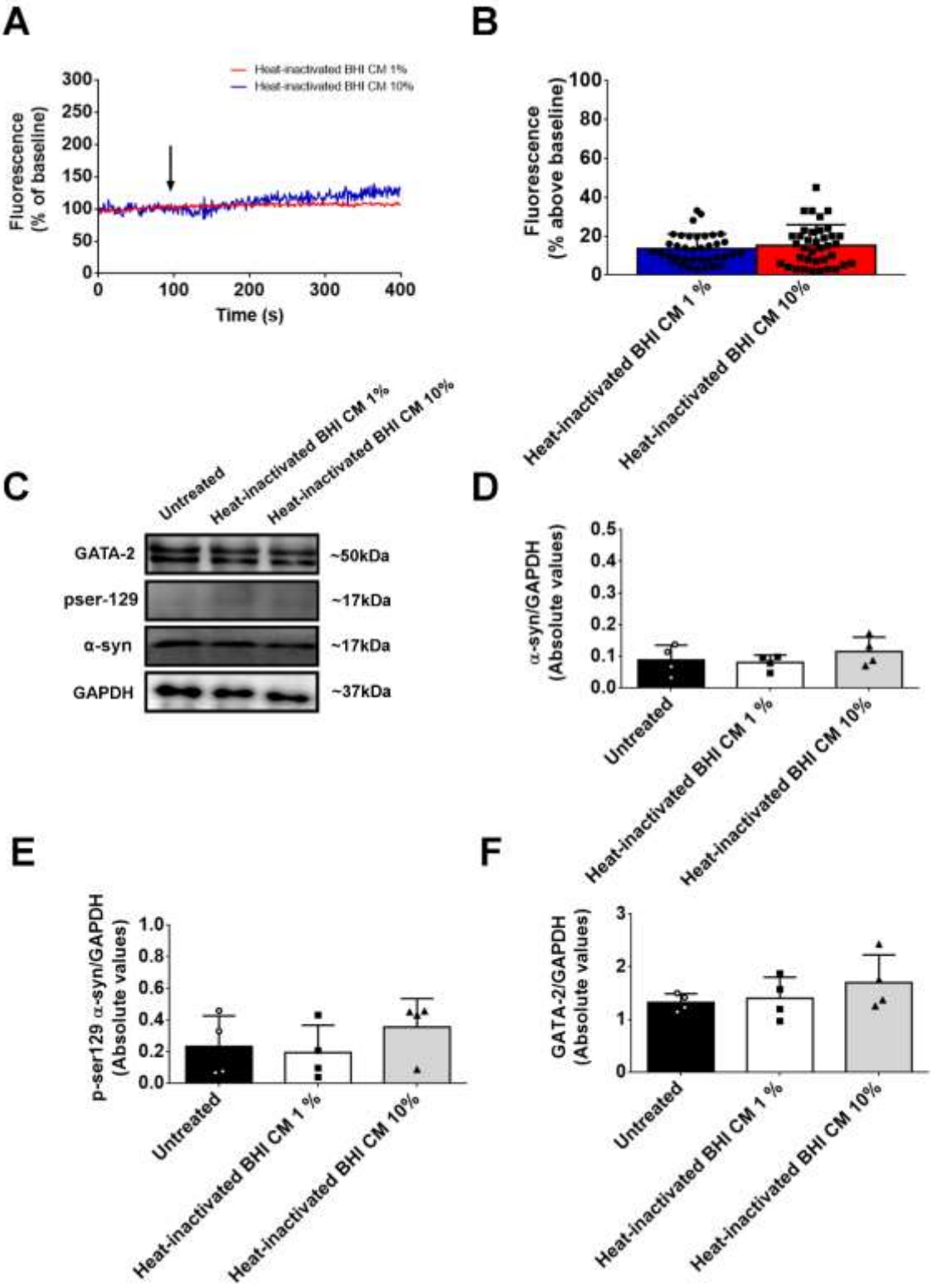

Figure S4. Heat-inactivation of *Akkermansia muciniphila* conditioned media completely suppress their intracellular calcium-inducing effects, related to Figure 2. (A) Representative time-course of total Ca^{2+} signal. Arrow indicates the moment when culture medium was applied. **(B)** Quantification of the peak fluorescence following stimulation with 1 or 10% >3kDa fraction of unconditioned mucin-free media (BHI) (Error bars indicate the media ± SEM; n= at least 25 cells for each group from 3 individual experiments, n.s.= not significant by unpaired Student's *t-test*). **(C)** Immunoblots of total cell lysates showing protein levels of pser129-αSyn, total αSyn and GATA-2 after 48 hours-incubation with 1-10% heat-inactivated protein-enriched fraction of conditioned media (BHI CM). Densitometric analysis shows no differential levels of any of the proteins analyzed [(**D)** pser129-αSyn; **(E)** αSyn**;** and **(F)** GATA-2] when compared to untreated group. (Data are represented as mean +/- SEM; n= 4 individual experiments, *p< 0.05 by two-way Student's *t-test*).

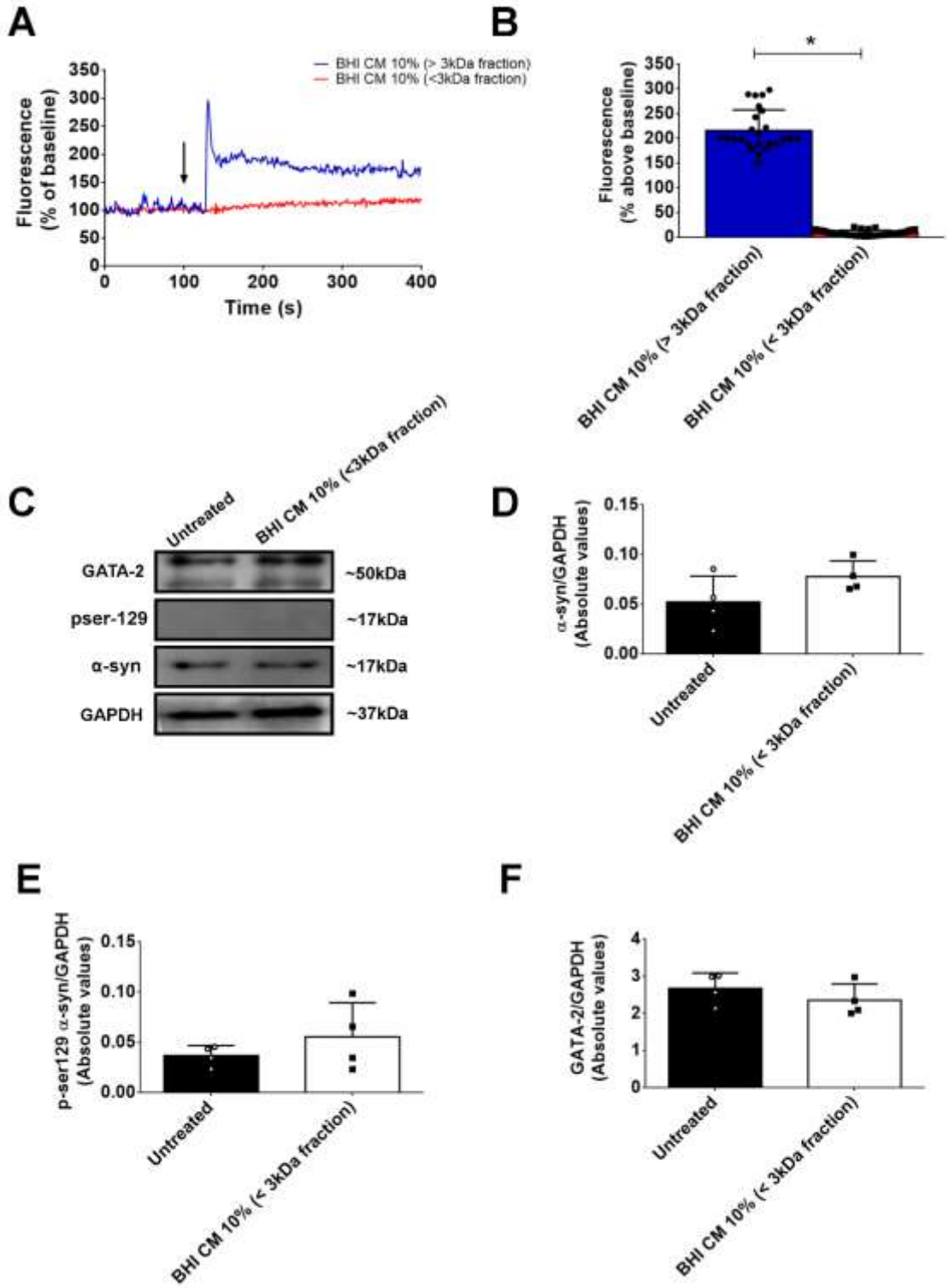

Figure S5. <3kDa fraction of *Akkermansia muciniphila* conditioned media does elicit intracellular calcium signaling and does not perturb α-synuclein homeostasis, related to Figure 2. (A) Representative time-course of total Ca^{2+} signal. Arrow indicates the moment when culture medium was applied. **(B)** Quantification of the peak fluorescence following stimulation with 10% < or > 3kDa fractions of conditioned mucin-free media (BHI CM) (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*). **(C)** Immunoblots of total cell lysates showing protein levels of pser129-αSyn, total αSyn and GATA-2 after 48 hours-incubation with 10% < 3kDa fraction of conditioned mucin-free media (BHI CM). Densitometric analysis shows no differential levels of any of the proteins analyzed [**(D)** αSyn; **(E)** pser129-αSyn; and **(F)** GATA-2] when compared to untreated group. (Data are represented as mean +/- SEM; n= 4 individual experiments, *$p< 0.05$ by two-way Student's *t-test*).

Figure S6

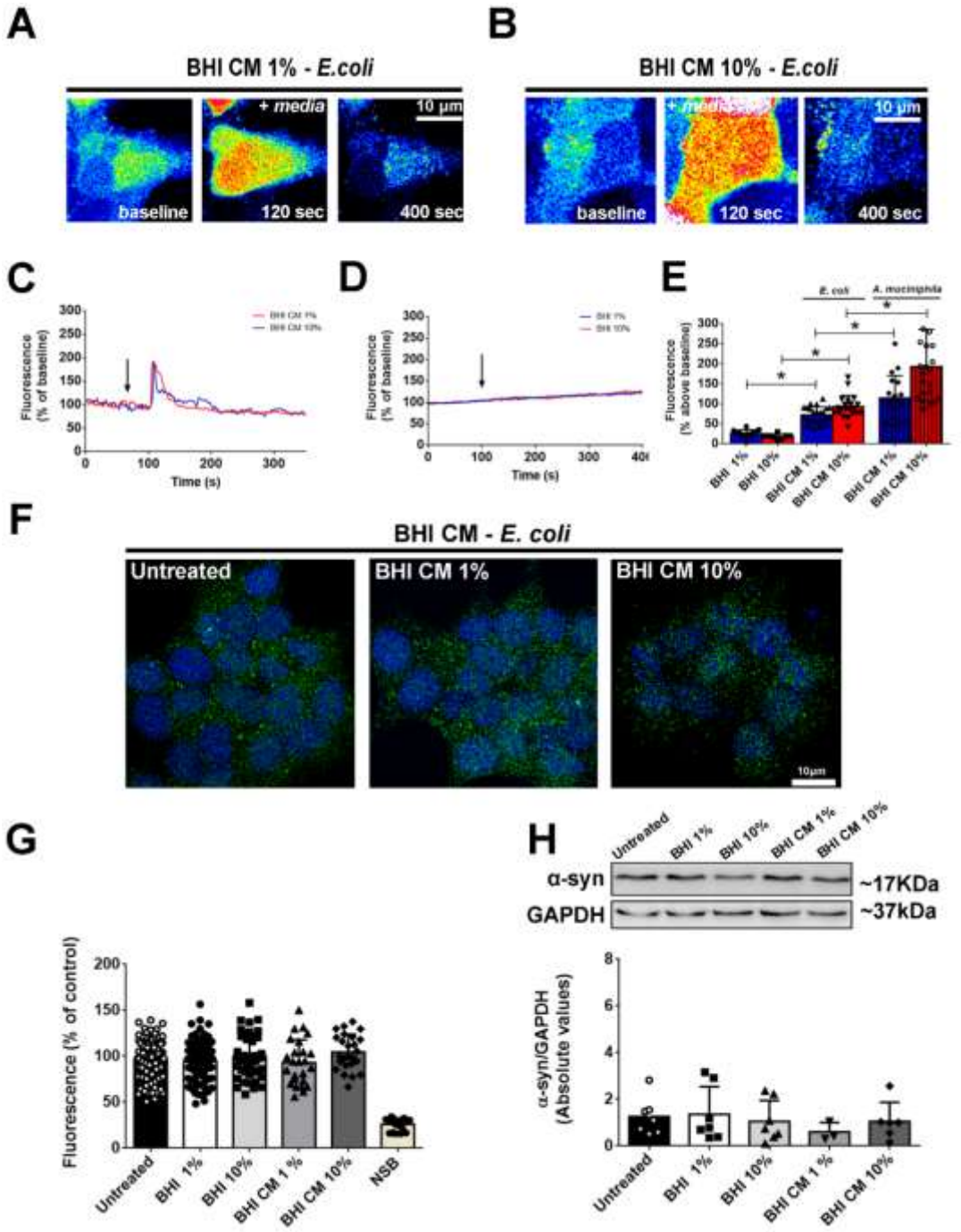

Figure S6. Although *Escherichia coli* conditioned medium induces intracellular calcium signals, it does not influence total levels of α-synuclein in STC-1 cells, related to Figure 2. (A-B) Confocal microscopy imaging of STC-1 cells incubated with Fluo-4/AM (6 µM) and stimulated with 1 **(A)** or 10% **(B)** >3kDa fraction of *E. coli* conditioned (BHI CM) media (scale bar: 10 µm). **(C-D)** Representative time-course of total Ca^{2+} signal. The arrow indicates the time when *E. coli* conditioned (BHI CM) **(C)** or unconditioned (BHI) **(D)** culture medium was applied. **(E)** Quantification of the peak fluorescence following stimulation with 1 or 10% *E. coli* and *A. muciniphila* conditioned (BHI CM) and unconditioned (BHI) media (Error bars indicate the media ± SEM; n= at least 35 cells for each group from 3 individual experiments, *p<0.05 by unpaired Student's *t-test*). **(F)** αSyn staining (green) in STC-1 cells after 48 hs incubation with 1-10% >3kDa fraction of *E. coli* conditioned (BHI CM) media demonstrating no alteration in the protein expression. Nuclei were stained with DAPI (blue) (scale bar: 10 µm). **(G)** Quantification of αSyn fluorescence intensity in images shown in **(F)** (Error bars indicate the media ± SEM; n= at least 35 cells for each group from 3 individual experiments, * p< 0.05 by unpaired Student's *t-test*). **(H)** Immunoblots (upper image) of total cell lysates showing expression of αSyn after 48 hours-incubation with 1-10% *E. coli* conditioned (BHI CM) or unconditioned media (BHI). Graphic representation of densitometric analysis showing expression of αSyn in 1/10% BHI CM or BHI media (Data are represented as mean +/- SEM; n= at least 3 individual experiments per group, * p< 0.05 by unpaired Student's *t-test*).

Figure S7

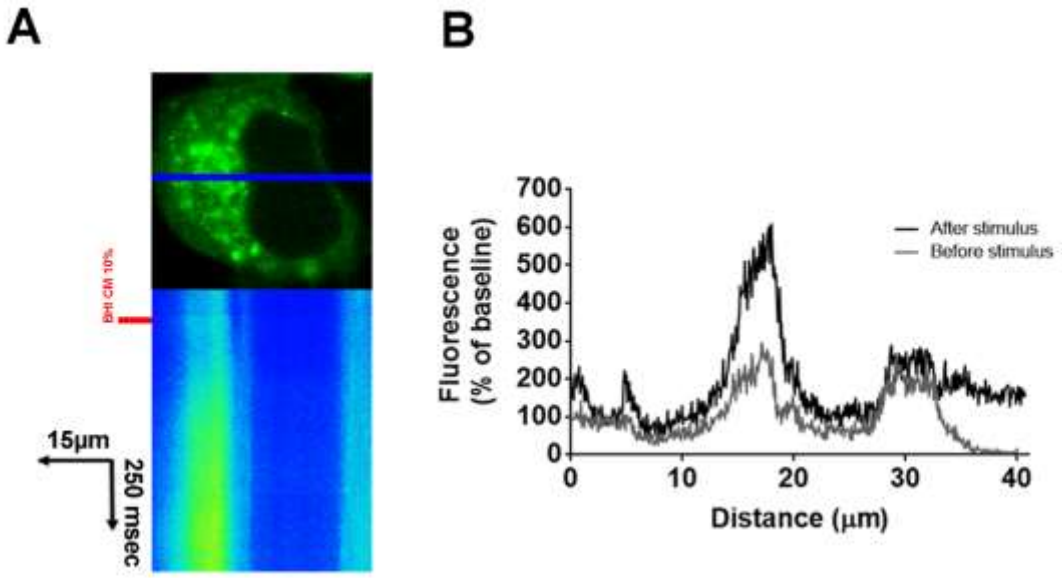

Figure S7. ***A. muciniphila*** **conditioned media promotes Ca^{2+} increase with greater intensity in the cytoplasm when compared to the nuclear region, related to Figure 3. (A)** Line scan of Ca^{2+} signal in STC-1 cells loaded with Fluo-4/AM. It is observed that *A. muciniphila* conditioned (BHI CM) media promotes Ca^{2+} increase with greater intensity in the cytoplasm when compared to the nuclear region. **(B)** Line tracing of the fluorescence Ca^{2+} intensity throughout a representative cell [(blue line in image (**A**)] shows increased fluorescence signal in the cytoplasm after stimulus (black line when compared to gray line). Data in **(B)** represent a representative tracing recorded from one individual STC-1 cell showed in **(A)**.

Figure S8

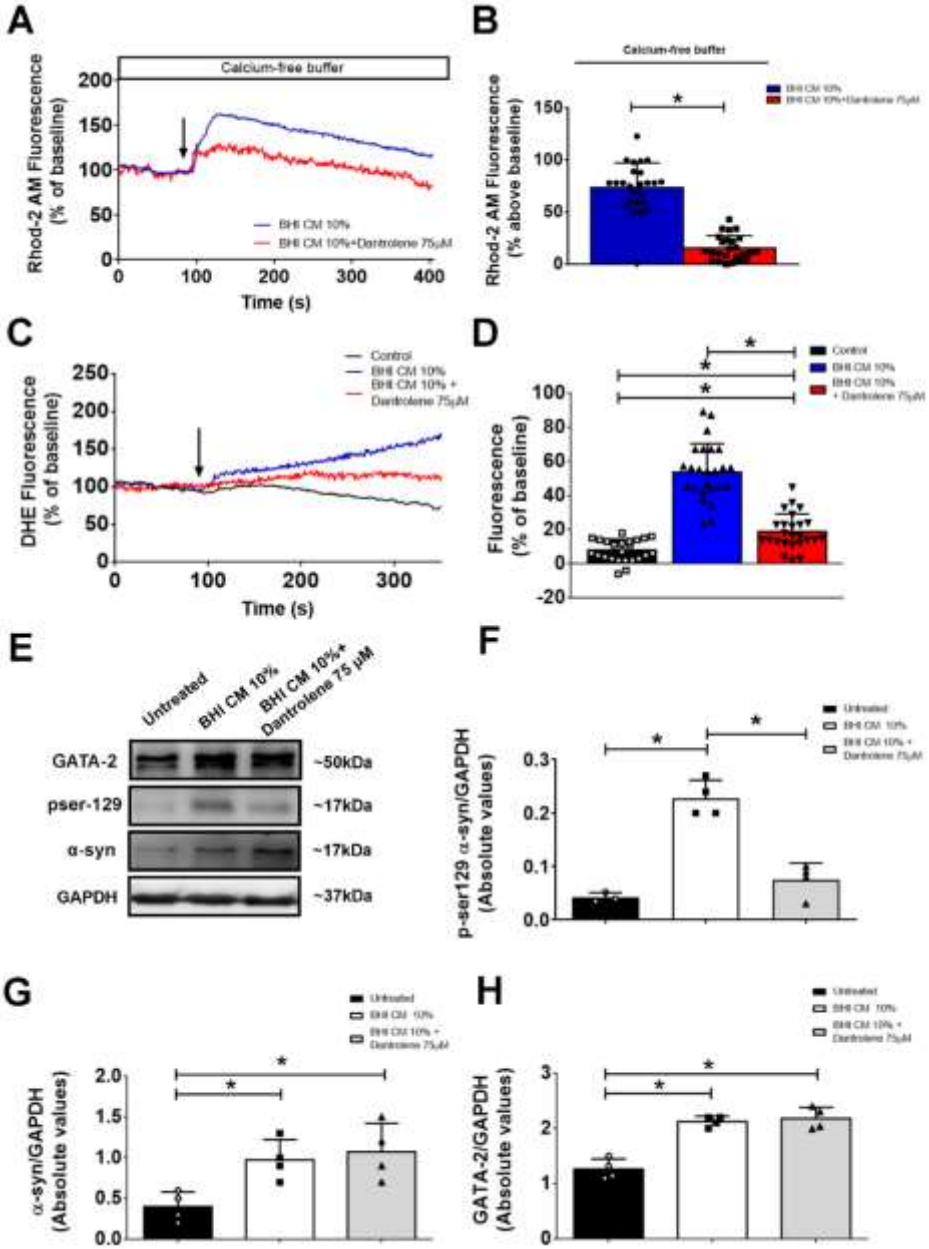

Figure S8. Block of the ryanodine receptor channel (RyR) reduces intracellular ROS levels and α-synuclein phosphorylation elicited by *Akkermansia muciniphila* conditioned medium but does not inhibit GATA-2-regulated α-synuclein expression, related to Figure 4 (A) Representative changes in mitochondrial Ca^{2+} signals over time are shown. Control cells and cells treated with dantrolene 75µM for 30 min were loaded with Rhod-2/AM and stimulated with 10% >3kDa fraction of unconditioned (BHI) or conditioned medium (BHI-CM) (arrow). Ca^{2+} signals were attenuated in cells expressing PV in mitochondria. **(B)** Peak Ca^{2+} signals were observed in three separate experiments for control or dantrolene-treated STC-1 cells. *p< 0.05 by unpaired Student's *t-test*. **(C)** Representative changes in intracellular ROS levels over time are shown. Cells were loaded with DHE and induced by 10% unconditioned (BHI) or conditioned medium (BHI-CM) (arrow). DHE fluorescence intensity was significantly reduced in cells treated with dantrolene. **(D)** Peak ROS signals were observed in three separate experiments for control or dantrolene-treated STC-1 cells. * p< 0.05 by unpaired Student's *t-test*. **(E)** Immunoblots of total cell lysates showing protein levels of pser129-αSyn, total αSyn and GATA-2 after 48 hours-incubation with 10% >3kDa fraction of conditioned media (BHI CM) with or without 75 µM dantrolene. Densitometric analysis shows no differential levels of any of the proteins analyzed [(**F**) pser129-αSyn; (**G**) αSyn; and (**H**) GATA-2] when compared to untreated group. (Data are represented as mean +/- SEM; n= 4 individual experiments, *p< 0.05 by two-way Student's t-test). Data in **(A)** and **(C)** represent a representative tracing recorded from one individual STC-1 cell of each group. Data in **(B)** and **(D)** represent the mean ± SEM of three independent experiments in which at least 25 individual cells were analyzed.

Figure S9

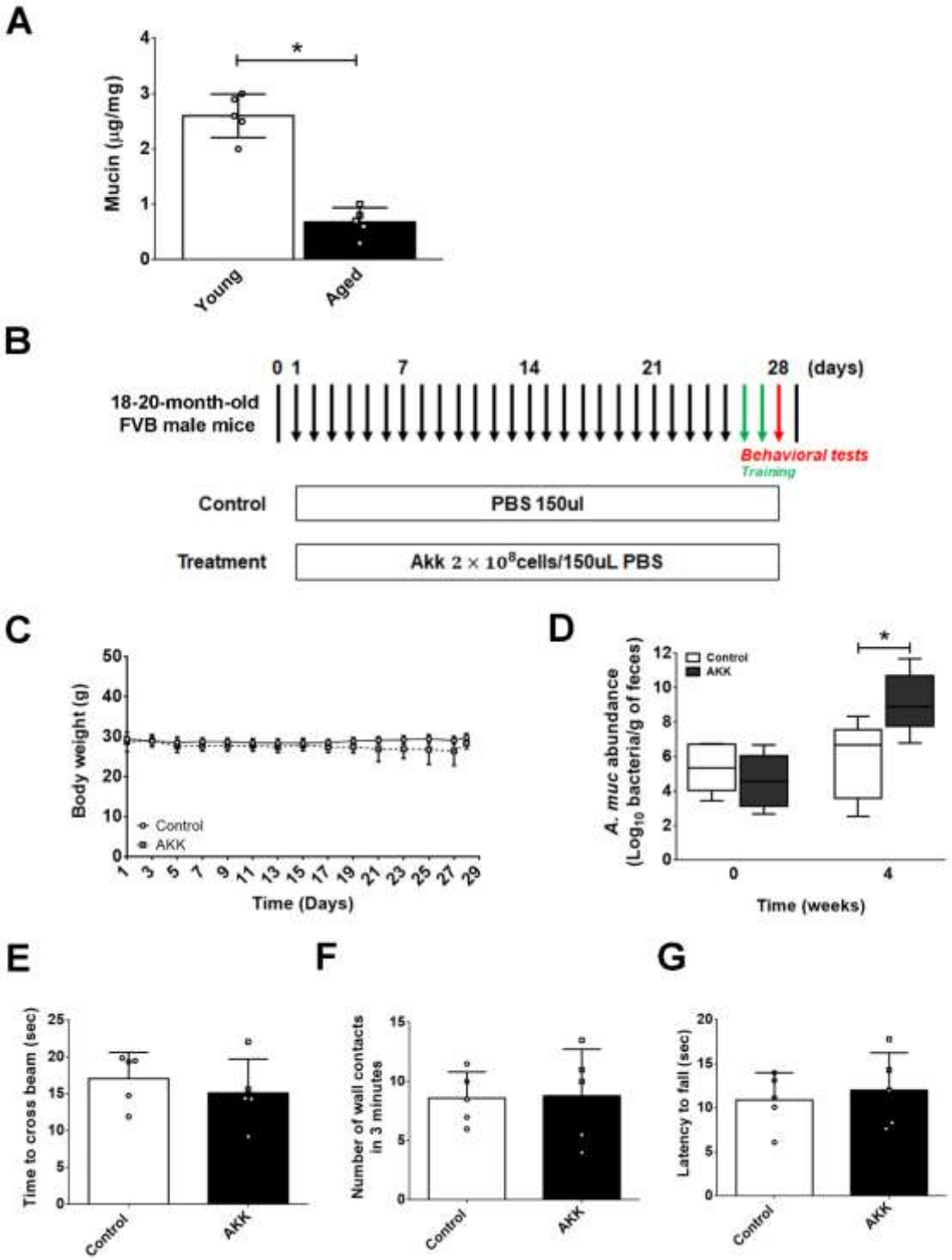

Figure S9. Administration of *Akkermansia muciniphila* by oral gavage for 28 days does not cause motor deficits in aged animals, related to Figure 8. (A) Volume concentration (µg of mucin/mg of feces) of young and aged mice in fecal mucin. **(B)** Study design: Aged mice were daily treated with 2 x 10^8 *A.muciniphila* cells in PBS or only PBS by oral gavage for 28 days. On day 26 and 27, animals were trained for the behavioral tests, performed on day 28. The mice were later euthanized and tissues were collected for different experiments. **(C)** Body weight evolution (g) over a 28-day period of *A. muciniphila* or vehicle administration. **(D)** Relative abundance of *A. muciniphila* from fecal samples collected before and after 4 weeks of *A. muciniphila* or PBS administration evaluated by quantitative qPCR (qPCR)**. (E)** Time to traverse beam apparatus. **(F)** Number of contacts in the cylinder wall during a 3-minute period. **(G)** Graph shows latency until the mice fell from the wire. (Data are represented as mean +/- SEM; Asterisk (*) shows significant difference ($p<0.05$) evaluated by *Student's t-test.* n= 5 animals per group).