The effect of saturated and unsaturated fatty acids on the production of outer membrane vesicles from *Bacteroides fragilis* and *Bacteroides thetaiotaomicron*

Zahra Sadat Mirjafari Tafti¹, Arfa Moshiri¹23, Fateme Etehad Marvasti¹, Samira Tarashi¹4, Seyyed Fatemeh Sadati Khalili¹, Atiyyeh Motahhary¹, Abolfazl Fateh¹4, Farzam Vaziri¹4, Sara Ahmadi Badi⁹, Seyed Davar Siadat¹4

¹Microbiology Research Center, Pasteur Institute of Iran, Tehran, Iran
²Gastrointestinal Cancer Department, Gastroenterology and Liver Diseases Research Center, Research Institute for Gastroenterology and Liver Diseases, Shahid Beheshti University of Medical Sciences, Tehran, Iran
³Experimental Therapy Unit, Laboratory of Oncology, G. Gaslini Children’s Hospital, Genoa, Italy
⁴Mycobacteriology and Pulmonary Research Department, Pasteur Institute of Iran, Tehran, Iran

**ABSTRACT**

**Aim:** The aim of present study is to investigate the effect of fatty acids on the outer membrane vesicles (OMVs) produced by *Bacteroides* spp.

**Background:** *Bacteroides* spp. is the important member of Gut microbiota that employ OMVs production for interact with host. Besides, dietary fatty acids could influence on determination of gut microbiota composition and immune response. In this regard, we evaluated the effect of fatty acids on the growth and OMVs production of *Bacteroides fragilis* and *Bacteroides thetaiotaomicron*.

**Methods:** *B. fragilis* and *B. thetaiotaomicron* were grown on BHI broth with and without palmitic and palmitoleic acids as saturated and unsaturated fatty acids, respectively. OMVs were extracted using multiple centrifugation and tris-ethylene diamine tetra acetic acid (EDTA)-Sodium deoxy cholate buffers. Physicochemical properties of OMVs were detected by electron microscopy (SEM), Bradford Coomassie brilliant blue assay and SDS-PAGE. Data were analyzed with One-way ANOVA using SPSS.

**Results:** The growths of both *Bacteroides* were significantly increased by palmitic acid. Nevertheless, palmitoleic acid had no significant effect on them. Palmitic acid significantly decreased and increased the production of *B. fragilis* OMVs at low and high concentration, respectively. However, the production of *B. thetaiotaomicron* OMVs was not significantly affected by palmitic acid. Although palmitoleic acid had a significant decreasing effect on the production of *B. fragilis* OMVs, it significantly increased the production of *B. thetaiotaomicron* OMVs at low concentration.

**Conclusion:** In conclusion we reported that palmitic acid had a stimulatory effect on the growth of *B. fragilis* and *B. thetaiotaomicron* and had a dose dependent effect on the production of *B. fragilis* OMVs. Also producing of *B. thetaiotaomicron* OMVs was affected by palmitoleic acid in a dose dependent manner.

**Keywords:** *Bacteroides fragilis*, *Bacteroides thetaiotaomicron*, Outer membrane vesicle, Palmitic acid, Palmitoleic acid.

*Please cite as: Mirjafari Tafti ZS, Moshiri A, Etehad Marvasti F, Tarashi S, Sadati Khalili SF, Motahhary A, et al. The effect of saturated and unsaturated fatty acids on the production of outer membrane vesicles from* *Bacteroides fragilis* and *Bacteroides thetaiotaomicron*. Gastroenterol Hepatol Bed Bench 2019;12(2):155-162.

**Introduction**

A diverse and complex microbial community colonizes human host, especially in the gastrointestinal tract (GIT) that refers as the “gut microbiota”. This microbial community has co-evolved with host to create a mutually beneficial relationship (1). Gut microbiota constantly interacts with the host and has determining roles in its functions due to having various physiological effects including maintenance of intestinal epithelium...
The effect of fatty acids on the production of Bacteroides spp. OMVs

The gut microbiota is affected by genetic background and environmental factors including toxins, drugs, diet and pathogens (6). It has been found that unfavorable altered gut microbiota composition that is called “dysbiosis”, disrupts gut microbiota-host interactions which increases prone to diseases. Dysbiosis is correlated with intestinal and extra-intestinal disorders like irritable bowel syndrome (IBS), coeliac disease, inflammatory bowel disease (IBD), asthma, allergy, cardiovascular disease (CVD), metabolic syndrome and obesity (6-10).

It has been suggested that diet is one of the most potent determinants of gut microbiota composition. It affects gut microbiota-host interactions through alternation of microbial metabolites, components and host metabolism. For example, dietary fatty acids have influential role on metabolic syndrome such as obesity, type 2 diabetes, hyper tension and rheumatoid arthritis (11-14). Dietary saturated fatty acids (SFAs) such as palmitic acid are able to activate inflammatory responses and promote metabolic syndrome. Conversely, polyunsaturated fatty acids (PUSAs) could suppress inflammatory responses (15).

The gut microbiota has significant roles on human physiology and metabolism. It produces essential metabolites from diet such as short-chain fatty acids (SCFAs) which act as source of energy for colonocytes, signaling molecules and epigenetic factor for modulation host functions (16, 17). Also immune system and host’s defenses are associated with composition and function of microbiota (16-18). One way to interact between gut microbiota and the host is to produce outer membrane vesicles (OMVs). OMVs are nano sized particles, 20 to 250 nm, which produced by gram negative bacteria (19, 20). The component of OMVs includes proteins, hydrolytic enzymes, toxin or lipopolysaccharide (LPS), DNA and RNA (21, 22). Recent studies demonstrated that Bacteroides spp. derived OMVs have significant role in maintenance of homeostasis and regulation of immune system. For example, OMVs containing capsular polysaccharide A (PSA) from B. fragilis modulate the immune system and tolerance to intestinal commensal bacteria. B. thetaetaotamicron OMVs modulate intestinal macrophages in a sulphatase dependent manner. Also hydrolytic enzymes that are packaged into Bacteroides spp. derived OMVs, contribute to maintenance of homeostasis (19-27). As mention above, the gut microbiota composition is affected by many factor especially diet, dietary SFAs and PUSAs. According to importance of dietary fatty acids and Bacteroides spp. and their OMVs, we evaluated the effect of palmitic and palmitoleic acids, as saturated and unsaturated fatty acids, on the growth and the production of OMVs from B. fragilis and B. thetaetaotamicron.

Methods

Bacterial strains and growth condition
B. fragilis ATCC 23745 and B. thetaetaotamicron ATCC 10774 were grown in brain heart infusion (BHI) broth supplemented with hemin (5µg/ml) and menadione (1µg/ml). These media incubated at 37 °C under anaerobic conditions provided 80% N2, 10% CO2 and 10% H2.

Fatty acids preparation and bacterial inoculation
Palmitic acid (PO500) and palmitoleic acid SIGMA-ALDRICH, GERMANY (P9417) solutions were prepared at 20 mg/ml concentration in absolute ethanol. Fatty acid solutions were sterilized using a 0.22µm-pore-size polyvinylidene difluoride filter (Millipore, Billerica, MA). Palmitic acid and palmitoleic acid solutions were added to BHI broth media at different concentration, 0, 125, 250 and 500 µg/L. B. fragilis and B. thetaetaotamicron were inoculated at 1.5×10⁸ CFU ml⁻¹ to BHI broth enriched with palmitic and palmitoleic acid and incubated under anaerobic conditions for an overnight. Finally, the optical density (OD) was measured by ELISA reader (Epoch Biotech ELX50).

OMVs purification
After an overnight incubation under anaerobic conditions as described previously, OMVs were isolated (28). Briefly, five hundred milliliters of bacterial cultures were centrifuged at 5000g at 4 °C. The cell pellets were washed twice by phosphate buffer solution (PBS). Then cell pellet was re-suspend in sodium chloride 9% solution. The cell suspension was homogenized and concentrated by centrifugation for 1 hour at 6500g at 4 °C. The OMVs were isolated using
Tris-ethylene diamine tetra acetic acid (EDTA)-sodium deoxycholate (Sigma-Aldrich, USA) buffers and centrifugation for 90 minutes at 20000g, 4 °C. The OMVs concentrated were re-suspended in 3% sucrose solution.

**Scanning electron microscopy (SEM)**

To determine morphology and size of OMVs, these vesicles were fixed using 2.5% glutaraldehyde and 2% paraformaldehyde in PBS. Gold coated samples were prepared by sputter coater (KYKY Technology, China) and examined using SEM (KYKY Technology, China).

**Quantitative and qualitative determination of protein**

After the extraction, the protein concentration of OMVs was measured by Bradford protein assay. Determination of OMVs proteins by Bradford Coomassie brilliant blue assay was confirmed by measuring absorbance at 590 nm (29). To determine protein profile electrophoresis of OMV was performed.

![Graph shows the OD values of *B. fragilis* ATCC23745 and *B. taiotaomicron* ATCC 10774 growths in BHI broth treated with palmitic acid. The horizontal and vertical axes show the concentration of Palmitoleic acid and the OD values of bacterial growth, respectively.](image1)

**Figure 1.** Graph shows the OD values of *B. fragilis* ATCC23745 and *B. taiotaomicron* ATCC 10774 growths in BHI broth treated with palmitic acid. The horizontal and vertical axes show the concentration of Palmitoleic acid and the OD values of bacterial growth, respectively.

![Graph shows the OD values of *B. fragilis* ATCC23745 and *B. thetaiotaomicron* ATCC 10774 growths in BHI broth treated with Palmitoleic acid. The horizontal and vertical axes show the concentration of Palmitoleic acid and the OD values of bacterial growth, respectively.](image2)

**Figure 2.** Graph shows the OD values of *B. fragilis* ATCC23745 and *B. thetaiotaomicron* ATCC 10774 growths in BHI broth treated with Palmitoleic acid. The horizontal and vertical axes show the concentration of Palmitoleic acid and the OD values of bacterial growth, respectively.
The effect of fatty acids on the production of Bacteroides spp. OMVs

by slab gel containing 12% SDS-PAGE. Then separated proteins were stained with Coomassie brilliant blue and were de-stained by acid acetic 1%(30).

Statistical Analyses

Data were analyzed with One-Way ANOVA based on LSD test using SPSS version 24.0 (SPSS Inc., Chicago, IL, USA). All results demonstrate as mean±Standard deviation (SD). In all experiments, P<0.05 was considered statistically significant.

Results

The effect of palmitic and palmitoleic acids on B. fragilis and B. thetaiotaomicron growth

To evaluate the effect of palmitic and palmitoleic acid on the growth of the B. fragilis and B. thetaiotaomicron, different concentration of both fatty acids was added to BHI broth. The results showed that palmitic acid significantly increased the growth of B. fragilis (P value < 0.05) and B. thetaiotaomicron (P value < 0.05). Palmitic acid had most stimulatory effect on these bacteria at the high concentration, 500µg/L. Interestingly, the growth of B. fragilis was more stimulated compared with B. thetaiotaomicron by palmitic acid (figure 1). In contrast, palmitoleic acid had no significant stimulatory effect on the growth of the B. fragilis and B. thetaiotaomicron (figure 2).

The effect of palmitic acid and palmitoleic acid on the production of B. fragilis and B. thetaiotaomicron derived-OMVs

To evaluate the effect of palmitic acid and palmitoleic acid on the OMVs production of B. fragilis and B. thetaiotaomicron, these particles were extracted in BHI broth supplemented with different concentrations of fatty acids examined by SDS and Bradford methods that results of the experiments are shown in the figures (figure 3 and 4). Although palmitic acid significantly decreased the OMVs production of B. fragilis at low concentration (P value < 0.01) but significantly increased it at high concentration (P value < 0.002). Based on statistical analyzes; it was showed that palmitic acid effect on the production of OMVs from B. fragilis was dose dependent due to the presence of a significant increasing effect at high concentration (P value < 0.001). The production of B. thetaiotaomicron OMVs was not significantly affected by palmitic acid at both concentrations (figure 3 and 4). On other hand, palmitoleic acid had a decreasing effect on the production of B. fragilis OMVs and the low concentration was statistically significant (P value < 0.03). We reported that the palmitoleic acid had a significant increasing effect on the production of B. thetaiotaomicron OMVs at low concentration (P value < 0.03) (Figure 5 and 6). Also, we identified a dose dependent effect of palmitoleic acid on the B. thetaiotaomicron OMVs production because of the high concentration compared with low concentration had a significant reducing effect (P value < 0.01) in this regard.

Discussion

Bacteroides spp. have significant roles in the gut microbiota-host interactions through various mechanisms including OMVs production. These particles influence the regulation of immune system and homeostasis (31, 32). On the ther hand, diet-derived saturated and unsaturated fatty acids have inflammatory and anti-inflammatory properties, respectively (33, 34). Here, we aimed to evaluate the effects of palmitic and palmitoleic acids (as saturated and unsaturated fatty acids) on the growth and the production of OMVs from B. fragilis and B. thetaiotaomicron.

It has been shown that diet has substantial role on the gut microbiota composition (35). In this regard, some studies focused on the role of high fat diet as an important factor on the pattern of gut microbiota (36, 37). Also, the effect of dietary fatty acids on the intestinal bacterial growth was assessed (38). For example, fatty acids derived from human and cow milk inhibits the growth of Lactobacillus bifidus (39). Furthermore, the growth of Bacteroides ruminicola, one species of rumen bacteria, was decreased in the presence of palmitic acid (40). Some researches indicated that unsaturated long-chain fatty acids (C18) have stimulation effect on the growth of microorganisms in low concentration (41). In present study, palmitic acid acts as a significant stimulant for the growth of both B. fragilis and B. thetaiotaomicron, while palmitoleic acid has no significant effect on the growth of these bacteria. The stimulatory effect of unsaturated fatty acids on the growth of bacteria decreases along with the increase in the length of the fatty acid chain. Also, fatty acids which
have induction effect on the bacterial growth might have opposite effect at other concentration. Therefore, fatty acids can be considered as a stimulator or inhibitor agent on the bacterial growth depending on their structure and concentration (41).
The effect of fatty acids on the production of Bacteroides spp. OMVs

B. fragilis and B. thetaiotaomicron are two important members of gut microbiota due to having important potentials such as regulating of immune responses and homeostasis (42, 43). It has been demonstrated that, B. fragilis and B. thetaiotaomicron employ OMVs production to exert their effects in gut microbiota-host interactions (44, 45). Considering the important roles of Bacteroides spp. derived OMVs, the effects of palmitic acid and palmitoleic acid which are components of diet, on the production of these vesicles were investigated for the first time. Our results illustrate that palmitic acid has a significant stimulatory effect on the production of B. fragilis derived OMVs at the high concentration but it significantly decreased production of B. fragilis OMVs at low concentration. However, palmitic acid had no significant effect on the production of B.
thetaiotaomicron OMVs. Palmitoleic acid, as an unsaturated fatty acid, significantly decreased the OMVs production from B. fragilis and significantly increased the production of B. thetaiotaomicron OMVs at low concentration. It can be concluded that the effect of palmitic acid on the production of OMVs from B. fragilis and also the effect of palmitoleic acid on the production of OMVs from B. thetaiotaomicron was dose dependent. Therefore, fatty acids can act as a stimulator or inhibitor agent on the production of OMVs depending on their concentration. However, further molecular investigations and animal models are required to illustrate in details the effect of palmitic and palmitoleic acid on the growth of Bacteroides spp. and OMVs production.

Acknowledgment
We thank all the personnel of Mycobacteriology and Pulmonary Research Department, Pasteur Institute of Iran for their assistance in this project. This project was supported by Iran Biotech Fund grant No. 94/10243 and National Institute for Medical Research Development grant No. 942995.

Conflict of interests
The authors declare that they have no conflict of interest.

References
1. Turnbaugh PJ, Ley RE, Hamady M, Fraser-Liggett CM, Knight R, Gordon JI. The human microbiome project. Nat 2007;449:804.
2. Brown EM, Sadarangani M, Finlay BB. The role of the immune system in governing host-microbe interactions in the intestine. Nat Immunol 2013;14:660.
3. Geurts L, Neyrinck AM, Delzenne NM, Knauf C, Cani PD. Gut microbiota controls adipose tissue expansion, gut barrier and glucose metabolism: novel insights into molecular targets and interventions using prebiotics. Benef Microbes 2014;5:3-17.
4. Turnbaugh PJ, Ley RE, Mahowald MA, Magrini V, Mardis ER, Gordon JI. An obesity-associated gut microbiome with increased capacity for energy harvest. Nat 2006;444:1027.
5. Marchesi JR. Prokaryotic and eukaryotic diversity of the human gut. Advanc App Microbiol 2010;43-62.
6. Tanoue T, Umesaki Y, Honda K. Immune responses to gut microbiota-commensals and pathogens. Gut Microbes 2010;1:224-33.
7. Ley RE, Turnbaugh PJ, Klein S, Gordon JI. Microbial ecology: human gut microbes associated with obesity. Nat 2006;444:1022.
8. Ley RE, Bäckhed F, Turnbaugh P, Lozupone CA, Knight RD, Gordon JI. Obesity alters gut microbial ecology. Proc Natl Acad Sci U S A. 2005;102:11070-5.
9. Zhang H, DiBaise JK, Zuccolo A, Kudrna D, Braidotti M, Yu Y, et al. Human gut microbiota in obesity and after gastric bypass. Proc Natl Acad Sci U S A 2009;106:2365-70.
10. Turnbaugh PJ, Hamady M, Yatsunenko T, Cantarel BL, Duncan A, Ley RE, et al. A core gut microbiome in obese and lean twins. Nat 2009;457:80.
11. Lorente-Cebrián S, Costa AG, Navas-Carretero S, Zabala M, Martínez JA, Moreno-Alía MJ. Role of omega-3 fatty acids in obesity, metabolic syndrome, and cardiovascular diseases: a review of the evidence. J Physiol Biochem 2013;69:633-51.
12. Riediger ND, Othman RA, Suh M, Moghadassian MH. A systemic review of the roles of n-3 fatty acids in health and disease. J Am Dietetic Assoc 2009;109:668-79.
13. Claesson MJ, Jeffery IB, Conde S, Power SE, O'connor EM, Cusack S, et al. Gut microbiota composition correlates with diet and health in the elderly. Nat 2012;488:178.
14. Nicholson JK, Holmes E, Kinross J, Burcelin R, Gibson G, Jia W, et al. Host-gut microbiota metabolic interactions. Sci 2012;336:1262-7.
15. Lee JY, Plakidas A, Lee WH, Heikkinen A, Chummunag P, Bray G, et al. Differential modulation of Toll-like receptors by fatty acids preferential inhibition by n-3 polyunsaturated fatty acids. J lipid Res 2003;44:479-86.
16. Bäckhed F, Ley RE, Sonnenburg JL, Peterson DA, Gordon JI. Host-bacterial mutualism in the human intestine. Sci 2005;307:1915-20.
17. Hooper LV, Midtvedt T, Gordon JI. How host-microbial interactions shape the nutrient environment of the mammalian intestine. Ann Rev Nut 2002;22:283-307.
18. Kamada N, Seo SU, Chen GY, Núñez G. Role of the gut microbiota in immunity and inflammatory disease. Nat Rev Immunol 2013;13:321.
19. Shen Y, Torchia MLG, Lawson GW, Karp CL, Ashwell JD, Mazmanian SK. Outer membrane vesicles of a human commensal mediate immune regulation and disease protection. Cell Host Microb 2012;12:509-20.
20. Bonnington KE, Kuehn MJ. Protein selection and export via outer membrane vesicles. Biochim Biophys Acta 2014;1843:1612-9.
21. Schwechheimer C, Kuehn MJ. Outer-membrane vesicles from Gram-negative bacteria: biogenesis and functions. Nat Rev Microbiol 2015;13:605.
The effect of fatty acids on the production of Bacteroides spp. OMVs

22. Unal CM, Schaar V, Riesbeck K. Bacterial outer membrane vesicles in disease and preventive medicine. Semin Immunopathol 2011;33:395-408.

23. MacDonald IA, Kuehn MJ. Offense and defense: microbial membrane vesicles play both ways. Res Microbiol 2012;163:607-18.

24. Lee EY, Choi DS, Kim KP, Gho YS. Proteomics in gram-negative bacterial outer membrane vesicles. Mass Spectrom Rev 2008;27:535-55.

25. Kulp A, Kuehn MJ. Biological functions and biogenesis of secreted bacterial outer membrane vesicles. Annu Rev Microbiol 2010;64:163-84.

26. Kulkarni HM, Jagannadham MV. Biogenesis and multifaceted roles of outer membrane vesicles from Gram-negative bacteria. Microbiol 2014;160:2109-21.

27. Ellis TN, Kuehn MJ. Virulence and immunomodulatory roles of bacterial outer membrane vesicles. Microbiol Mol Biol Rev 2010;74:81-94.

28. Daliri SS, Kafil HS, Aghazadeh M, Fateh A, Yousefi M, Siadat SD. Extraction and Biological Evaluation of the Membrane Vesicles of Mycobacterium tuberculosis (CRBIP7.11) as Adjuvant and Vaccine Candidate. Jundishapur J Microbiol 2017;10.

29. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976;72:248-54.

30. Claassen I, Meylis J, Van Der Ley P, Peeters C, Brons H, Robert J, et al. Production, characterization and control of a Neisseria meningitidis hexavalent class I outer membrane protein containing vesicle vaccine. Vaccine 1996;14:1001-8.

31. Fábrega MJ, Aguilera L, Giménez R, Varela E, Alexandra Cañas M, Antolin M, et al. Activation of immune and defense responses in the intestinal mucosa by outer membrane vesicles of commensal and probiotic Escherichia coli strains. Front Microbiol 2016;7:705.

32. Lin L, Zhang J. Role of intestinal microbiota and metabolites on gut homeostasis and human diseases. BMC Immunol 2017;18:2.

33. Nicholls SJ, Lundman P, Harmer JA, Cutri B, Griffiths KA, Rye K, et al. Consumption of saturated fat impairs the anti-inflammatory properties of high-density lipoproteins and endothelial function. J Am College Cardio 2006;48:715-20.

34. Calder PC, Grimble RF. Polyunsaturated fatty acids, inflammation and immunity. Europe J Clin Nutr 2002;56:S14.

35. Maslowski KM, Mackay CR. Diet, gut microbiota and immune responses. Nat Immunol 2010;12:5.

36. Den Besten G, Van Eunen K, Groen AK, Venema K, Reijngoud DJ, Bakker BM. The role of short-chain fatty acids in the interplay between diet, gut microbiota, and host energy metabolism. J lipid Res 2013;54:2325-40.

37. Hildebrand MA, Hoffmann C, Sherrill–Mix SA, Keibbaugh SA, Hamady M, Chen YY, et al. High-fat diet determines the composition of the murine gut microbiome independently of obesity. Gastroenterol 2009;137:1716-24.

38. Kau AL, Ahern PP, Griffin NW, Goodman AL, Gordon JL. Human nutrition, the gut microbiome and the immune system. Nat 2011;474:327.

39. Tomarelli RM, Norris R, Rose C, Gyorgy P. The effect of fatty acids on the growth of strains of Lactobacillus bifidus. J Biol Chem 1950;187:197-204.

40. Maczulak A, Dehority B, Palmquist D. Effects of long-chain fatty acids on growth of rumen bacteria. App Environ Microbiol 1981;42:856-62.

41. Nieman C. Influence of trace amounts of fatty acids on the growth of microorganisms. Bacteriol Rev 1954;18:147.

42. Round JL, Mazmanian SK. The gut microbiota shapes intestinal immune responses during health and disease. Nat Rev Immunol 2009;9:313.

43. Ivanov II, Litman DR. Modulation of immune homeostasis by commensal bacteria. Curr Opin Microbiol 2011;14:106-14.

44. Ahmadi Badi S, Moshiri A, Fateh A, Rahimi Jamnani F, Sarshar M, Vaziri F, et al. Microbiota-Derived extracellular vesicles as new systemic regulators. Front Microbiol 2017;8:1610.

45. Ahmadi Badi S, Khatami SH, Irani SH, Siadat SD. Induction Effects of Bacteroides fragilis Derived Outer Membrane Vesicles on Toll Like Receptor 2, Toll Like Receptor 4 Genes Expression and Cytokines Concentration in Human Intestinal Epithelial Cells. Cell J 2019;21:57-61.

46. Ahmadi Badi S, Khatami SH, Irani SH, Siadat SD. Induction Effects of Bacteroides fragilis Derived Outer Membrane Vesicles on Toll Like Receptor 2, Toll Like Receptor 4 Genes Expression and Cytokines Concentration in Human Intestinal Epithelial Cells. Cell J 2019;21:57-61.