The Effect of *Lactobacillus casei* Derived Extracellular Vesicles on the Expression of Toll-Like Receptor 2 Gene

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Abstract

Research Article

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Introduction: In Gram-positive bacteria, the production of extracellular vesicles has been neglected to date due to the presence of a thick peptidoglycan cell wall. But in recent years more studies have been done on these nanoparticles.
Materials & Methods: The purpose of this study is to show that *Lactobacillus casei* has the ability to produce extracellular vesicles. Since extracellular vesicles (EVs) are an important agent to mediate the expression of genes. In this scientific research, we have examined to find out the effect of EVs derived from *Lactobacillus casei* on the expression of *Toll-like receptor 2* gene. The EVs were purified from the conditioned medium of *Lactobacillus casei* using ultracentrifugation and confirmed by scanning electron microscopy (SEM). The Caco2 cells were treated with different concentrations of purified extracellular vesicles.
Results: The electron microscopy showed spherical vesicles that had an average diameter of 200nm. The extracted protein content was 2.4 in the ultracentrifugation method. It was determined that the extracellular vesicles of this bacterium at concentrations of 150 µg/ml had no significant effect on the expression of *Toll-like receptor-2* gene expression in comparison with the control (Sucrose), whereas the expression of this gene in the treatment of EVs at concentrations of 50 and 100 µg/ml decreased.
Conclusion: Our result creates a paradigm for future studies of the functional component from gut microbiota as a new possible dietary supplement instead of probiotic.
Keywords: *Toll-like receptor, Lactobacillus casei, Extracellular Vesicles, Gene*

Introduction

Lactobacilli are a genus of Gram-positive bacteria which belonging to the Lactic Acid Bacteria (LAB) group[1]. They are known as healthy bacteria and therefore are usually used for production of foods all over the world. Generally, probiotics include a wide range of microbial organisms like Bifidobacterium spp. and Lactobacillus spp. which have health-promoting properties[2]. Among the species of Lactobacillus, is one of the best-documented, considered to be a probiotic with industrial applications. It has beneficial effects on human health such as significant improvements in immunity[3-5], allergies [3], cholesterol levels [3] and some studies have shown that *L. casei* improves the pattern of gut microbiota[4] and the symptoms of arthritis[5] and type II diabetes[6] and it has anti-cancer properties [7, 8]. In addition, Lactobacilli have immunomodulatory effects[9]. Castillo *et al.*

(2011) showed that administration of *Lactobacillus casei* modulates cytokine production and *TLR* expression in the experimental mice[10]. *TLR2* expression is critical for the recognition of many diverse microbial structures. Extracellular vesicles (EVs) are endogenous nano-particles which are secreted by eukaryotic and prokaryotes cells. Recent studies indicate that both Gram-positive and Gram-negative bacteria secrete EVs under different environmental conditions. EVs are released by Gram-negative bacteria known as outer membrane vesicles (OMVs) [11]. In Gram-positive bacteria, although there is no outer membrane and a thick peptidoglycan cell wall surrounding bacterial cells, EVs is also secreted as the vesicles of the membrane (MV)[12, 13].

EVs consist of lipid bilayers that are in the range of 20–500 nm in diameter. These structures generally consist of variety components, such as toxins, lipoproteins, nucleic acids, and communication signals, and are really important in microbial pathogenesis and physiology [12.]

Today, we know that EVs of eukaryotic cells is an essential intercellular delivery system that transmits different kinds of signals between different cells. In fact, EVs affect immune responses and have immunomodulatory effects for the purpose of therapeutic goals. EVs play an important role in various types of vital processes, such as inhibition, promotion, regulation of gene expression, differentiation and proliferation in cells that receive them. Among the EVs, those with micro-RNA molecules play a greater role in gene expression throughout the binding to mRNA molecules and manipulate the protein translation process. Interestingly, the EVs will be consistent with the receptor cell membrane and molecules such as RNA molecules are released as metabolites in the body fluids. This feature makes the EVs an effective and attractive structure [14]. Based on the information given above, here we tried to find out the effect of EVs secreted by *L. casei* as a probiotic on the expression of TLR2 in Caco2 cell line.

Material and Methods

Bacterial strain and culture condition

L. casei (ATCC 393 strains) were prepared from the Iranian Biological Resource Center (Tehran, Iran). The bacteria were grown on De Man, Rogosa and Sharpe agar (MRS) (Sigma-Aldrich, USA). In order to obtain more biomasses, the bacteria were inoculated in MRS broth at 37°C for 24 hours.

EVs extraction

The ultracentrifuge differential method was performed for EV extraction[15]. By using multiple centrifugation and ethylenediamine-tetraacetic acid (EDTA)-sodium deoxycholate buffers bacterial EVs were extracted. For EV purification, sequential centrifugation was performed at 20000 g for 40 minutes, and then, ultracentrifugation was conducted for 2 hours at 40000 g. the amount of protein was measured by a Nanodrop 2000 spectrophotometer (Thermo-Scientific, USA) at the wavelength of 280 nm.

SEM analysis

Size and morphological characteristics of the purified EVs were recognized by scanning electron microscopy (SEM). The samples were coated with 5 nm of gold (HIT 4160 02).

Cell culture

The provided Caco2 cell lines from Iranian Biological Resource Center (Tehran, Iran) were inoculated in DMEM high glucose (Inoclon,

Tehran, Iran) culture medium. The medium was enriched by supplemental compositions, including fetal bovine serum (FBS) (10%) (Inoclon, Tehran, Iran), 100 mg/ml streptomycin and 100 U/ml penicillin (Inoclon, Tehran, Iran).

The cell growth was carried out in the presence of 5% CO_2 atmosphere at 37 °C.

Gene expression analysis

For determining the effect of EVs on the expression of *TLR-2* gene and a housekeeping gene of GAPDH, Real-time PCR was performed. Therefore, the cells were treated with 50, 100, 150 mg/ml EV within 48 hrs. Then, the total mRNA molecules were extracted using the RNX-Plus kit (Cinnagen, Tehran, Iran). The harvested mRNA molecules were then treated with isopropanol. An amount of 20 ml diethylpyrocarbonate (depc) was added into the mRNAs to obtain a suspension. A Nanodrop 2000 spectrophotometer (Thermo-Scientific, USA) was recruited to measure the absorbance of related mRNA molecules.

In another step, a kit (Bioneer, Takara, Japan) was recruited for cDNA synthesis from the harvested mRNA molecules. Oligo dT, Random Hexamers, M-MLV reverse transcriptase were necessary for converting mRNAs into cDNAs during the RT reactions. On the other hand, the specific primers for *TLR*-2 gene were used to amplify *TLR*-2 gene and housekeeping gene of GAPDH exploiting Real-time PCR. The GAPDH gene was expressed and assumed as internal control. Real-time PCR was performed. The relative level of gene expression was assessed by comparing *TLR*-2 gene with the housekeeping gene of GAPDH.

Statistical analysis

In order to analyze the significant differences between outcomes of test and control groups, statistical analysis was performed using student's T-test and Microsoft Excel version 2013 software and the p-value lesser than 0.05 was propounded as a significant discrepancy.

Results

SEM features

We found that *L. casei* produces EVs with specific physicochemical properties. The electron microscopy showed spherical vesicles that had an average diameter between 20–500 nm.

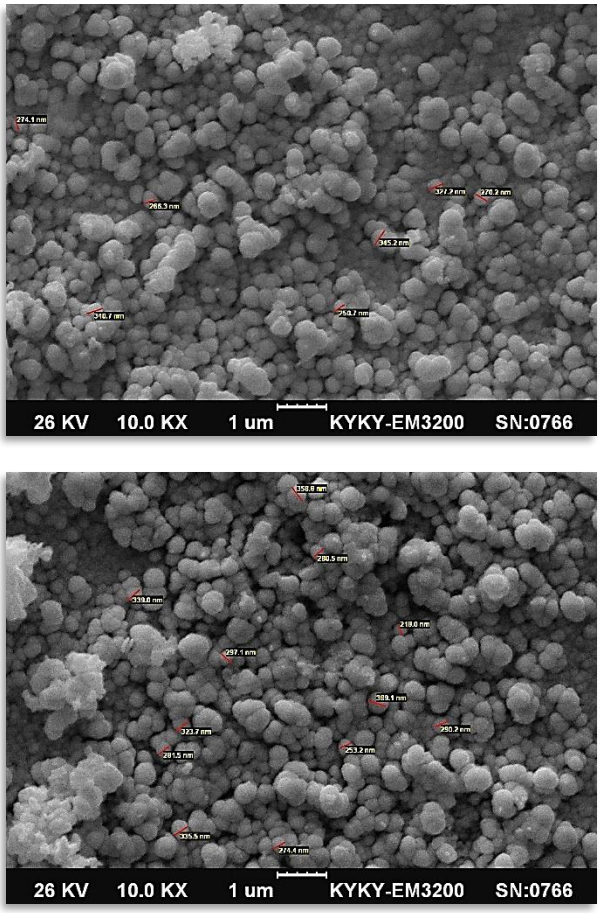

Fig. 1: SEM micrographs of the EV preparations by the ultra-centrifugation technique

TLR-2 gene expression

To survey the effect of probiotic EVs on the expression of *TLR*-2 three different concentrations of EVs separated from *L. casei*

were chosen. Three concentrations of EVs were 50, 100, and 150 were treated with the cell line. First, for all the samples for GAPDH and *TLR*-2 genes, Real-time PCR performed. The reaction was carried out in a final volume of 20 µl using the forward and reverse primers of the genes mentioned in the LightCycler® 96 SW 1.1 device. Then, the difference in expression of these genes in each trait was calculated in comparison to the expression of the genetic expression GAPDH gene (housekeeping gene). As illustrated in Fig. 2 By analyzing the results, it was determined that the EVs of this bacterium at concentrations of 150 µg/ml had no significant effect on the expression of *TLR*2 gene expression in comparison with the control (Sucrose), whereas the expression of this gene in treatment of the EVs at concentrations of 50 and 100 µg/ml decreased.

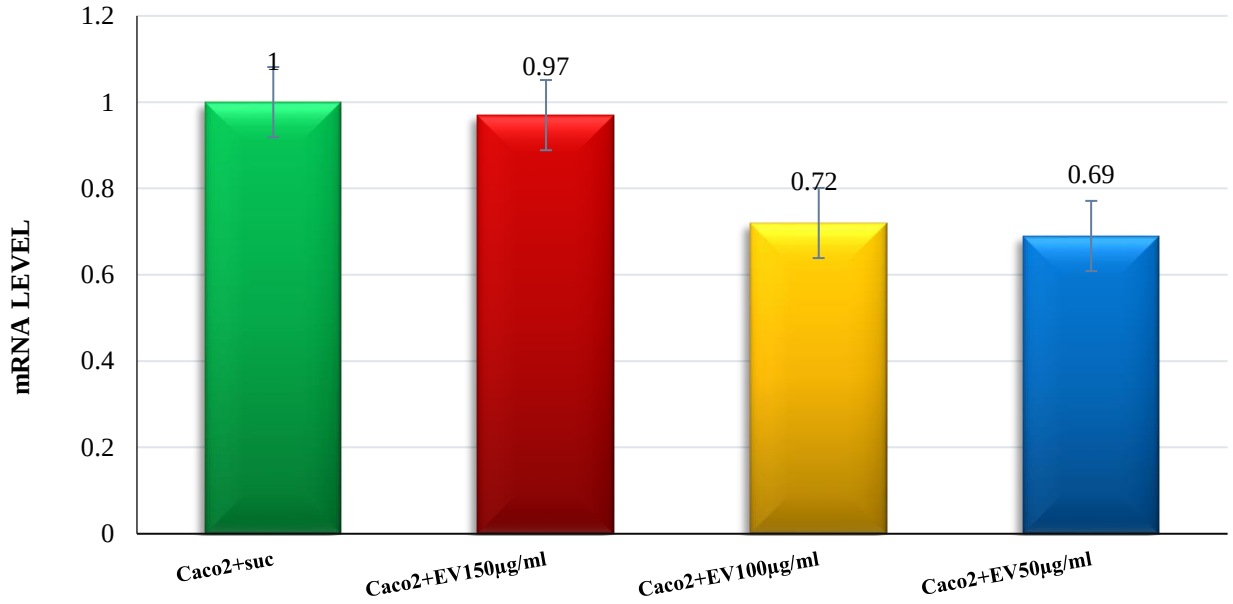

Fig. 2: *TLR*-2 gene expression after treatment of Caco2 cells with different concentrations of EVs derived from *Lactobacillus casei* indicates the p-value lesser than 0.05.

Discussion

Human and microorganisms have significant correlation during evolution. The human body has been colonized with a complex, diverse, and dynamic microbial community called Microbiota. The gastrointestinal tract is the largest and most diverse microbial community in the world. The digestive microbiota provides a variety of benefits to humans, including the production of important vitamins and metabolites, such as SCFAs, regulate the body's signaling pathways, modulation of the immune system [16].

Lactobacillus is a probiotic bacterium and one of the gastrointestinal bacteria. Because of the importance of probiotics in basic studies and commercial uses, we decided to study EVs from this group of bacteria. A large number of publications devoted to probiotics have shown that this group of bacteria has a significant positive effect on human health. At the same time, production of functional foods based on probiotics has increased. Furthermore, scientists have proven that probiotics and their

components can have positive effects on health; for example, components from Lactobacillus spp. can down-regulate the proinflammatory signaling pathways [17]. Therefore, determining the advantages of molecules derived from probiotics can be used to develop a new generation of functional compounds.

One of the molecular components of bacteria is EV. The formation and delivery of different ingredients, via EVs, are common among Gram-negative bacteria. But there are few types of research on EV production in Gram-positive bacteria. It is obvious that EVs serve as carriers for many different cargoes. According to a large-scale study, the morphological features of EVs from Gram-positive bacteria are in common with OMVs obtained from Gram-negative bacteria. OMVs and EVs consist of lipid bilayers that are in the range of 20–500 nm in diameter. The role of these vesicles in both groups of bacteria is to protect molecules like proteins, nucleic acids, lipoproteins, communication signals and to release these molecules to the target cells [18.]

It is repeatedly demonstrated that some of the probiotic bacteria, particularly lactobacillus species such as *L. casei*, *L. reuteri*, and *L. rhamnosus* affect the cellular mechanisms and signaling pathways. Probiotic bacteria and their bioactive components have direct interaction with epithelial cells in the GI tract [10]. These bioactive particles are able to transfer to other organs and it can have positive effects on the GI tract. EVs are physiologically active nano-particles and show different biological effect without the existence of whole bacteria [19]. Although Gram-positive bacteria have thick cell walls but, secretion of extracellular vesicles occurs in these bacteria. On the other hand, the mechanism of generation and the release of EVs are poorly realized [12]. As shown in our study, *L. casei* can produce vesicles.

The TLR2 expression is critical for the recognition of many diverse microbial structures. *TLR2* responds to lipoproteins and peptidoglycan from Gram-positive bacteria (20). Since the peptidoglycan structure is present in Gram-positive bacteria, it can, therefore, affect the *TLR*-2 gene. The purpose of this study was to determine whether EVs can affect the expression of *TLR*-2 gene.

Herein, we observed that EVs derived from *L. casei* can change the expression of a number of genes such as *TLR*-2. In this study, we isolated the EVs as one of the bioactive components from conditioned medium of *L. casei* and found their effects on gene expression.

Castillo *et al.* showed that oral administration of a probiotic Lactobacillus modulates cytokine production and *TLR* expression [10]. It was shown that extracellular vesicles of Lactobacillus can modulate immune function altering *TLR2* activity and phagocytosis[21]. It has been demonstrated that these probiotic-derived EVs are able to alter gene expression. Our findings revealed that the EVs of this bacterium at concentrations of 150 µg/ml had no significant effect on the expression of *TLR2* gene expression in comparison with the control (Sucrose), whereas the expression of this gene in the treatment of the EVs at concentrations of 50 and 100 µg/ml decreased.

Our result creates a paradigm for future studies of the functional component from gut microbiota as a new possible dietary supplement instead of probiotic. But further studies are required, in order to understand the precise details concerning the mechanisms and properties of these vesicles.

Conflict of interest

The authors declare no conflicts of interest.

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