Original Article

Evaluation of efficiency of Methods used for extraction of *Bifidobacterium bifidum*-Derived Extracellular Vesicles: An experimental study

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Abstract

Background & Aims: Bacteria naturally secret nano-scale vesicles containing a wide range of biomolecules, such as proteins, DNA, and RNA. These vesicles are called extracellular vesicles (EVs). EVs play important roles in host-microbiota interactions. For isolating EVs, different methods have been proposed and each method has its advantages and also limitations. Therefore, in the current study, efficacy of two methods used for extraction of EVs was investigated.

Materials & Methods: For this purpose, *Bifidobacterium bifidum* was cultured in MRS broth under anaerobic conditions. In the first isolation protocol, ultra-centrifugation was used (Ultra-method) and in the second protocol, ultra-centrifugation (Non-Ultra method) was not used. After isolation, protein content was measured by the NanoDrop system. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) technique was utilized to compare protein pattern of the EVs. Scanning electron microscopy (SEM) images of the EVs̀ samples were taken and size of the EVs was evaluated by Digimizer software.

Results: The results showed that the EVs isolated by the Ultra-method had significantly higher vesicle-associated protein content compared to those isolated by the Non-Ultra method (3.42 and 0.26 mg/ml, respectively). More and larger EVs (up to 235 nm and with frequent size ranging between 100 – 125 nm) were isolated by the Ultra-method compared to the Non-Ultra method (up to 117 nm and with frequent size ranging between 50–75 nm). Also, protein patterns of the EVs were similar in both methods and protein bands were observed at 25 to 250 KDa in both methods.

***Conclusion*:** Our results showed that ultra-centrifugation method is a more proper method for isolation of *B. bifidum*-derived EVs and produces a higher amount of EVs with higher protein content and proper sizes. However, further studies are required to confirm our results.

***Keywords**: Bifidobacterium bifidum*, Extracellular Vesicles, Ultra-Centrifugation, Isolation Methods

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Introduction

Most cells including Gram-positive and Gram-negative bacteria are able to produce and release very small (in nanoscales) membrane vesicles called extracellular vesicles (EVs). These vesicles are sphere-shaped consisting of two proteolipid membranes, which contain specific types of biomolecules, such as protein, lipid, metabolites, and nucleic acids. EVs of Gram-positive bacteria were discovered for the first time in supernatants of *Staphylococcus aureus* and *Bacillus subtilis* cultures (1). Further studies have revealed that EVs of *S. aureus* are involved in pathogenesis of the bacterium and contain various pathogenic proteins including β-lactamase, coagulase, and hemolysin. After then, many studies have been performed to identify EVs of other bacteria and the results have illustrated that many Gram-positive bacteria, such as *Bacillus*, *Streptomyces*, *Listeria*, *Clostridium*, and *Streptococcus* are able to release EVs (2-7). Bacterial microbiota also produces EVs involved in host-microbiota interactions and communications. The microbiota-derived EVs may contain wide range of bioactive molecules having important roles in immunomodulation and triggering specific signaling pathways (8). In recent years, EVs have received a great deal of attention due to their immunomodulatory properties. Studies have revealed that EVs have high potential to be considered as new generation of adjuvants and vaccines (9-13). In addition, they have been introduced as delivery systems of specific biomolecules and drugs to treat diseases (14).

Bifidobacteria are Gram-positive, anaerobic, and non-motile bacteria colonizing the infants' intestinal tract in the early days of life. These bacteria have been shown to have important protective roles and can prevent some diseases or improve recovery of some illnesses. It has been indicated that administration of *Bifidobacteria* (as a probiotic) could improve healing process of inflammatory bowel disease (IBD), positively influence colon regularity, and prevent gastrointestinal infections (15-17). Similar to other Gram-positive bacteria, *Bifidobacteria* also produce and release EVs. Studies have shown that *Bifidobacteria*-derived EVs can trigger apoptosis in intestinal mast cells and subsequently, suppress allergic diarrhea (18).

Purification of EVs by different methods has remained a challenging process due to diverse levels of purity and obtained amounts of EVs. Various methods are used for isolation of EVs. The most common methods include differential centrifugation, sucrose gradient centrifugation, and ultra-centrifugation. Also, various novel techniques have been proposed for isolation and purification of EVs including magnetic beads coated with antibodies, microfluidic devices, and precipitation and filtration technologies (19). On the other hand, using an individual method may result in different levels of yield and purity of EVs in diverse species of bacteria. This event may be related to the properties of EVs, such as size, buoyant density, and factors influencing separation of EVs from bacterial cells through centrifugation. The first step for purification of EVs in many protocols includes separating the cell's derbies and intact bacteria by low-speed centrifugation. After that, the aggregated biomolecules, apoptotic bodies, and structures with higher buoyant density than the EVs should also be separated. But, as mentioned above, these steps may not be similar for all the bacterial species and different methods are required to achieve the best results (14, 20, 21). Totally, each method has some limitations and advantages as well. For example, results of a study showed that EVs of *S. aureus* could not be isolated by density gradient ultra-centrifugation method while the EVs could be purified by other methods, such as sucrose cushion ultra-centrifugation and filtration. Furthermore, the results of another study showed that *B. anthracis*-derived EVs could be isolated only by ultra-

centrifugation method. Various methods have been proposed for isolation of EVs in Gram-positive bacteria, such as filtration, ultra-centrifugation, sucrose cushion centrifugation, and density gradient centrifugation (6, 22). Ultra-centrifugation uses high-speed centrifugal force to isolate the EVs in the sufficient time and repeated centrifugation steps. However, the repeated centrifugation phases result in the reduced amount of non-EVs particles, which may also damage the structure of the EVs. In precipitation techniques, polymers are utilized to decrease solubility of EVs and cause precipitation of proteins and particles of the same size. Nevertheless, for decreasing the amount of co-precipitated proteins in polymer-based methods, utilizing a protein removal kit is highly recommended. Generally, it can be said that each bacterial species requires a specific method for isolation of its EVs and achieving the best results (23). To the best of our knowledge, no previous study has compared the mentioned methods to isolate EVs of *B. bifidum* yet. So, in the current study, the efficacy of two methods used for isolation of *Bifidobacterium bifidum*-derived EVs was compared to determine the method with better results.

Materials & Methods

Preparation *of B. bifidum*:

In this experimental study, for performing the required experiments, *B. bifidum* (BIA-7), as a probiotic bacterium was purchased from Tak-Gen-Zist Pharmaceutical Company (Tehran, Iran). The bacterium was cultured in De Man, Rogosa and Sharpe (MRS) Agar (Merck, Germany) supplemented with 0.05% L-cysteine (Sigma-Aldrich, USA) under anaerobic conditions (CO2: 5%, H2: 5%, N2: 90%) using Anoxomat anaerobic jar (Advanced Instruments, USA) for 24- 48 h at 37 °C (18, 24).

Isolation Methods of Evs:

After growth, the bacteria were collected and the same amount of bacteria was inoculated into 2 containers (500 ml) containing MRS broth (Merck, Germany) supplemented with 0.05% L-cysteine through mild shaking (150 rpm) under anaerobic conditions (CO2: 5%, H2: 5%, N2: 90%). Optical density (OD) of the broth cultures was measured at OD_{600} using a spectrophotometer (SP-3000 plus, Optima Co., Tokyo, Japan). After 20 h of incubation, OD of the cultures reached the value of 1 and then, the cultures were transferred on ice to stop proliferation (25).

In the first isolation protocol, where ultra-centrifugation (Ultra method) was used, bacterial pellets were removed by centrifugation (11,000 g for 20 min) and were washed twice with phosphate buffer saline (PBS) solution and then, supernatant was used for extraction of EVs. The supernatant was filtered (pore size: 0.22 μm) and then, it was ultra-centrifuged at 200,000 g for 2 h at 4°C. The sediment was resuspended in PBS solution and was kept at -80°C (26).

In the second isolation protocol, in which ultra-centrifugation was not utilized (Non-Ultra method), the bacteria were cultured for 20 h under anaerobic conditions as described previously. After that, the culture medium was centrifuged at 6,000 g at 4°C. The bacterial pellet was washed twice with PBS solution and was resuspended in 9% NaCl solution and then, was centrifuged for 1 h (6,000 g, 4°C). A sequential centrifugation was utilized to extract the EVs. In this step, the suspension was centrifuged for 90 min (20,000 g, 4°C) utilizing EDTA-sodium deoxycholate buffer (Sigma-Aldrich, USA) and the extracted EVs were stored at -80°C (27).

Protein Yield in Extraction Methods:

Protein content was assessed as an index regarding efficacy of isolation methods. For measuring protein yield of the methods, a spectrophotometry method was used via a NanoDrop system (Thermo Scientific, Lite, USA) (28).

SDS-PAGE Technique:

After extraction of EVs, sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) technique was used to determine protein weight of the extracted EVs by comparing them with a protein ladder. Electrophoresis was performed in a vertical gel electrophoresis device with a gel in the size of 13.8 × 13 cm and thickness of 0.7 mm. Polyacrylamide gels were prepared from stacking gel (4%) and resolving gel (10%) and were run for 45 min at voltage of 200 V. Each well was loaded with about 10 µL of bacterial EVs. After that, the gel was stained using 0.01% Coomassie blue R-250 solution (Sigma, USA), glacial acetic acid (10% v/v), and methanol (45% v/v) for 2 h and then was unstained using methanol (10% v/v) and acetic acid (1% v/v) for 1 h (29).

Scanning Electron Microscopy (SEM):

A fixative solution was prepared to fix the EVs̀ samples. For this purpose, 2.5% glutaraldehyde (v/v) and 2% paraformaldehyde (v/v) were added to PBS solution. The samples were washed and then were dried at room temperature. Then, the samples were coated with gold by physical vapor deposition method and using sputter coater device (KYKY Technology, China). Finally, the samples were assessed by the SEM (KYKY Technology, China) (30).

Measurement of the EVs̀ Size:

Size of the EVs was determined by Digimizer software (version 5.3.5, MedCalc Software, Belgium) and size distribution plot of the EVs was obtained by defining 30 vesicles in each measurement. Three different images were used for each method and error bars were determined by means ± SDs obtained from independent measurements in triplicate (31).

Results

Protein Yield of Extraction Methods:

The results indicated that the Ultra method yielded more amount of proteins compared to Non-Ultra method. So that, Ultra method could yield 3.42 mg/ml of protein while the other method yielded only 0.26 mg/ml of protein.

Determination of the EVs̀ Size:

As shown in Fig. 1(A), the most frequent size of the EVs extracted by Ultra method was observed in range of 100 – 125 nm while the greatest size of the EVs extracted by Non-Ultra method was identified in range of 50-75 nm (Fig. 1(B)).

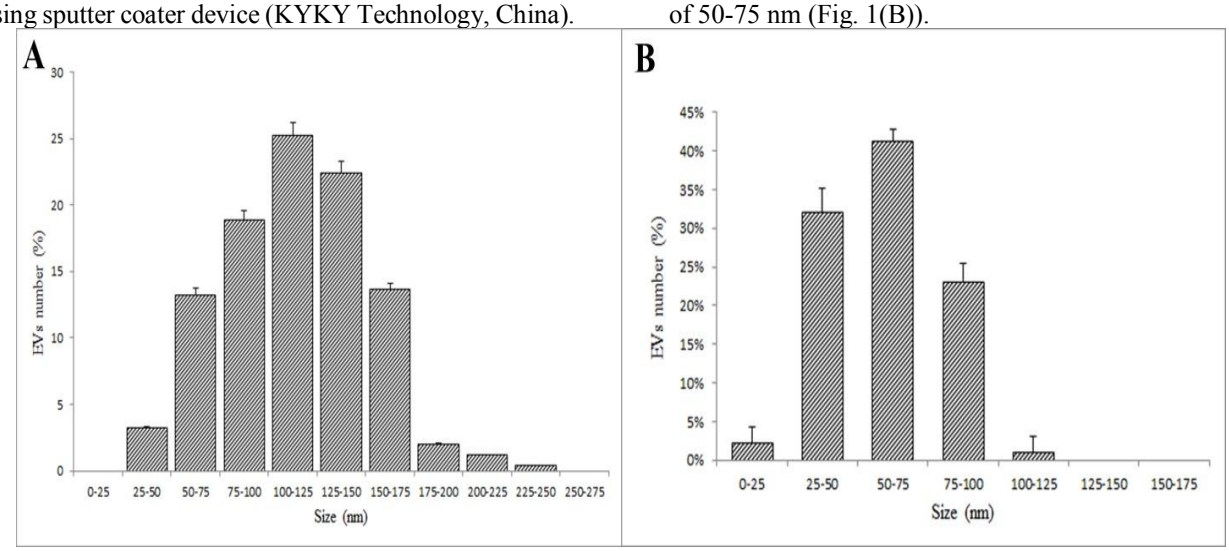

Fig 1. Determination of the EVs̀ size. (A)The EVs̀ size obtained by Ultra method. The largest size of the EVs was observed in range of 100 – 125 nm. (B) The EVs̀ size obtained by Non- Ultra method. The largest size of the EVs was observed in range of 50 – 75 nm.

SDS-PAGE Technique:

Analyzing the results obtained from SDS-PAGE technique showed protein bands ranging from 25 to about 250 kDa. Both methods showed similar patterns; however, the bands in Non-Ultra method were very weak compared to those of the Ultra-method (Fig. 2).

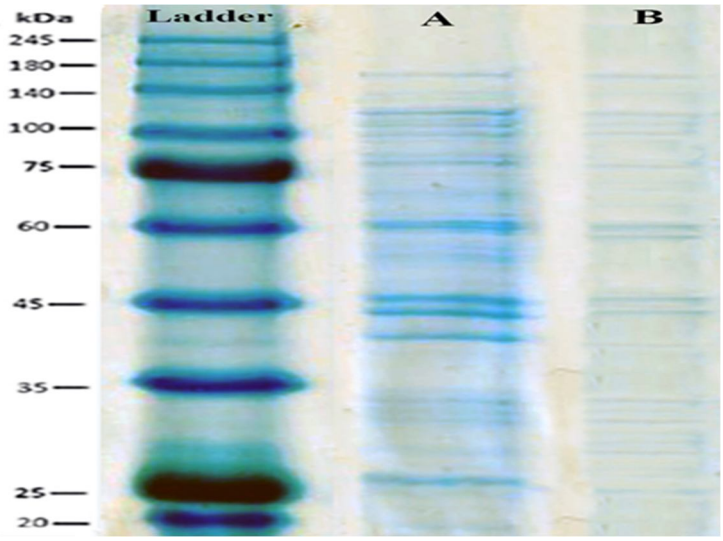

Fig 2. SDS-PAGE analysis on *B. bifidum*-derived EVs extracted by Ultra method (A) and Non-Ultra method (B). In both methods, protein bands were observed from 25 to about 250 kDa. Although, both methods showed similar protein patterns, the protein bands in Ultra method were much stronger than those in Non-Ultra method indicating low protein content of the extracts obtained from Non-Ultra method.

SEM Analysis:

SEM images showed that the EVs had spherical shapes (Fig. 3). No differences were observed between the two extraction methods except for number of the EVs. Number of the EVs in Ultra method was higher compared to Non-Ultra method. The EVs in both methods had spherical shapes but number of the EVs was higher in Ultra-method visually.

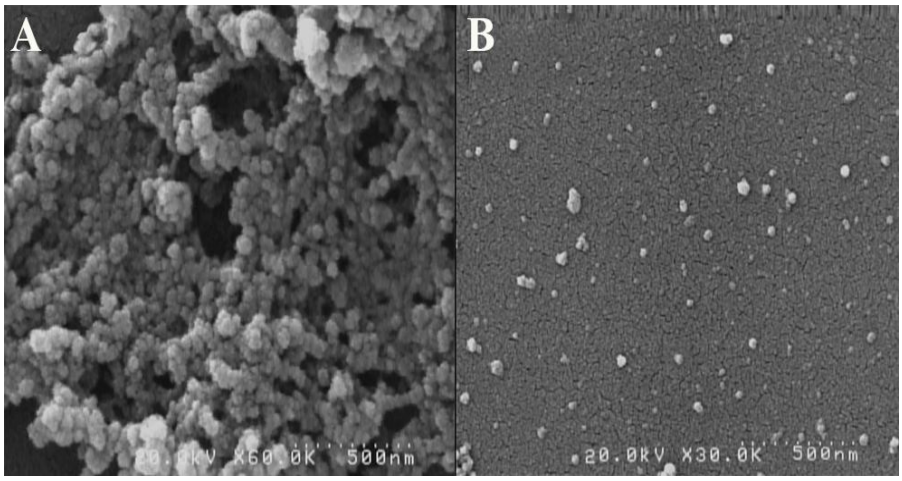

Fig 3. SEM image of *B.bifidum*-derived EVs. As illustrated, the EVs had spherical shapes. There was no noticeable difference between the two methods except for number of the EVs. (A) The bacterium-derived EVs extracted by Ultra method. (B) The bacterium-derived EVs extracted by Non-Ultra method.

Discussion

In the present study, efficacy of two methods (with and without ultra-centrifugation) used for extraction of *B. bifidum*-derived EVs was compared to determine the method providing more appropriate products.

Since discovery of bacterial EVs, different methods and techniques have been proposed for their purification among which, ultra-centrifugation is the most commonly utilized technique to separate the EVs. This method uses multiple steps to remove cell debris, biopolymers, and apoptotic bodies as unwanted materials (32). Overall, each purification method has some advantages and disadvantages. In the case of ultra-centrifugation method, disadvantages, such as contamination during preparation of EVs and time-consuming steps have been reported. Instead, this method has some advantages including requiring small amounts of reagents. Ultra-centrifugation is considered as a gold standard method for extraction of EVs due to its advantages (21).

According to the results obtained from SEM images, natural form of the *B.bifidum*-derived EVs has been conserved during extraction processes in both methods. The EVs had spherical shape in both Ultra and Non-Ultra methods. Jafari et al. (2017) isolated and characterized the EVs of *Faecalibacterium prausnitzii.* They used ultra-centrifugation method to purify *F. prausnitzii*-derived EVs. The extracted EVs were evaluated by SEM and they had spherical shapes with various sizes ranging from 30 to 250 nm (33). Li et al., in another study extracted *Lactobacillus plantarum*-derived EVs. They extracted the EVs using a Non-Ultra method and precipitation protocol and evaluated their characteristics by transmission electron microscopy (TEM). Their results showed that the bacterium-derived EVs had spherical shapes and sizes between 30–300 nm (34). Moreover, Ashrafian et al., assessed the effects of *Akkermansia muciniphila*-derived EVs on toll-like receptors and tight junction expression. The EVs from *A. muciniphila* were obtained by ultra-centrifugation method and were studied visually by SEM. Their results showed that the EVs had spherical shapes and their size varied between 40 – 150 nm (26). In all the above-mentioned studies, natural forms of the EVs were spherical and they were conserved during extraction processes, which are in line with our results.

In the current study, the outcome of different isolation methods varied in terms of protein content. The results of measuring protein yield illustrated that Ultra method could yield about 17 times more protein compared to Non-Ultra method. Also, different size patterns of the EVs were observed utilizing extraction methods. The EVs had diameters ranging from 40 to 235 nm in Ultra method while their diameters ranged from 10 to 117 nm in Non-Ultra method. This difference may be due to different materials used in extraction protocols. Also, centrifugation rotating force may have influenced size of the isolated EVs.

The results of SDS-PAGE technique showed that both methods had almost similar protein patterns; however, protein bands in Non-Ultra methods were very weak. Weaker bands could appear due to low protein content of the samples, which in turn results from low number of the EVs. Low number of the EVs in Non-Ultra method was also observed visually while taking the SEM images. Considering the fact that methods used for isolation of bacterial EVs may have different results in different bacteria, it seems that the results obtained in the current study cannot be generalized to other bacterial species; however, further studies are required.

One of the limitations of this study was the lack of access to a high-speed centrifugation tool in our laboratory and transferring the samples from our laboratory to the laboratory at the University of Tehran to be analyzed by an ultra-centrifuge device.

In conclusion, our results indicated that the ultra-centrifugation caused isolation of more amounts of *B. bifidum*–derived EVs with higher protein content and proper sizes. According to the results of the current study, the ultra-centrifugation method is a more proper method to isolate *B.bifidum*-derived EVs compared to the other protocols where ultra-centrifugation is not used. However, more studies are required to confirm our results.

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