The impact of Akkermansia muciniphila and its Extracellular Vesicles in the regulation of Serotonergic Gene Expression in a small intestine of mice “Probiotics and Antimicrobial Proteins”

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Abstract

A better understanding of host-microbe interaction as a cross-talk between the gastrointestinal (GI) tract and the gut microbiota by improving the maintenance of GI homeostasis can help treat and prevent GI disorders. Gut microbiota can affect signaling molecules like serotonin, which regulate endocrine systems through the GI tract. While studying the importance of gut microbiota effects in the small intestine is also pivotal in humans’ GI health. Here, we investigated the potential role of *Akkermansia muciniphila* as a next-generation probiotic and its Extracellular Vesicles (EVs) as a post-biotic in regulating the serotonin-related gene system in the duodenum and ileum of the small intestine of mice. *A. muciniphila* significantly affected the mRNA expression of genes involved in the serotonin system (*Tph1, Slc6a4a, Mao, Htr3B, Htr4, and Htr7*) in the duodenum and ileum of mice (*P* < 0.05). Moreover, *A. muciniphila*-derived EVs could impact the expression of major genes involved in the serotonin system (*Tph1, slc6a4a, Mao, Htr3B, Htr4, and Htr7*) in the duodenum and ileum of mice (*P* < 0.05). This study may pave the way for further investigation of the effects of strain-specific probiotics on the serotonergic system, which is currently in its infancy.

1. Introduction

Serotonin [5-hydroxytryptamine (5-HT)], as a functional signaling molecule, has a vital role in regulating the gastrointestinal tract (GI), including motility, secretion, and inflammation (1). Serotonin is synthesized from the essential amino acid tryptophan (Trp) by the rate-limiting enzyme tryptophan hydroxylase 1 (*Tph1*) via enterochromaffin (EC) cells in the epithelium of the GI tract (2). Since the serotonin system (i.e., synthesis, release, and clearance) have a fundamental role in maintaining intestinal homeostasis, there is a correlation between the pathogenesis of gut disorders and serotonin pathways impairment (3, 4). Alterations in peripheral serotonin transmission may underlie the pathological symptoms (3). Indeed, the regulation of serotonin system-related genes in human epithelial cells play a central role in GI functions (5).

The signaling of serotonin is initiated by interacting with various serotonergic receptors and terminated by catalysis via mono-amino oxidase (MAO) of the 5-HT oxidative degradation (6). The serotonin in the gut is uptake by the serotonin-selective reuptake transporter (SERT), which removes serotonin from the interstitial space, so most of the bioavailability of serotonin in the gastrointestinal tract is modulated by SERT (7). Therefore, the SERT function plays a pivotal role in maintaining the serotonin system's homeostasis in the gut; thus, any functional impairment is associated with GI disorders pathology (8). Reducing serotonin uptake by SERT and increasing the level of serotonin in the gut may play an essential role in the pathogenesis of inflammatory bowel disorder (8). Since most of the serotonin in the body (about 90%) is synthesized in the gut, the GI is considered the main reservoir of serotonin (9, 10). Hence, the connection between the regulation of the serotonin system and GI activity is a critical key to identifying the gut disorders associated with this interaction. Meantime, the human GI has a complex bacterial community that regulates endocrine systems and influences hormone signaling and neurotransmitter like serotonin (11, 12). Microbial endocrinology reveals the capability of microbiota to produce and regulate neurotransmitter signaling in the host they inhabit, representing the interaction of microbiology and neurobiology (13). New evidence suggests that modulation of serotonin signaling via intestinal microbiota can modify the risk and severity of inflammatory bowel disease (IBD) (14). Since the microbiome modulates the serotonin system, the increase in probiotic-based interventions could be considered a way to manipulate serotonergic signaling.

Since the small intestine is the first phase of the digestive system, where most nutrient assimilation and absorption occurs, studying this ecosystem is principally relevant to digestive health (15). Despite growing studies defining the association between microbiota residing in the colon with health and disease, few studies have analyzed the small intestine microbiome. Recent literature reveals the role of enteric serotonin as the most abundant neurotransmitter in
the intestine in increasing small intestinal mucosal function (16). Our previous research highlights the relationship between the Akkermansia muciniphila as a next-generation probiotic and the serotonergic system behind the interaction between the gut and brain in the mice model (17). However, our data showed that the bacterium and its EVs could modulate serotonin signaling/metabolism in the colon; it remains unknown whether the serotonin system regulated by A. muciniphila also affects intestinal function throughout the small intestine.

The gut microbiota modulates host biology in multiple ways; notably, substantial contribution to this regulation comes from microbiota-derived metabolites. Microbiota-released EVs as a remarkable mediator, can modulate the intercellular signaling mechanism involved in microbiota-host communications (18). Due to much pancreatic juice, bile acids, pH 6.0–7.0, and digestive enzymes (including lipases, proteases, and amylases) in the small intestine can impact probiotic viability (19). In vitro studies have demonstrated that EVs as post-biotics have inherent stability under different body conditions and are more biocompatible than bacteria (20). Thus, given the role of EVs in the signaling processes, we hypothesized that A. muciniphila-derived EVs might affect the serotonin system-related genes in the small intestine of mice.

Almost all ingested food substrate is absorbed in the small intestine, where essential amino acids like Trp are absorbed in the small intestine. Meanwhile, the majority of Trp is the sole precursor of serotonin absorbed in the small intestine. The small intestine is the first tissue exposed to diet-derived nutrients; recognizing the role of environmental factors like microbe-microbe and host-microbe interactions may benefit human health. In line with this notion, we aimed to examine the impact of A. muciniphila and its EVs on the serotonin system in the duodenum and ileum of the small intestine of mice.

2. Materials And Methods

2.1. Approval for animal experiments

All animal studies were carried out in relevant guidelines and regulations of the National Institute of Health guide for the care and use of laboratory animals (NIH Publication No_ 8023, revised 1978). The Ethics Committee of the Pasteur Institute of Iran (IR.PII.REC.1399.004) approved all experimental animal protocols. Male C57BL/6J mice (age: eight weeks) were obtained from the Pasteur Institute of Iran (Tehran, Iran) and maintained under the same conditions 12 h light, 22–23°C, and 40% humidity; all mice were fed with a standard antibiotic-free diet and autoclaved water ad libitum.

2.2. Bacterial culture

A. muciniphila MucT (ATCC BAA-835) (DSMZ Institute, Germany) was cultured in a basal mucin-based medium in brain heart infusion (BHI) medium (Quelab, Canada) under anaerobic conditions (80% N₂, 10% H₂, and 10% CO₂) at 37°C for 3–7 days.

2.3. Extraction and characterization of A. muciniphila-derived EV

EV isolation and purification from A. muciniphila were performed, according to the study of Yaghoubfar et al (17). Briefly, A. muciniphila strain was routinely inoculated in BHI broth (Quelab, Canada), supplemented with 0.5% mucin (Sigma-Aldrich) under anaerobic conditions (150 rpm shaking) overnight at 37°C until an optical density (OD₆₀₀) of one was reached. Next, to separate the supernatant from bacterial pellets, the bacterial cells were pelleted by centrifugation at 11,000 g for 20 minutes at 4°C and washed twice with phosphate-buffered saline (PBS). Then, the
supernatant is used to extract EVs. For this purpose, the supernatant was filtered through a 0.22-mm filter (Millipore, USA). EVs were extracted with ultracentrifugation (200,000 g for 2 h at 4°C). The vesicle pellets were resuspended in sterile PBS and stored at -80°C until used. The size and morphology of extracted EVs were evaluated by Transmission Electron Microscopes (TEM) (ZEISS EM. 109 / EM 900).

### 2.4. Experimental design and sample collection

Mice (n = 30) were randomly divided into three groups (10 mice per group); group I was orally gavaged 200 µL of PBS (PBS as a control group). Mice within group II was orally gavaged 10⁹ colony-forming units (CFU)/200 µL of viable A. muciniphila, suspended in PBS (A. muciniphila group), and group III was orally gavaged 10 µg of protein/200 µL of EVs (A.m-EV group), once daily for 4 weeks. The body weight of each group was measured once a week. After four weeks of treatment, at the end of the experiment, all mice were sacrificed via cervical dislocation, and their samples and biopsies (organ collection) were collected. Afterward, the duodenum and ileum content was emptied, and the tissue segments were collected in RNase-free Eppendorf tubes, snap-frozen with liquid nitrogen, and stored at -80°C for subsequent analyses.

### 2.5. Tissue RNA extraction and quantitative real-time PCR (qPCR) assay

Total RNA was isolated and purified from tissues according to the protocol of Yaghoubfar et al. (17). In brief, frozen duodenum and ileum tissues were weighed and then homogenized via ultrasonication using the MSE Soniprep 150 Plus disintegrator in appropriate volumes of phenylmethylsulfonyl fluoride (PMSF; Sigma-Aldrich, Catalog Number: P7626, as a general inhibitor of a serine protease and the total RNA, was extracted according to the manufacturer’s instructions. RNA was treated with DNase-I (Qiagen, Germany) to remove genomic DNA, and then total RNA was converted to cDNA using PrimeScript RT Reagent Kit (Takara, USA). The qPCR amplification was also carried out using SYBR Premix Ex Taq II (Takara, USA) in triplicate; validated oligonucleotide primer sequences used in this study are presented in Table 1.

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>Product size (bp)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rpl-13A</td>
<td>AGCCTACCAGAAAGTTTGCTTAC</td>
<td>GCTTCTTCTTCCGATAGTCAT</td>
<td>129</td>
<td>[21]</td>
</tr>
<tr>
<td>β-actin</td>
<td>GAGGTATCCTGACCCCTGAAGTA</td>
<td>CACACGCACGGTATGGTTGA</td>
<td>104</td>
<td>[22]</td>
</tr>
<tr>
<td>Tph1</td>
<td>ACTGCGACATCAGGCAGGAA</td>
<td>CGCAGAGTCAGGCAGCAATC</td>
<td>162</td>
<td>[23]</td>
</tr>
<tr>
<td>slc6a4a</td>
<td>TATCCAATGGGTACTCCGAG</td>
<td>CCGTTCCCTTGGTGATCTT</td>
<td>110</td>
<td>[24]</td>
</tr>
<tr>
<td>Mao</td>
<td>GGAGAAGGCCAGATCAGAAG</td>
<td>GAACCAAGACATTAATTTTGATGAC</td>
<td>113</td>
<td>[25]</td>
</tr>
<tr>
<td>Htr3B</td>
<td>CTGTCTACCTGGACACCTTGC</td>
<td>AACTCATCGTCCAAACCTTC</td>
<td>103</td>
<td>This study</td>
</tr>
<tr>
<td>Htr4</td>
<td>GATGCTAATGGTGACACCTTGGA</td>
<td>CAGCAGGTTGCCCAAGATG</td>
<td>105</td>
<td>This study</td>
</tr>
<tr>
<td>Htr7</td>
<td>TGGCGGGAGCAGATCAACTA</td>
<td>GACAAAGACACCCGAGATC</td>
<td>120</td>
<td>[26]</td>
</tr>
</tbody>
</table>

**Table 1**
The oligonucleotide sequence of primers of target genes in mice.
2.6. Statistical analysis

The means and standard deviation (SD) of the data are displayed. The relative gene expression was analyzed by the ΔΔCT method, and Rpl-13A and β-actin genes in the tissue of mice were used as the endogenous control. For calculating changes in gene expression and statistical analysis, GraphPad Prism 8.0 (GraphPad Software Inc, CA, USA) was exploited. The statistical significance of differences in the expression of target genes was analyzed using a one-way analysis of variance (ANOVA) followed by Tukey’s post hoc test (for multiple comparisons between more than two groups). P-values of less than 0.05 were considered statistically significant.

3. Results

3.1. Characterization and morphology of *A. muciniphila*-derived EVs

As shown in (Fig. 1), in electron microscopic observations, multiple spherical vesicles were observed, ranging between 20 and 200 nm.

3.2. *A. muciniphila* and its EVs induced changes in the expression of genes involved in the serotonin system in the duodenum

The findings demonstrated that, in comparison to the control group, *A. muciniphila* and its EVs (A.m-EV) affect on expression of the following genes involved in intestinal serotonin signaling: Slc6a4, Tph1, Mao, Htr2B, Htr3, Htr4, and Htr7. The results showed that both *A. muciniphila* and EV treatments significantly increased the gene expression of Tph1 (P = 0.0001 and P < 0.0001, respectively) (Fig. 2A). These experiments showed that the A.m-EV significantly increased the expression of the Slc6a4 (P = 0.0006) gene compared to the control group (Fig. 3B). In contrast, the *A. muciniphila* group did not significantly impact the Slc6a4 gene (Fig. 3B). Following treatment with *A. muciniphila* and its EVs, Mao gene reduced consider, compared to the control group (P < 0.0001, P < 0.0001, respectively) (Fig. 2C). Both *A. muciniphila* and EV treatments significantly reduced the gene expression of Htr3B (P < 0.0001 and P = 0.0059, respectively) (Fig. 2D). Compared with the control group, A.m-EV treatments significantly enhanced the expression of the Htr4 gene (P < 0.0001) (Fig. 2E). On the contrary, the *A. muciniphila* group did not significantly impact the Htr4 gene (Fig. 2E). Following treatment with the bacterium, the Htr7 gene expression was significantly reduced compared to the control group (P = 0.0021) (Fig. 2F). In contrast, the A.m-EV group did not show a significant response to the treatment (Fig. 2F).

3.3. Administration of *A. muciniphila* and its EVs affected the mRNA expression of serotonin system-related genes in the ileum of mice.

In response to treatment, the finding showed that both *A. muciniphila* and its EVs could impact the mRNA expression of serotonin system-related genes. In comparison with the control group, the A.m-EV group, the mRNA expression of Tph1 and Slc6a4 significantly increased (P < 0.0001, P < 0.0001, respectively) (Fig. 3A and 3B); on the contrary, the *A. muciniphila* group did not significantly impact the Tph1 and Slc6a4 genes (Fig. 3A and 3B). After treatment, a decrease in the expression level of the Mao gene was observed in A.m-EV (P = 0.0003) (Fig. 3C). In contrast, no significant response was seen in the *A. muciniphila* group (Fig. 3C). In comparison with the control group, both *A. muciniphila* and A.m-EV treatments significantly reduced the Htr3B, Htr4, and Htr7 genes expression (P = 0.0002, P = 0.0078, P < 0.0001, P = 0.0002, P < 0.0001 P < 0.0001, respectively) (Fig. 3D,3E,3F).

4. Discussion
The effects of the gut microbiome on the host serotonin system have been demonstrated in some studies. The gut microbiota-serotonin system axis is involved in the pathogenesis of several GI disorders, but little is known about this interaction in gut disorder etiology (9).

While serotonin synthesizes from Trp and most of Trp is absorbed in the small intestine, focusing on the serotonin system in the small intestine ecosystem may have an essential role in digestive health. That is why we looked closely at the expression of serotonin system-related genes in the small intestine. In addition, there is significant interaction between gut microbiota and the serotonin system. The interaction between serotonin signaling and microbiota composition in the small intestine is less well characterized because of challenges in sampling this segment of the digestive tract. In this study, we focused on one abundant gut microbiota as a promising probiotic, like *A. muciniphila*, to explain its relationship with the serotonin system in the small intestine.

The serotonin system homeostasis plays an essential role in maintaining intestinal health and GI homeostasis, so any disturbance in this system is associated with gastrointestinal disorders. Most of the bioavailability of serotonin in the gastrointestinal tract is modulated by SERT; therefore, the SERT function is critical in maintaining the serotonin system's homeostasis. Previous studies have shown that the dysfunction of SERT is associated with the pathogenesis of several gastrointestinal disorders like IBS, celiac disease, and diarrheal disease (21, 22). In the present study, our results indicated that the expression of *Slc6a4* mRNA was seemingly upregulated in the EV group. Dissimilar to the EVs, the bacterium did not affect the expression of *Slc6a4*, and we observed the independent effects of the bacterium and its EVs in the small intestine of mice. Meanwhile, this difference could be accounted for by harsh conditions in the small intestine, where the effect of live bacteria is diminished. Similarly, we observed this effect in our previous study in the colon of mice, and it can be concluded that the EVs had a similar impact on the expression of the *Slc6a4* gene in the different conditions of the GI tract. SERT function is correlated with metabolic disorders, while Singhal et al. study revealed that the mice with the deletion of SERT, metabolic syndromes, especially diabetes, occurred. They also showed that the composition of microbiota was changed, and the abundance of *A. muciniphila* as a beneficial bacterium was decreased, so there is a correlation between SERT function and gut microbial homeostasis (22).

Since SERT is highly expressed on the surface of nearly all IECs, in our previous laboratory studies, we investigated the effect of *A. muciniphila* and its EVs in the colorectal epithelial adenocarcinoma (Caco-2) cell line as a model to represent intestinal cells (5). In line with the abovementioned results, we showed that both *A. muciniphila* and its EVs led to the induction of *Slc6a4* mRNA expression in the Caco-2 cell line (5). In the Caco-2 cell, we showed that both *A. muciniphila* and its EVs led to the induction of *Slc6a4* mRNA expression. Despite the differences between small intestine (in vivo) and Caco-2 cells (in vitro) conditions, EVs groups showed similar results; it can be assumed that EVs as non-viable microbial metabolites may have a direct effect on the *Slc6a4* gene expression. In contrast, our observations showed that *A. muciniphila* did not affect *Slc6a4* in mice's duodenum or ileum, which indicates that bacteria may not have similar functions in different conditions. In terms of enhancing the SERT function, we worked on another next-generation probiotic, *Faecalibacterium prausnitzii* (*F. prausnitzii*), and its EVs in our previous study. As such, our results indicated that *F. prausnitzii* and its EVs showed the same effect as *A. muciniphila*, and its EVs could impact the expression of the *Slc6a4* gene in the Caco-2 cell line (5). Inconsistent with our research, several studies showed that probiotics like *Lactobacillus acidophilus* and *Bifidobacterium longum* enhanced the *Slc6a4* gene expression in Intestinal epithelial cells and mice intestinal tissues (23, 24). Moreover, previous in vivo studies reported the induction of SERT function after supernatant *F. prausnitzii* administration in mice with chronic low-grade inflammation (25). Besides, in our last experiment, we carried out a comparative study that compared the effects of *A. muciniphila* and *F. prausnitzii* supernatant in the gene expression of *Slc6a4* in the Caco-2 cell line (5). Altogether,
our present study and previous studies revealed that microbial metabolites or supernatants substantially affect the SERT function in addition to bacteria.

One of the genes involved in peripheral serotonin that system affected by gut microbiota is Tph1.

Our results showed that oral administration of A. muciniphila and its EVs had a significant effect on the induction of the Tph1 gene expression in the duodenum of mice. Our results showed that oral administration of A. muciniphila and its EVs had a significant effect on the induction of the Tph1 gene expression in the duodenum of mice. Meanwhile, EVs had better results in comparison with the bacterium.

Of interest, only EVs could improve Tph1 gene expression in the ileum, and A. muciniphila did not significantly affect on expression of the Tph1 gene. Given that the Tph1 enzyme is intracellular, it can be hypothesized that EVs may access the cells by passing through the inner mucus layer and could affect the expression of this gene. MAO is another intracellular enzyme of the serotonin system that regulates the catalysis of oxidative serotonin degradation. In the same vein, our results showed that EVs decreased the MaoA expression compared to A. muciniphila in the ileum of mice. It is noteworthy that A. muciniphila affected Tph1 gene expression in the duodenum compared to the ileum; the histological differences in the ileum and duodenum sections of the small intestine may explain this difference. Overall, these initial data suggest that enteric serotonin-related genes could be affected by commensal bacteria and their metabolites in the small intestine. However, it remains unknown whether these changes occur throughout the complex microenvironment of the small intestine, which requires further study.

Serotonin in the GI mediates its effects by binding to various serotonergic receptors (5-HTR1 to 5-HTR7) on intestinal epithelial cells and enteric neurons (6). To explore the impact of A. muciniphila and its EVs on the serotonin system function, we also evaluated the expression of Htr3B, Htr4, and Htr7 receptors in the duodenum and ileum of mice. The 5- Htr3 and 5- Htr4 are implicated in various physiological functions in GI, and they have been studied more in the literature compared to other serotonin receptors (6). In the duodenum, the expression of Htr3B and Htr7 genes decreased, whiles the expression of the Htr4 gene increased following EVs treatment.

Our findings also showed that the gene expression of Htr3B, Htr4, and Htr7 receptors decreased in the ileum of mice. The possible explanation for our conclusion is that the changes in the expression of these receptors can be explained by feedback regulation.

Altogether, probiotics-host crosstalk, through complex interactions along the intestinal tract, can maintain intestinal homeostasis and improve a wide range of disorders, possibly benefiting the host's health. In the context described above, here we carried out preliminarily in vivo studies of the effect of the next-generation strain on the serotonin system-related genes through a pilot study performed in different intestinal sites like the duodenum and ileum of mice. Our study provided the basis for subsequent studies to determine the signal transduction pathway.

Declarations

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Author Contributions
R.Y: carried out the experiments and wrote the first draft of the manuscript. A.K, F.A, and A.SH: had a role in the acquisition of data; E.Z.B.K: analyzed the data; M.D: laid out the experiment’s protocol; A.F: read and approved the final manuscript; S.D.S and SH.KH: designed and supervised the study and read and approved the manuscript. All authors were involved in the preparation of the manuscript.

**Data Availability**

The authors declare that all data generated or analyzed during this study are included in this published article.

**Compliance with Ethical Standards**

**Conflict of Interests**

The authors declare no competing both financial and non-financial interests.

**Ethical Approval**

This work does not contain any studies related with human participants or animals

**References**


Figures
Figure 1

Characterization of EVs isolated from *A. muciniphila*. The TEM image of negatively stained EVs indicated different sizes (range: 20-200 nm) and vesicle-like structures of EVs.
**Figure 2**

The effects of treatment with *A. muciniphila* and its EVs on the expression level of serotonin system-related genes in the duodenum of mice. The relative mRNA expression in the duodenum of mice was affected by both the bacterium and its EVs. Expression of **A)** *Tph1*, **B)** *Slc6a4*, **C)** *Mao* **D)** *Htr3B* **E)** *Htr4*, and **F)** *Htr7*. Data are normalized using *RPL13a* and *β-actin* as control genes. One-way ANOVA was used to compare values. The difference between mice groups was indicated as a p-value. *P < 0.05, **P < 0.01 were considered statistically significant.***
Figure 3

Administration of *A. muciniphila* and its EVs induced changes in the expression of the ileum serotonin system-related gene in mice. Expression of **A**) *Tph2** B) *Slc6a4** C) *Mao** D) *Htr3B** E) *Htr4** F) *Htr7*. Data are normalized using *RPL13a* and *β-actin* as control genes. One-way ANOVA was used to compare values. The difference between mice groups was indicated as a p-value. *P < 0.05, **P < 0.01 were considered statistically significant.