TITLE PAGE
- Food Science of Animal Resources -
Upload this completed form to website with submission

ARTICLE INFORMATION	Fill in information in each box below
Article Type	Short Communication
Article Title	Bovine colostrum-derived extracellular vesicles may accelerate the growth of Akkermansia muciniphila by regulating energy metabolism in intestinal anaerobic coculture system
Running Title (within 10 words)	BCEVs influence on Akkermansia in the gut environment
Author	Daye Mun1†, Sangdon Ryu2†, Hye Jin Choi1, An Na Kang1, Dong-Hyun Lim3, Sangnam Oh4*, and Younghoon Kim1*
Affiliation	1 Department of Agricultural Biotechnology and Research Institute of Agriculture and Life Science, Seoul National University, Seoul 08826, Korea. 2 Honam National Institute of Biological Resources, Mokpo 58762, Korea. 3 Dairy Science Division, National Institute of Animal Science, Rural Development Administration, Cheonan 31000, Korea. 4 Department of Functional Food and Biotechnology, Jeonju University, Jeonju 55069, Korea.
Special remarks – if authors have additional information to inform the editorial office	
ORCID (All authors must have ORCID) https://orcid.org	Daye Mun (0000-0002-3470-7632) Sangdon Ryu (0000-0001-5338-8385) Hye Jin Choi (0000-0002-5977-2780) Anna Kang (0000-0003-0208-6234) Dong-Hyun Lim (0000-0002-8575-0198) Sangnam Oh (0000-0002-2428-412x) Younghoon Kim (0000-0001-6769-0657)
Conflicts of interest List any present or potential conflict s of interest for all authors. (This field may be published.)	The authors declare no potential conflict of interest.
Acknowledgments State funding sources (grants, funding sources, equipment, and supplies). Include name and number of grant if available. (This field may be published.)	This research was supported by a National Research Foundation of Korea Grant, funded by the Korean government (MEST) (NRF-2021R1A2C3011051) and Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (2022M3A9I5018286).
Author contributions (This field may be published.)	Conceptualization: Mun D, Ryu S, Choi HJ, Kang A, Lim DH, Oh S, Kim Y Data curation: Mun D, Ryu S, Choi HJ, Kang A Formal analysis: Mun D, Ryu S, Choi HJ, Kang A Methodology: Mun D, Ryu S, Choi HJ, Kang A Software: Mun D, Ryu S, Choi HJ, Kang A Validation: Mun D, Ryu S, Choi HJ, Kang A, Lim DH, Oh S, Kim Y Investigation: Oh S, Kim Y Writing - original draft: Mun D, Ryu S, Choi HJ, Kang A, Lim DH, Oh S, Kim Y Writing - review & editing: Mun D, Ryu S, Choi HJ, Kang A, Lim DH, Oh S, Kim Y (This field must list all authors)
Ethics approval (IRB/IACUC) (This field may be published.)	

CORRESPONDING AUTHOR CONTACT INFORMATION

For the <u>corresponding</u> author (responsible for correspondence, proofreading, and reprints)	**Fill in information in each box below**
First name, middle initial, last name	Younghoon Kim
Email address – this is where your proofs will be sent	ykeys2584@snu.ac.kr

Secondary Email address	
Postal address	Department of Agricultural Biotechnology and Research Institute of Agriculture and Life Science, Seoul National University, Seoul 08826, Korea
Cell phone number	
Office phone number	+82-02-880-4808
Fax number	+82-02-873-2271

7
8

Running title: BCEVs influence on *Akkermansia* in the gut environment

Bovine colostrum-derived extracellular vesicles may accelerate the growth of *Akkermansia muciniphila* by regulating energy metabolism in intestinal anaerobic coculture system

Daye Mun[1†], Sangdon Ryu[2†], Hye Jin Choi[1], An Na Kang[1], Dong-Hyun Lim[3], Sangnam Oh[4]*, and Younghoon Kim[1]*

[1] Department of Agricultural Biotechnology and Research Institute of Agriculture and Life Science, Seoul National University, Seoul 08826, Korea.

[2] Honam National Institute of Biological Resources, Mokpo 58762, Korea.

[3] Dairy Science Division, National Institute of Animal Science, Rural Development Administration, Cheonan 31000, Korea.

[4] Department of Functional Food and Biotechnology, Jeonju University, Jeonju 55069, Korea.

[†] These authors contributed equally.

***Corresponding author:**

Sangnam Oh: osangnam@jj.ac.kr

Younghoon Kim: ykeys2584@snu.ac.kr

Abstract

Extracellular vesicles derived from milk are known to play a significant role in regulating gut microbiota. However, few studies have focused on the effects of these vesicles on specific bacterial species. This study aimed to investigate how bovine colostrum-derived extracellular vesicles (BCEVs) affect the growth and viability of commensal bacteria, specifically *Akkermansia muciniphila*. BCEVs and *A. muciniphila* were co-cultured to measure growth rates using spectrophotometry, and cell viability was assessed at the endpoints. Additionally, to determine whether BCEVs enhance the survival of *A. muciniphila* in the presence of Caco-2 cells, an anaerobic co-culture experiment was conducted to determine the specific interaction between intestinal epithelial cells and gut microbiota using a Transwell system. The results showed that co-culture with BCEVs increased the growth rate and viability of *A. muciniphila*. Consistent with this, increased viability of *A. muciniphila* was observed when it was co-cultured with Caco-2 cells. Transcriptomic analysis revealed that BCEVs regulate nitrogen metabolism in *A. muciniphila*, enhancing the growth rate and viability. Thus, regulating beneficial gut bacteria, such as *A. muciniphila*, through BCEVs presents a novel biological approach that positively impacts human health.

Keywords: Extracellular vesicles, Bovine colostrum, *Akkermansia muciniphila*, Intestinal anaerobic coculture system

Introduction

Extracellular vesicles (EVs) are small membrane-enclosed vesicles released into the extracellular environment (Choi et al., 2023; Robbins and Morelli, 2014). EVs play an important role in cell-to-cell communication by delivering proteins, lipids, RNA, and DNA to recipient cells (Bok et al., 2024; Tkach and Thery, 2016). Since these characteristics, EVs are involved in various physiological processes and play a role in cancer and immune diseases (van Niel et al., 2018). EVs can be categorized into exosomes, microvesicles, and apoptotic bodies based on their origin, size, and mode of release (Zaborowski et al., 2015). In addition, they are increasingly being studied as biomarkers and therapeutics for diseases because of the specificity of their internal miRNAs (Chae et al., 2023; Lee et al., 2023a; McKelvey et al., 2015; Park and Kim, 2023; Zhang et al., 2023).

A growing body of evidence suggests that milk-derived EVs can benefit gut health by reshaping the gut microbiota (Lee et al., 2023b). Oral administration of milk-derived EVs to C57BL/six mice for 8 weeks increased the abundance of Ruminococcaceae and Lachnospiraceae in the large intestine (Tong et al., 2020). The same researchers reported that the oral administration of milk-derived EVs to a mouse model of colitis increased *Akkermansia* in the intestine (Tong et al., 2021). The oral administration of milk-derived EVs to mice with dextran sodium sulfate-induced chronic colitis increased *Bifidobacterium*, *Lachnoclostridium*, and *Lachnospiraceae* in the gut (Du et al., 2022). Furthermore, feeding milk-derived EVs to mice with osteoarthritis increased Ruminococcaceae and Akkermansiaceae and decreased Proteobacteria in the gut (Liu et al., 2023).

Akkermansia, specifically *Akkermansia muciniphila*, is a bacterium that belongs to the next generation probiotics and is beneficial for gut health (Karamzin et al., 2021; Park et al., 2024). *A. muciniphila* degrades mucin, a major component of the mucus layer of the gut (Kim et al., 2023). This promotes mucin production by the goblet cells, which improves the integrity of the intestinal barrier (Kim et al., 2021; Kwak et al., 2024). Notably, *A. muciniphila* has been reported to be low in abundance in the gut of patients with inflammatory bowel disease or metabolic disorders, and may modulate anti-inflammatory activity (Derrien et al., 2017). However, little is known regarding the

regulation of gut microbiota by colostrum-derived EVs and its mechanisms. Therefore, this study aimed to investigate whether colostrum-derived EVs could regulate the growth of commensal bacteria, specifically *A. muciniphila*.

Materials and Methods

Samples

Colostrum samples from healthy Holstein cows were collected within 3 days after delivery. After milking, the milk samples were immediately refrigerated and transported to the laboratory for further analysis.

Isolation and characterization of BCEVs

The colostrum was centrifuged at 1,200×g for 10 min for defatting, and then at 16,000×g for 1 h to pellet the cell debris. The supernatant was centrifuged at 50,000×g for 1 h to generate the whey fraction. The whey fraction was ultracentrifuged at 100,000×g to remove large particles. The supernatant was ultracentrifuged at 135,000×g for 90 min to pellet the EVs. The EVs were then resuspended in phosphate-buffered saline (PBS), washed under the same conditions, and frozen at -80 ℃ until use (Fig. 1a). Transmission electron microscopy (TEM) was used to confirm the morphology of BCEVs, as previously described (Choi et al., 2023). The average size and particle concentration of the isolated EVs were measured using nanoparticle tracking analysis (NTA; Malvern NanoSight NS300, Malvern Technologies, Malvern, UK). For further characterization of extracellular vesicles, the expression of CD63 was determined using a western blot assay, as previously described (Shimizu et al., 2021).

Bacterial culture

A. muciniphila strain KCTC 15667 was cultured in brain heart infusion (BHI) broth (BD Difco, USA) with 0.5% mucin type II (Sigma, St. Louis, USA) and *Bacteroides intestinalis* KCTC 5441, *Bacteroides fragilis* KCTC 5013, *Phocaeicola massiliensis* KCTC 5470 were cultured in BHI-supplemented (BHIS) medium (Melissa K. Bacic 2008) in anaerobic chamber (COY Laboratories, Grass Lake, USA). For bacterial inoculation, *A. muciniphila* was cultured for two days, whereas *Bacteroides* and *Phocaeicola* were cultured for 1 d before use in the experiment. To evaluate the

growth curves of each bacterial strain supplemented with BCEVs, 1×10^9 or 1×10^{10} particles/mL of BCEVs or PBS were added to the BHI or BHIS broth, and then each bacterial strain was inoculated. The absorbance (OD600) of the bacterial cultures was measured every 2 h for 2 d for *A. muciniphila* and the number of viable cells was measured at the endpoint. Cultures of *Bacteroides* and *Phocaeicola* were measured every 2 h for 1 d.

Small-scale anaerobic bacterial-aerobic cell line co-culture system

Caco-2 cells obtained from the American Type Culture Collection were grown in Dulbecco's modified Eagle's medium (HyClone, Logan, UT, USA) supplemented with 10% fetal bovine serum. Inserts with 0.4 μm pore size (apical part) were placed in a 12-well plate (basal part) and Caco-2 cells were seeded in the apical part. After culturing Caco-2 cells in a conventional aerobic 5% CO_2 incubator at 37 ℃ for 3 weeks, the apical part was inoculated with *A. muciniphila* and BCEVs in an anaerobic chamber (COY Laboratories). Apical inserts were sealed with airtight butyl rubber to maintain the anaerobic conditions, then the plates were incubated again in an aerobic 5% CO_2 at 37 ℃ for 2 d, and the survival of *A. muciniphila* in the apical part was checked. Dissolved oxygen concentration was measured using a Milwaukee MW600 PRO (Milwaukee Instruments Inc., Rocky Mount, NC, USA) according to the manufacturer's instructions.

Scanning electron microscopy (SEM)

For SEM analysis, the cultured cell culture inserts were fixed with Karnovsky's fixative overnight at 4 ℃ and stained with 1% osmium tetroxide (Sigma) for 1 h at room temperature. The membrane part of the insert was then gradually dehydrated using ethanol. The membrane was thoroughly dried with hexamethyldisilazane, platinum coated, and imaged using field emission SEM (Sigma; Carl Zeiss, UK).

Transcriptomic analysis

For transcriptome analysis, *A. muciniphila* cultured with BCEVs or PBS was grown to mid-log (OD600 = 0.35). The pellet was immediately harvested by centrifugation and RNA was isolated using TRIzol reagent (Invitrogen, USA) and an RNeasy Mini kit (Qiagen, Germany). The quantity and quality of the extracted RNA were measured using a spectrophotometer (SpectraMax ABS Plus; Molecular Devices, San Jose, CA, USA). Library construction and sequencing were performed by Macrogen (Seoul, South Korea). Libraries were prepared using the NEBNext rRNA Depletion (Bacteria) and TruSeq Stranded Total RNA Library Prep Gold Kit and sequenced on an Illumina NovaSeq platform. Preprocessed trimmed reads were mapped to known reference genomes using the Bowtie software. After read mapping, the HTSeq program was used to extract gene-specific read counts for each sample based on the gene annotation of the species. EdgeR was used to perform differentially expressed genes (DEGs) analysis to select DEGs between the two groups, and genes that met the conditions of |fold change (fc)| ≥ 1.5 and exactTest raw p-value <0.05 were extracted. If known gene information was available, functional annotation and gene-set enrichment analysis based on the Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) databases were performed on the DEGs (Munyaneza et al., 2024).

Statistical analysis

Comparisons between the two groups were performed using Student's t-test, and one-way analysis of variance was used to compare different treatment groups. Each group was compared using Tukey's test to assess any differences from the control and between the treatments, unless otherwise indicated. Growth rates were analyzed using the Growthcurver R package. Statistical analyses and visualizations were performed using GraphPad Prism 9.0 (San Diego, CA, USA).

Results

Characterization of BCEVs

Milk-derived EVs play a role in modulating the gut microbiota, particularly by increasing the abundance of *Akkermansia*, which has recently been considered a next-generation probiotic (Hanninen et al., 2018; Tong et al., 2021). Therefore, this study aimed to determine how BCEVs interact with gut microbiota, specifically with *A. muciniphila*. We isolated and identified BCEVs from colostrum by ultracentrifugation (Fig. 1), following the guidelines of the International Society for Extracellular Vesicles (Thery et al., 2018). TEM imaging confirmed their morphology (Fig. 1c), NTA determined their size and concentration (Fig. 1d), and western blotting assays identified CD63 as a protein marker for EVs, confirming the successful isolation of BCEVs (Fig. 1e). The average size of the BCEVs was 147.6 nm.

Growth rate and viability of commensal bacteria

To assess the effect of BCEVs on the growth rate of *A. muciniphila*, two concentrations of BCEVs were co-cultured with *A. muciniphila* and the growth rates were measured (Fig. 2a). Both concentrations significantly increased the growth rate of *A. muciniphila*. To verify the direct effects on the growth of *A. muciniphila*, cell viability was measured at the endpoint. Both concentrations significantly increased cell viability compared to the control, which was consistent with previous results (Fig. 2b). In contrast, co-culture of BCEVs did not affect the growth rate of the gut commensals *B. intestinalis* and *B. fragilis* and tended to decrease the growth rate of *P. massiliensis* (Fig. 2c–e). These results suggest that BCEVs can regulate the growth rate differently depending on the species and that they increase the growth rate of *A. muciniphila*.

Co-culture of *A. muciniphila* with Caco-2 cells

To assess whether BCEVs exerted a similar influence on the growth of *A. muciniphila* in the presence of intestinal epithelial cells, a co-culture system of anaerobic bacteria and Caco-2 cells was

established (Fig. 3a). The amount of dissolved oxygen was significantly lower in the apical portion of the wells with rubber than in those without rubber, with no significant difference in the basal portion (Fig. 3b). In addition, the number of colonies of *A. muciniphila* was measured, and it was found that *A. muciniphila* could not survive in the absence of rubber; the number of colonies was significantly higher when co-inoculated with *A. muciniphila* and BCEVs than when inoculated with *A. muciniphila* alone (Fig. 3c). SEM images also showed that more *A. muciniphila* was observed when co-cultured with BCEVs, which is consistent with previous results (Fig. 3d). These results confirmed the establishment of a co-culture system of anaerobic bacteria and cells and showed that treatment with BCEVs enhanced the viability of *A. muciniphila* even in the presence of intestinal epithelial cells.

Transcriptome analysis of *A. muciniphila*

After confirming that BCEVs promoted the growth of *A. muciniphila*, transcriptomic analysis was performed by extracting RNA from *A. muciniphila* cells co-incubated with PBS or BCEVs to identify the mechanism of increased growth. Of the 2,201 observed genes, 70 DEGs with $|fc| \geq 1.5$ & raw $p < 0.05$ were identified, and 23 and 47 genes were up- and downregulated, respectively, in the BCEVs-treated group compared to the PBS-treated group (Fig. 4a and Table 1). GO terms and KEGG pathway enrichment analyses were performed to predict the function of the identified DEGs. Among the 70 DEGs, 35 genes had no hits in GO analysis, 12 genes were involved in metabolic processes, and 10 genes were involved in cellular and biological processes (Fig. 4b). KEGG pathway analysis showed that the two-component system and nitrogen metabolism-related pathway was significantly increased when co-cultured with BCEVs, and protein export was decreased (Table 2). In particular, Amuc_1252 and Amuc_1253, genes related to glutamine synthetase, were upregulated following BCEVs treatment. Thus, it was predicted that BCEVs might promote the growth of *A. muciniphila* by regulating nitrogen metabolism, particularly of glutamine synthetase.

Discussion

This study confirmed the effect of BCEVs on *A. muciniphila* growth. Co-culture with BCEVs increased the growth rate and viability of *A. muciniphila*. Consistent with this, increased viability of *A. muciniphila* was observed when it was co-cultured with Caco-2 cells. Transcriptomic analysis revealed an increase in nitrogen metabolism-related genes in BCEV-treated *A. muciniphila*. These results suggest that treatment with BCEVs modulates the expression of genes in *A. muciniphila*, specifically by regulating energy metabolism, resulting in increased growth.

A. muciniphila has been found to be beneficial for gut health in several recent studies (Choi et al., 2024; Karamzin et al., 2021). Increased colonization by *A. muciniphila* enhances the expression of cyclic AMP-responsive element-binding protein H in the gut, which alleviates endoplasmic reticulum stress, intestinal barrier leakage, and endotoxemia in the blood caused by inflammatory stress (Wade et al., 2023). *A. muciniphila* ameliorates transmural colonic wall defects by promoting interleukin (IL)-22 secretion via myeloid differentiation primary response 88 (Bachmann et al., 2022). *A. muciniphila* also has a major effect on intestinal homeostasis, mediated by alpha kinase 1 in enterocytes *in vitro* (Martin-Gallausiaux et al., 2022). The outer membrane protein of *A. muciniphila* has been shown to exert a proinflammatory function by inducing the secretion of IL-10 (Ottman et al., 2017). Considering this, it is possible that BCEVs increase the growth *of A. muciniphila*, which may enhance the anti-inflammatory responses in the gut. This growth promotion may also strengthen the role of *A. muciniphila* in maintaining gut homeostasis and supporting the gut barrier. Further research is needed to investigate how BCEVs influence A. muciniphila within the broader gut microbiome and their potential therapeutic applications.

As research on the potential of various dietary extracellular vesicles to modulate the gut microbiome grows, the impact of EVs on bacterial growth and the mechanisms involved are increasingly being investigated. A previous study showed that milk-derived EVs promoted the growth of *Escherichia coli* MG1655 and *Lactobacillus plantarum* WCFS1, which was attributed to the bacterial gene regulation properties of milk-derived EVs (Yu et al., 2019). Milk-derived EVs are

taken up by bifidobacteria and promote the growth of bifidobacteria by promoting carbohydrate metabolism (Luo et al., 2023). Human urine-derived exosomes inhibit the growth of *E. coli* associated with urinary tract infections (Hiemstra et al., 2014). Furthermore, miRNAs have been shown to enter bacteria, regulate bacterial genes, and affect bacterial growth (Liu et al., 2016). In this study, treatment with BCEVs had a minimal impact on the growth of other commensal bacteria but significantly increased the growth rate and viability of *A. muciniphila*. These findings demonstrate that BCEVs can influence the growth rate and survival of gut commensal bacteria, particularly in a bacteria-specific manner. Further research involving a broader range of microbial species is necessary to elucidate the interactions between colostrum-derived EVs and microorganisms.

With the development of culturomic techniques and the growing importance of characterizing individual bacteria, the need to understand anaerobic bacterial–host interactions has increased (Na and Guan, 2022). Therefore, attempts have been made to develop a human gut mimetic system that can be co-cultivated with anaerobic bacteria (Kim, 2023; Moossavi et al., 2022; Sasaki et al., 2020). In a previous study, a system for co-culturing human organoids and anaerobic bacteria using a Transwell cell culture insert and butyl rubber was devised, demonstrating that commensal gut microbiota such as *A. muciniphila*, *Bifidobacterium adventitiae*, *Bacteroides fragilis*, and *Clostridium butyricum* could survive in this system and influence gene expression in human cells (Sasaki et al., 2020). Umehara and Aoyagi (2023) developed a system that allowed the co-culture of aerobic cells and anaerobic bacteria using a cell culture insert and liquid paraffin, and reported that Bifidobacterium bifidum had a higher growth rate in the upper section containing mineral oil (Umehara and Aoyagi, 2023). In the present study, to verify whether the growth-promoting effects of BCEVs on *A. muciniphila* persist in the presence of intestinal epithelial cells, we developed a simple anaerobic culture system using a Transwell cell culture insert to co-culture Caco-2 cells and anaerobic bacteria. The system effectively maintained a significantly reduced oxygen concentration in the apical insert sealed with butyl rubber, confirming the viability of *A. muciniphila* under these conditions. Additionally, it was observed that *A. muciniphila* viability increased when co-cultured with BCEVs in this system. These findings suggest that BCEVs could enhance the growth of *A. muciniphila* even in the presence of intestinal

epithelial cells. Further analysis is required to assess the impact of increased *A. muciniphila* on Caco-2 cells following BCEV treatment.

Transcriptomic analysis confirmed that glutamate metabolism in *A. muciniphila* was regulated by BCEVs. Glutamate is considered highly important in bacterial metabolism and is known for its role in linking nitrogen and carbon metabolism (Commichau et al., 2006; Commichau et al., 2008). Additionally, it is known to influence pathways, such as protein synthesis and the TCA cycle (Feehily and Karatzas, 2013), allowing us to predict that BCEV-treated *A. muciniphila* may have adapted to its environment and increased its energy metabolism, which increased its growth. However, the transcriptomic results in this study showed that the differences in gene expression were slight, and only a few genes were identified in the predicted pathways. Therefore, further analysis is needed to identify more precise mechanisms.

Conclusion

This study demonstrates that BCEVs significantly enhance the growth rate and viability of *A. muciniphila*, in the presence and absence of host cells. This effect appears to be partially mediated by the modulation of genes involved in nitrogen metabolism. To the best of our knowledge, this is the first transcriptomic analysis exploring the impact of colostrum-derived EVs on the growth of *A. muciniphila*. Considering our initial findings, further studies are needed to elucidate the specific roles of colostrum-derived EVs and microRNAs in bacterial growth dynamics.

Acknowledgements

This research was supported by a National Research Foundation of Korea Grant, funded by the Korean government (MEST) (NRF-2021R1A2C3011051) and Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (2022M3A9I5018286).

References

Bachmann R, Van Hul M, Baldin P, Leonard D, Delzenne NM, Belzer C, Ouwerkerk JP, Repsilber D, Rangel I, Kartheuser A, Brummer RJ, De Vos WM, Cani PD. 2022. Akkermansia muciniphila reduces peritonitis and improves intestinal tissue wound healing after a colonic transmural defect by a myd88-dependent mechanism. Cells 11.

Bok E-Y, Seo SY, Lee HG, Kim EJ, Cho A, Jung Y-H, Hur T-Y, So K-M, Lee S-L, Wimalasena SH. 2024. Exosomes isolation from bovine serum: Qualitative and quantitative comparison between ultracentrifugation, combination ultracentrifugation and size exclusion chromatography, and exoeasy methods. Journal of Animal Science and Technology.

Chae J-W, Choi Y-H, Lee J-N, Park H-J, Jeong Y-D, Cho E-S, Kim Y-S, Kim T-K, Sa S-J, Cho H-C. 2023. An intelligent method for pregnancy diagnosis in breeding sows according to ultrasonography algorithms. Journal of Animal Science and Technology 65:365.

Choi H, Mun D, Ryu S, Kwak M-J, Kim B-K, Park D-J, Oh S, Kim Y. 2023. Molecular characterization and functionality of rumen-derived extracellular vesicles using a caenorhabditis elegans animal model. Journal of Animal Science and Technology 65:652.

Choi Y, Lee W, Kwon J-G, Kang A, Kwak M-J, Eor J-Y, Kim Y. 2024. The current state of phage therapy in livestock and companion animals. Journal of Animal Science and Technology 66:57.

Commichau FM, Forchhammer K, Stulke J. 2006. Regulatory links between carbon and nitrogen metabolism. Curr Opin Microbiol 9:167-172.

Commichau FM, Gunka K, Landmann JJ, Stulke J. 2008. Glutamate metabolism in bacillus subtilis: Gene expression and enzyme activities evolved to avoid futile cycles and to allow rapid responses to perturbations of the system. J Bacteriol 190:3557-3564.

Derrien M, Belzer C, De Vos WM. 2017. *Akkermansia muciniphila* and its role in regulating host functions. Microb Pathog 106:171-181.

Du C, Wang K, Zhao Y, Nan X, Chen R, Quan S, Xiong B. 2022. Supplementation with milk-derived extracellular vesicles shapes the gut microbiota and regulates the transcriptomic landscape in experimental colitis. Nutrients 14.

Feehily C, Karatzas KA. 2013. Role of glutamate metabolism in bacterial responses towards acid and other stresses. J Appl Microbiol 114:11-24.

Hanninen A, Toivonen R, Poysti S, Belzer C, Plovier H, Ouwerkerk JP, Emani R, Cani PD, De Vos WM. 2018. Akkermansia muciniphila induces gut microbiota remodelling and controls islet autoimmunity in nod mice. Gut 67:1445-1453.

Hiemstra TF, Charles PD, Gracia T, Hester SS, Gatto L, Al-Lamki R, Floto RA, Su Y, Skepper JN, Lilley KS, Karet Frankl FE. 2014. Human urinary exosomes as innate immune effectors. J Am Soc Nephrol 25:2017-2027.

Karamzin AM, Ropot AV, Sergeyev OV, Khalturina EO. 2021. Akkermansia muciniphila and host interaction within the intestinal tract. Anaerobe 72:102472.

Kim HW, Lee SY, Hur SJ, Kil DY, Kim JH. 2023. Effects of functional nutrients on chicken intestinal epithelial cells induced with oxidative stress. Journal of Animal Science and Technology 65:1040.

Kim R. 2023. Advanced organotypic in vitro model systems for host-microbial coculture. Biochip J:1-27.

Kim S, Shin YC, Kim TY, Kim Y, Lee YS, Lee SH, Kim MN, O E, Kim KS, Kweon MN. 2021. Mucin degrader *akkermansia muciniphila* accelerates intestinal stem cell-mediated epithelial development. Gut Microbes 13:1-20.

Kwak M-J, Ha D-J, Park MY, Eor JY, Whang K-Y, Kim Y. 2024. Comparison study between single enzyme and multienzyme complex in distiller's dred grains with soluble supplemented diet in broiler chicken. Journal of Animal Science and Technology 66:398.

Lee S, Kang S, Heo J, Hong Y, Vu TH, Truong AD, Lillehoj HS, Hong YH. 2023a. Microrna expression profiling in the lungs of genetically different ri chicken lines against the highly pathogenic avian influenza h5n1 virus. Journal of Animal Science and Technology 65:838.

Lee SY, Yun SH, Lee J, Park J, Choi Y, Han D, Kim JS, Joo S-T, Hur SJ. 2023b. Study on the current research trends and future agenda in animal products: An asian perspective. Journal of Animal Science and Technology 65:1124.
Liu Q, Hao H, Li J, Zheng T, Yao Y, Tian X, Zhang Z, Yi H. 2023. Oral administration of bovine milk-derived extracellular vesicles attenuates cartilage degeneration via modulating gut microbiota in dmm-induced mice. Nutrients 15.
Liu S, Da Cunha AP, Rezende RM, Cialic R, Wei Z, Bry L, Comstock LE, Gandhi R, Weiner HL. 2016. The host shapes the gut microbiota via fecal microrna. Cell Host Microbe 19:32-43.
Luo Y, Bi J, Lin Y, He J, Wu S, Zhang Y, Wang Y, Song S, Guo H. 2023. Milk-derived small extracellular vesicles promote bifidobacteria growth by accelerating carbohydrate metabolism. Lwt 182.
Martin-Gallausiaux C, Garcia-Weber D, Lashermes A, Larraufie P, Marinelli L, Teixeira V, Rolland A, Beguet-Crespel F, Brochard V, Quatremare T, Jamet A, Dore J, Gray-Owen SD, Blottiere HM, Arrieumerlou C, Lapaque N. 2022. Akkermansia muciniphila upregulates genes involved in maintaining the intestinal barrier function via adp-heptose-dependent activation of the alpk1/tifa pathway. Gut Microbes 14:2110639.
Mckelvey KJ, Powell KL, Ashton AW, Morris JM, Mccracken SA. 2015. Exosomes: Mechanisms of uptake. J Circ Biomark 4:7.
Moossavi S, Arrieta MC, Sanati-Nezhad A, Bishehsari F. 2022. Gut-on-chip for ecological and causal human gut microbiome research. Trends Microbiol 30:710-721.
Munyaneza JP, Kim M, Cho E, Jang A, Choo HJ, Lee JH. 2024. Genome-wide association studies of the fatty acid composition of korean native chicken breast meat. Journal of Animal Science and Technology.
Na SW, Guan LL. 2022. Understanding the role of rumen epithelial host-microbe interactions in cattle feed efficiency. Anim Nutr 10:41-53.
Ottman N, Reunanen J, Meijerink M, Pietila TE, Kainulainen V, Klievink J, Huuskonen L, Aalvink S, Skurnik M, Boeren S, Satokari R, Mercenier A, Palva A, Smidt H, De Vos WM, Belzer C. 2017. Pili-like proteins of akkermansia muciniphila modulate host immune responses and gut barrier function. PLoS One 12:e0173004.
Park T, Yoon J, Yun Y, Unno T. 2024. Comparison of the fecal microbiota with high-and low performance race horses. Journal of Animal Science and Technology 66:425.
Park Y-B, Kim J-M. 2023. Identification of long non-coding rna-mrna interactions and genome-wide lncrna annotation in animal transcriptome profiling. Journal of Animal Science and Technology 65:293.
Robbins PD, Morelli AE. 2014. Regulation of immune responses by extracellular vesicles. Nat Rev Immunol 14:195-208.
Sasaki N, Miyamoto K, Maslowski KM, Ohno H, Kanai T, Sato T. 2020. Development of a scalable coculture system for gut anaerobes and human colon epithelium. Gastroenterology 159:388-390 e385.
Shimizu A, Sawada K, Kobayashi M, Yamamoto M, Yagi T, Kinose Y, Kodama M, Hashimoto K, Kimura T. 2021. Exosomal cd47 plays an essential role in immune evasion in ovarian cancer. Molecular Cancer Research 19:1583-1595.
Thery C, Witwer KW, Aikawa E, Alcaraz MJ, Anderson JD, Andriantsitohaina R, Antoniou A, Arab T, Archer F, Atkin-Smith GK, Ayre DC, Bach JM, Bachurski D, Baharvand H, Balaj L, Baldacchino S, Bauer NN, Baxter AA, Bebawy M, Beckham C, Bedina Zavec A, Benmoussa A, Berardi AC, Bergese P, Bielska E, Blenkiron C, Bobis-Wozowicz S, Boilard E, Boireau W, Bongiovanni A, Borras FE, Bosch S, Boulanger CM, Breakefield X, Breglio AM, Brennan MA, Brigstock DR, Brisson A, Broekman ML, Bromberg JF, Bryl-Gorecka P, Buch S, Buck AH, Burger D, Busatto S, Buschmann D, Bussolati B, Buzas EI, Byrd JB, Camussi G, Carter DR, Caruso S, Chamley LW, Chang YT, Chen C, Chen S, Cheng L, Chin AR, Clayton A, Clerici SP, Cocks A, Cocucci E, Coffey RJ, Cordeiro-Da-Silva A, Couch Y, Coumans FA, Coyle B, Crescitelli R, Criado MF, D'souza-Schorey C, Das S, Datta Chaudhuri A, De Candia P, De Santana EF, De Wever O, Del Portillo HA, Demaret T, Deville S, Devitt A, Dhondt B, Di Vizio D, Dieterich LC, Dolo V, Dominguez Rubio AP,

399 Dominici M, Dourado MR, Driedonks TA, Duarte FV, Duncan HM, Eichenberger RM,
400 Ekstrom K, El Andaloussi S, Elie-Caille C, Erdbrugger U, Falcon-Perez JM, Fatima F, Fish
401 JE, Flores-Bellver M, Forsonits A, Frelet-Barrand A, et al. 2018. Minimal information for
402 studies of extracellular vesicles 2018 (misev2018): A position statement of the international
403 society for extracellular vesicles and update of the misev2014 guidelines. J Extracell Vesicles
404 7:1535750.
405 Tkach M, Thery C. 2016. Communication by extracellular vesicles: Where we are and where we need
406 to go. Cell 164:1226-1232.
407 Tong L, Hao H, Zhang X, Zhang Z, Lv Y, Zhang L, Yi H. 2020. Oral administration of bovine milk-
408 derived extracellular vesicles alters the gut microbiota and enhances intestinal immunity in
409 mice. Mol Nutr Food Res 64:e1901251.
410 Tong L, Hao H, Zhang Z, Lv Y, Liang X, Liu Q, Liu T, Gong P, Zhang L, Cao F, Pastorin G, Lee CN,
411 Chen X, Wang JW, Yi H. 2021. Milk-derived extracellular vesicles alleviate ulcerative colitis
412 by regulating the gut immunity and reshaping the gut microbiota. Theranostics 11:8570-8586.
413 Umehara Y, Aoyagi H. 2023. Development of a simple cultured cell–anaerobic microbial co-culture
414 system using liquid paraffin. Journal of bioscience and bioengineering 135:487-492.
415 Van Niel G, D'angelo G, Raposo G. 2018. Shedding light on the cell biology of extracellular vesicles.
416 Nat Rev Mol Cell Biol 19:213-228.
417 Wade H, Pan K, Duan Q, Kaluzny S, Pandey E, Fatumoju L, Saraswathi V, Wu R, Harris EN, Su Q.
418 2023. Akkermansia muciniphila and its membrane protein ameliorates intestinal
419 inflammatory stress and promotes epithelial wound healing via crebh and mir-143/145. J
420 Biomed Sci 30:38.
421 Yu S, Zhao Z, Xu X, Li M, Li P. 2019. Characterization of three different types of extracellular
422 vesicles and their impact on bacterial growth. Food Chem 272:372-378.
423 Zaborowski MP, Balaj L, Breakefield XO, Lai CP. 2015. Extracellular vesicles: Composition,
424 biological relevance, and methods of study. Bioscience 65:783-797.
425 Zhang J, Zhao C, Yao M, Qi J, Tan Y, Shi K, Wang J, Zhou S, Li Z. 2023. Transcriptome sequencing
426 reveals non-coding rnas respond to porcine reproductive and respiratory syndrome virus and
427 haemophilus parasuis concurrent infection in lungs of kele piglets. Journal of Animal Science
428 and Technology.
429

430

Figure Legends

Figure 1. Characterization of BCEVs.

(a) Schematic of the isolation of BCEVs from colostrum sample. (b) Photograph of isolated BCEVs. (c) TEM images of BCEVs. Scale bar: 200 nm. (d) NTA of BCEVs. (e) Western blot analysis of EV markers, CD63. White arrows indicate BCEVs. BCEVs, bovine colostrum-derived extracellular vesicles; TEM, transmission electron microscopy; NTA, nanoparticle tracking analysis.

Figure 2. Growth rate and viability of commensal bacteria treated with BCEVs.

a–b) Growth rate (a) and cell viability at the end point (b) of *A. muciniphila* co-incubated with PBS or BCEVs (1×10^9 and 1×10^{10} particles/mL). c–e) Growth rates of *B. intestinalis* (c), *B. fragilis* (d), and *P. massiliensis* (e) co-cultured with PBS or BCEVs (1×10^9 and 1×10^{10} particles/mL). All data are presented as the mean ± standard deviation. The two groups were compared using Student's t-test, and one-way analysis of variance and Dunnett's test analyzed the differences among multiple groups. Asterisks represent statistical significance at $p < 0.05$ (*), $p < 0.01$ (**). Growth rate was calculated using the Growthcurver package of R. PBS, phosphate-buffered saline control; BCEVs, bovine colostrum-derived extracellular vesicles.

Figure 3. Establishment of a small-scale anaerobic bacterial-aerobic cell line co-culture system.

(a) Schematic of a small-scale anaerobic bacterial-aerobic cell line co-culture system. (b) Amount of dissolved oxygen in the Transwell plate. (c) Viability of *A. muciniphila* in the apical part. (d) SEM image of the apical part. Orange arrow indicates *A. muciniphila*. All data are presented as the mean ± standard deviation. The two groups were compared using Student's t-test, and one-way analysis of variance and Dunnett's test analyzed the differences among multiple groups. Asterisks represent statistical significance at $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***). BCEVs, bovine colostrum-derived extracellular vesicles; SEM, scanning electron microscopy.

Figure 4. Transcriptomic analysis of *A. muciniphila* influenced by BCEVs.

(a) Volcano plot showing differential gene expression in *A. muciniphila* treated with BCEVs compared to PBS control. Significant upregulation is indicated by blue dots (FC ≥1.5, $p < 0.05$) and significant downregulation by orange dots (FC ≤-1.5, $p < 0.05$). Grey dots represent genes with no significant changes. (b) Pie chart illustrating the distribution of biological processes affected in *A. muciniphila* after BCEVs treatment. BCEVs, bovine colostrum-derived extracellular vesicles; PBS, phosphate-buffered saline control; FC, fold change.

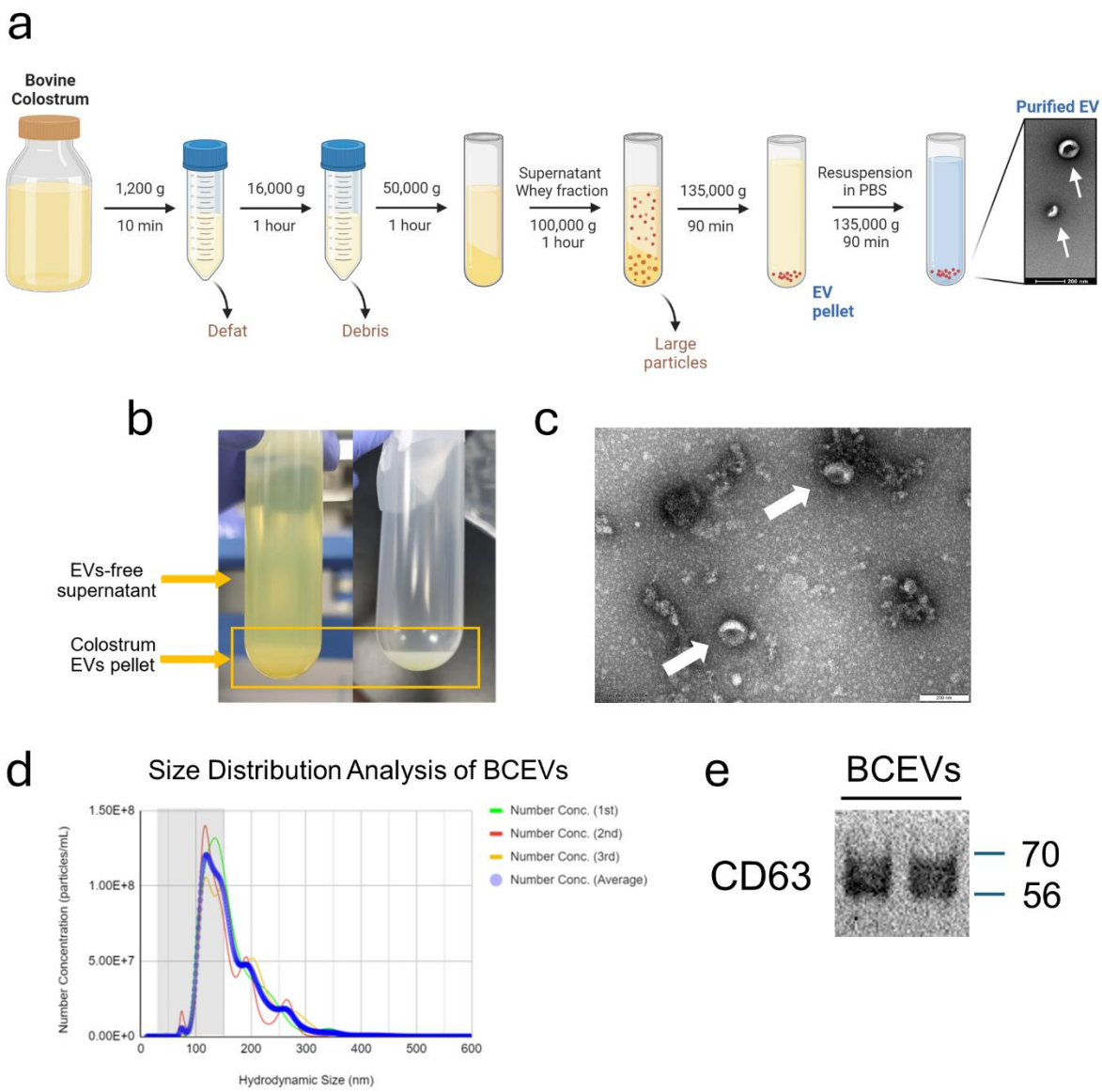
a
Bovine Colostrum
1,200 g
10 min
Defat
16,000 g
1 hour
Debris
50,000 g
1 hour
Supernatant Whey fraction
100,000 g
1 hour
Large particles
135,000 g
90 min
EV pellet
Resuspension in PBS
135,000 g
90 min
Purified EV
b
EVs-free supernatant
Colostrum EVs pellet
c
d
Size Distribution Analysis of BCEVs
Number Concentration (particles/mL)
1.50E+8
1.00E+8
5.00E+7
0.00E+0
0
100
200
300
400
500
600
Hydrodynamic Size (nm)
Number Conc. (1st)
Number Conc. (2nd)
Number Conc. (3rd)
Number Conc. (Average)
e
BCEVs
CD63
70
56

Figure 1.

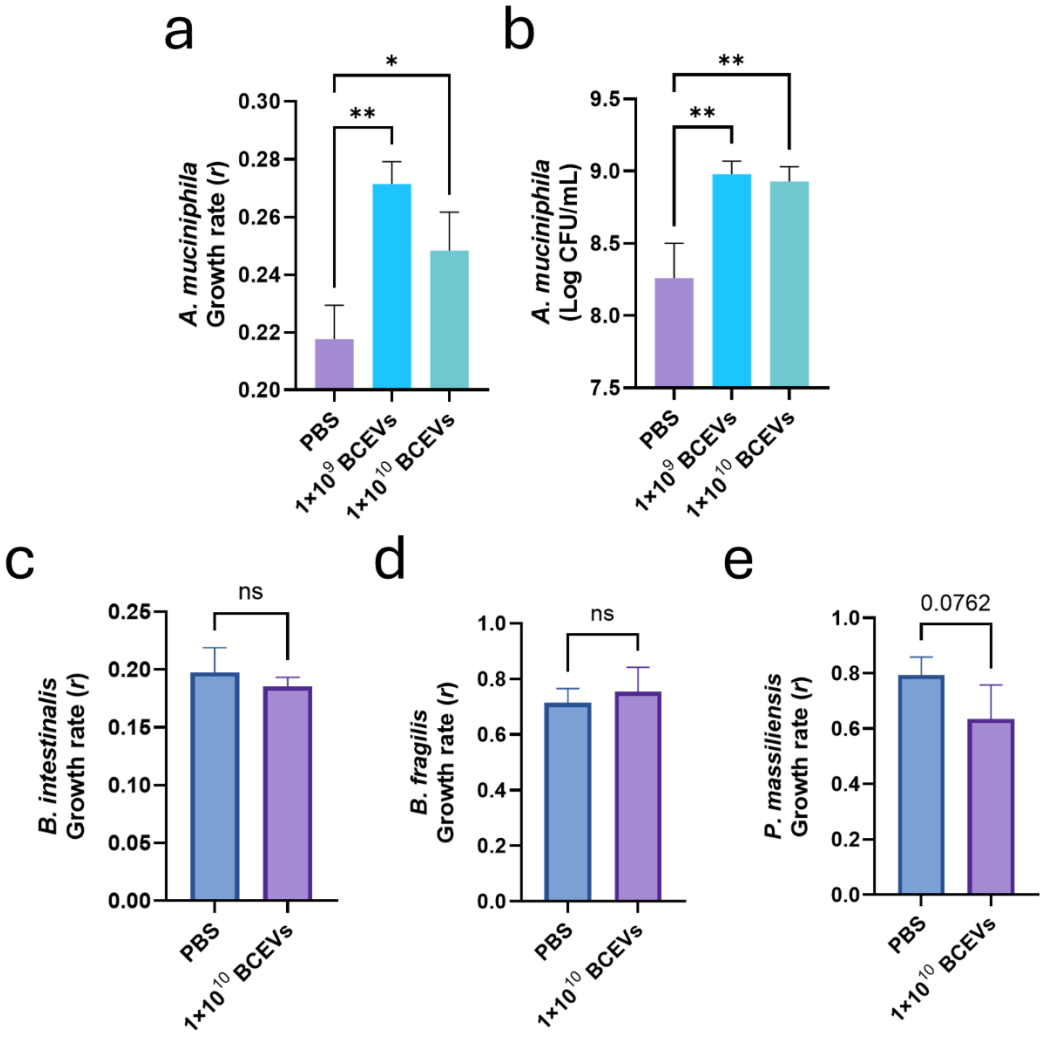
a
b
c
d
e
A. muciniphila Growth rate (r)
A. muciniphila (Log CFU/mL)
B. intestinalis Growth rate (r)
B. fragilis Growth rate (r)
P. massiliensis Growth rate (r)
PBS
1×10^9 BCEVs
1×10^10 BCEVs
*
**
ns
0.0762

Figure 2.

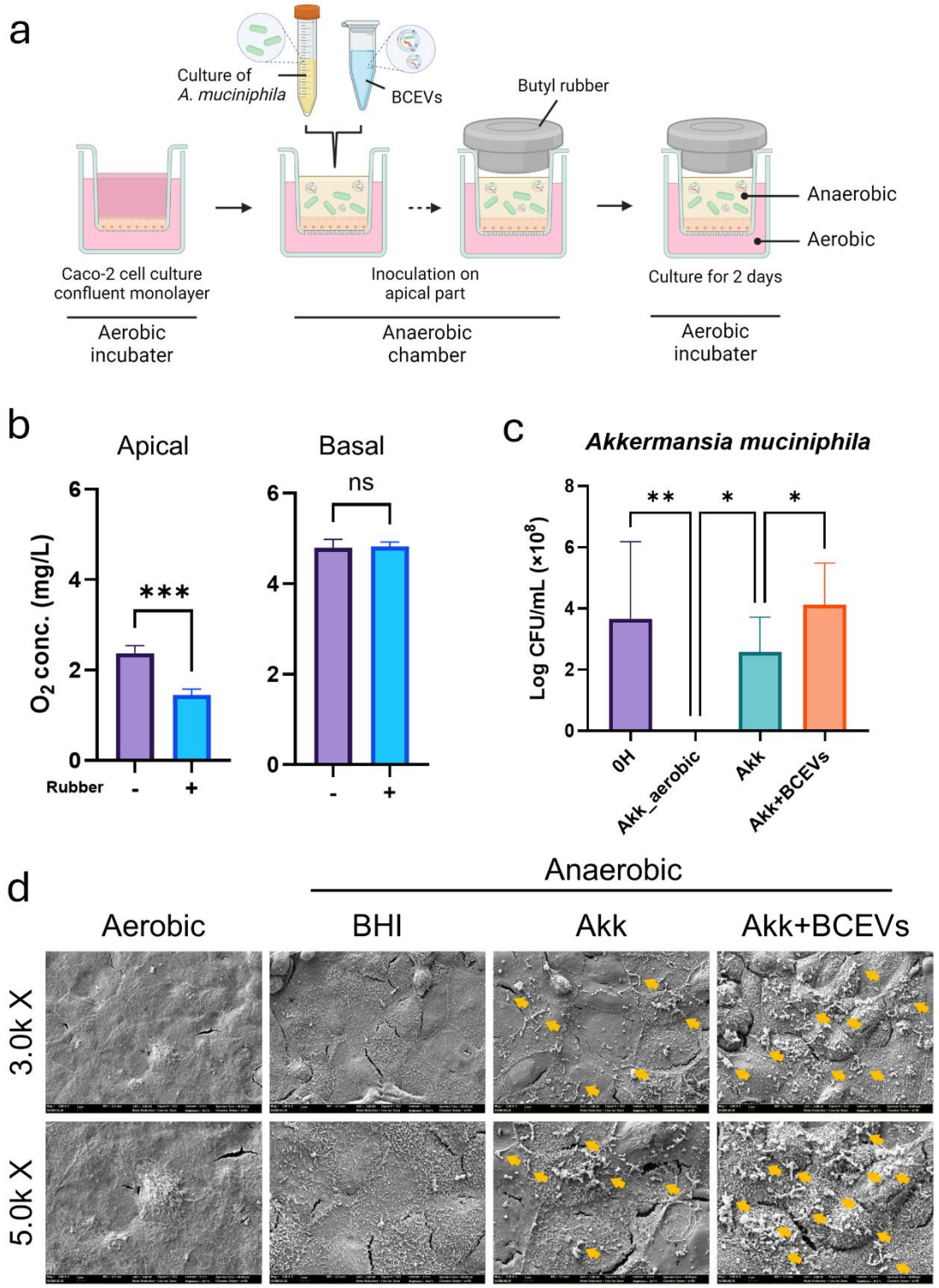
a
Culture of
A. muciniphila
BCEVs
Butyl rubber
Anaerobic
Aerobic
Caco-2 cell culture
confluent monolayer
Inoculation on
apical part
Culture for 2 days
Aerobic
incubater
Anaerobic
chamber
Aerobic
incubater
b
Apical
Basal
ns
***
O_2 conc. (mg/L)
Rubber
c
Akkermansia muciniphila
**
*
*
Log CFU/mL (×10^8)
0H
Akk_aerobic
Akk
Akk+BCEVs
d
Anaerobic
Aerobic
BHI
Akk
Akk+BCEVs
3.0k X
5.0k X

Figure 3.

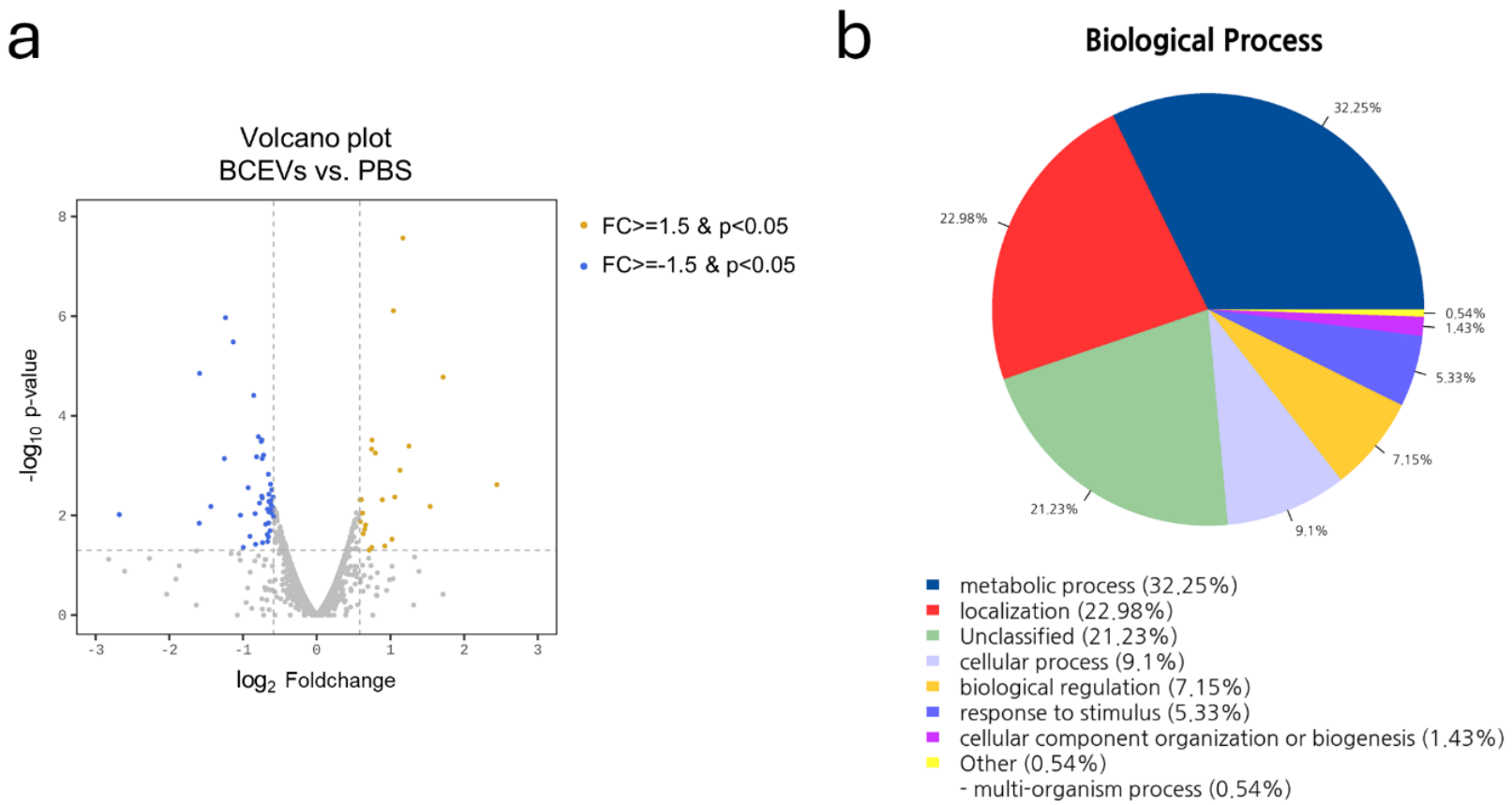
a
Volcano plot
BCEVs vs. PBS
FC>=1.5 & p<0.05
FC>=-1.5 & p<0.05
-log10 p-value
log2 Foldchange
b
Biological Process
32.25%
22.98%
21.23%
9.1%
7.15%
5.33%
1.43%
0.54%
metabolic process (32.25%)
localization (22.98%)
Unclassified (21.23%)
cellular process (9.1%)
biological regulation (7.15%)
response to stimulus (5.33%)
cellular component organization or biogenesis (1.43%)
Other (0.54%)
- multi-organism process (0.54%)

Figure 4.

Table 1. Top five differentially expressed genes between negative control and bovine colostrum-derived extracellular vesicles treated *Akkermansia muciniphila*

Upregulated			Downregulated		
Gene	Definition	FC	Gene	Definition	FC
Amuc_1300	Adenylylsulfate reductase, thioredoxin dependent	5.446	Amuc_1922	Putative ferredoxin	-2.192
Amuc_1253	Glutamate synthase (ferredoxin)	2.249	Amuc_0875	Glycoside hydrolase family 16	-1.760
Amuc_0910	Transcriptional regulator, LysR family	2.188	Amuc_2028	Transcriptional regulator, MarR family	-1.685
Amuc_1252	Glutamine synthetase catalytic region	2.057	Amuc_0095	Ribosomal protein L33	-1.678
Amuc_1153	Potassium-transporting ATPase, C subunit	1.897	Amuc_0860	Anaerobic ribonucleoside-triphosphate reductase activating protein	-1.676

FC, fold change

Table 2. KEGG pathway analysis of differentially expressed genes between negative control and bovine colostrum-derived extracellular vesicles treated *Akkermansia muciniphila*

Upregulated			Downregulated		
Gene	Description	FC	Gene	Description	FC
Two-component system			**Protein export**		
Amuc_0736	Protein tyrosine phosphatase	1.678	Amuc_0240	Twin-arginine translocation protein, TatA/E family subunit	-1.567
Amuc_0830	Potassium-transporting ATPase, A subunit	1.569	Amuc_1047	Preprotein translocase, SecE subunit	-1.670
Amuc_1153	Potassium-transporting ATPase, C subunit	1.897			
Nitrogen metabolism					
Amuc_1252	Glutamine synthetase catalytic region	2.057			
Amuc_1253	Glutamate synthase (ferredoxin)	2.249			

KEGG, Kyoto Encyclopedia of Genes and Genomes; FC, fold change