JOURNAL OF MICROBIOLOGY, EPIDEMIOLOGY AND IMMUNOBIOLOGY. 2021; 98(3)
DOI: https://doi.org/10.36233/0372-9311-86
ORIGINAL RESEARCHES

Original article
https://doi.org/10.36233/0372-9311-86

Mechanism of persistence of indigenous bifidobacteria under the impact of acetate in the human colon biotope

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Abstract

Aim. To determine the role of the acetate in the persistence of indigenous bifidobacteria in the colon biotope through the lysozyme resistance in model conditions of the acetylation–deacetylation of peptidoglycan.

Materials and methods. The study was performed on 16 strains of the two indigenous bifidobacteria species: *Bifidobacterium bifidum и Bifidobacterium longum subsp. longum*. Bifidobacteria was cultivated in the 0.6% O_2 and 9% CO_2 atmosphere at the temperature 37ºC in CO_2 incubator for 48 hours. The production of the acetate by the bifidobacteria was determined by gas chromatography. The effect of the acetate on the lysozyme resistance of non-indigenous gram-positive bacteria was determined on the *Listeria monocytogenes* ICIS-280 model strain by the cultivation in LB-Lennox broth with ammonium acetate added in the concentration range matching the concentrations produced by the studied bifidobacteria, in lysozyme serial dilutions at final concentrations 5 μg/ml to 40 μg/ml within 24 hours.

Results. It was found that the acetate release of *Bifidobacterium longum subsp. longum* was on average two times higher that of *Bifidobacterium bifidum* (27.0 and 14.7 mmol/liter, respectively) and was quite consistent with the concentrations of acetic acid determined in the intestinal contents (up to 50 mmol/liter). Cultivation of bifidobacteria in a medium with lysozyme, ammonium acetate and their combination did not have a significant impact on their growth parameters at the maximum used concentrations of these substances. In the test strain, the addition of ammonium acetate in the range created by bifidobacteria caused a decrease in the minimum inhibitory concentration of lysozyme by more than two times — from 40 μg/ml to less than 20 μg/ml. In the control medium without lysozyme, no inhibition of the growth of the indicator culture was observed up to the maximum concentrations of ammonium acetate.

Conclusion. The mechanism of persistence (survival) of indigenous bifidobacteria in the human intestinal biotope has been identified, which is associated with the production of acetic acid at a level that selectively suppresses lysozyme resistance of non-indigenous gram-positive microbiota viareversible deacetylation of peptidoglycan. This allows indigenous bifidobacteria to maintain a stable dominant position in the biotope.

Keywords: *bifidobacteria, bacterial persistence, peptidoglycan, lysozyme resistance, acetate, intestinal microbiota, microbiocenosis*

Ethics approval. The study was conducted with the informed consent of the patients. The research protocol was approved by the Local Bioethics Committee of the Institute for Cellular and Intracellular Symbiosis (Approval No. 1, 21.04.2020).

Funding source. This study was not supported by any external sources of funding.

Conflict of interest. The authors declare no apparent or potential conflicts of interest related to the publication of this article.

For citation: Bukharin O.V., Andryuschenko S.V., Perunova N.B., Ivanova E.V. Mechanism of persistence of indigenous bifidobacteria under the impact of acetate in the human colon biotope. *Journal of microbiology, epidemiology and immunobiology = Zhurnal mikrobiologii, èpidemiologii i immunobiologii.* 2021;98(3):276–282.
DOI: https://doi.org/10.36233/0372-9311-86

Научная статья
https://doi.org/10.36233/0372-9311-86

Механизм персистенции индигенных бифидобактерий под действием ацетата в кишечном биотопе человека

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Аннотация

Цель исследования — определить роль ацетата в персистенции индигенных бифидобактерий в кишечном биотопе через лизоцимрезистентность в модельных условиях ацетилирования–деацетилирования пептидогликана.

Материалы и методы. Исследовано по 16 штаммов двух видов индигенных бифидобактерий: *Bifidobacterium bifidum* и *Bifidobacterium longum subsp. longum*. Бифидобактерии культивировали в CO_2-инкубаторе при содержании O_2 0,6%, CO_2 9% и температуре 37ºС в течение 48 ч. Продукцию уксусной кислоты (ацетата) бифидобактериями выявляли методом газовой хроматографии. Влияние ацетата на устойчивость неиндигенных грамположительных бактерий к лизоциму определяли на модели штамма *Listeria monoytogenes* ICIS-280 путём культивирования в бульоне LB-Lennox с добавлением ацетата аммония в диапазоне концентраций, продуцируемых исследуемыми бифидобактериями, в серии разведений лизоцима в конечных концентрациях от 5 до 40 мкг/мл в течение 24 ч.

Результаты. Установлено, что у *Bifidobacterium longum subsp. longum* выделение ацетата в среднем было в 2 раза выше, чем у *Bifidobacterium bifidum* (14,7 и 27 ммоль/л соответственно), и вполне соответствовало концентрациям уксусной кислоты, определённым в кишечном содержимом (до 50 ммоль/л). Культивирование бифидобактерий в среде с лизоцимом, ацетатом аммония и их сочетанием не оказало существенного влияния на их показатели роста при максимальных использованных концентрациях данных веществ. У тест-штамма добавление ацетата аммония в диапазоне, создаваемом бифидобактериями, вызывало снижение минимальной подавляющей концентрации лизоцима более чем в 2 раза — от 40 до менее 20 мкг/мл. В контрольной среде без лизоцима не отмечено ингибирования роста индикаторной культуры вплоть до максимальных концентраций ацетата аммония.

Заключение. Выявлен механизм персистенции (выживания) индигенных бифидобактерий в кишечном биотопе человека путём продукции ацетата, избирательно подавляющего лизоцимрезистентность неиндигенных грамположительных бактерий, за счёт обратимости деацетилирования пептидогликана, что позволяет индигенным бифидобактериям сохранять стабильное доминантное положение в биотопе.

Ключевые слова: *бифидобактерии, персистенция, пептидогликан, лизоцимрезистентность, ацетат, кишечная микробиота, микробиоценоз*

Этическое утверждение. Исследование проводилось при информированном согласии пациентов. Протокол исследования одобрен локальным комитетом по биоэтике ИКВС УрО РАН (протокол № 1 от 21.04.2020).

Источник финансирования. Авторы заявляют об отсутствии внешнего финансирования при проведении исследования.

Конфликт интересов. Авторы декларируют отсутствие явных и потенциальных конфликтов интересов, связанных с публикацией настоящей статьи.

Для цитирования: Бухарин О.В., Андрющенко С.В., Перунова Н.Б., Иванова Е.В. Механизм персистенции индигенных бифидобактерий под действием ацетата в кишечном биотопе человека. *Журнал микробиологии, эпидемиологии и иммунобиологии.* 2021;98(3):276–282.
DOI: https://doi.org/10.36233/0372-9311-86

Among the biotopes of the human body, the large intestine is distinguished by an exceptionally high number and variety of microorganisms. In this regard, data on their ecological structure, mechanisms and conditions of its formation turn out to be important for medical microbiology, although they are still characterized by significant incompleteness and inconsistency. In this biotope, attention is drawn to bacteria of the genus Bifidobacterium, which, as it is well known, are important for the normal functioning of the human digestive tract [1] and discrimination in the body of pathogens [2].

The main final metabolite of indigenous bifidobacteria is acetic acid [3]. It was previously demonstrated that it was the production of acetic acid by indigenous bifidobacteria that prevented lethal infection with enteropathogenic *E. coli* [4]. At the same time, the mechanism of the protective effect of bifidobacteria metabolites against pathogenic or conditionally pathogenic gram-positive microbiota has not yet been studied.

The formation of symbiotic interactions between bacteria and the host is based on the phenomenon of persistence (long-term survival of microorganisms in

DOI: https://doi.org/10.36233/0372-9311-86

the host body), where the key biotarget of immunity is the cell wall — its peptidoglycan (PG) [5], and the lysozyme enzyme is a universal method for destroying peptidoglycan. In the case of gram-positive bacteria, the peptidoglycan is open to the host immune system (not protected). The only mechanism described to date to ensure the resistance of gram-positive bacteria to the enzymatic action of lysozyme is the modification of peptidoglycan [6].

There are two most common ways of such modification:

1) O-acetylation of PG occurring in both gram-positive and some gram-negative bacteria [7], providing tolerance to PG lysozyme of *Bifidobacterium longum* [8];

2) de-N-acetylation of PG, detected only in gram-positive bacteria.

We paid attention to the fact that peptidoglycan de-N-acetylases were detected mainly in microorganisms that could not be attributed to the resident human microbiota, in particular: bacilli, clostridia [9], and listeria. It was this mechanism that provided the main contribution to their lysozyme resistance [10]. Both mechanisms involved acetic acid but in a multidirectional quality. In the case of O-acetylation, its residue (acetate) is added to the residue of N-acetylmuramic acid of PG. In the case of de-N-acetylation, it is cleaved from the N-acetylglucosamine residue of PG. In the latter case, the enzyme de-N-acetylase of peptidoglycan, belonging to the 4th family of carbohydrate esterases, like chitin deacetylase, is also reversible [11]. Therefore, excess acetate in the medium can shift the equilibrium of the deacetylation reaction towards intact peptidoglycan. In other words: free acetate can block the resistance to lysozyme of gram-positive bacteria foreign to the human intestine.

The objective of the study was to investigate the role of acetate in the persistence of indigenous bifidobacteria in the intestinal biotope through lysozyme resistance in modelling of peptidoglycan acetylation-deacetylation.

Materials and methods

Research objects

At the first stage of the research, 16 strains of the two most common species of indigenous bifidobacteria were selected: *Bifidobacterium bifidum* and *Bifidobacterium longum subsp. longum*. They were previously isolated from conventionally healthy individuals (one strain from each subject) in the laboratory of biomonitoring and molecular genetic studies during examination for intestinal dysbiosis and identified by cultural and morphological characteristics, biochemical properties using ANAEROtest-23 kits (LaChema, Czech Republic) and protein profile by MALDI-TOF mass spectrometer "Microflex" ("Bruker Daltonics", Germany).

Determination of acetate production by bifidobacteria

To establish the range of acetate concentrations created by indigenous intestinal bifidobacteria, the level of its production *in vitro* was determined by gas chromatography. Bifidobacteria were introduced in an amount of 10^8 CFU — in a volume of 0.1 ml of bacterial suspension in 0.9% NaCl solution in 4.9 ml broth Shaedler (HiMedia, India) and cultured in Binder CO_2 incubator (Germany) at oxygen content of 6%, carbon dioxide content of 9% and a temperature of 37°C for 48 hours. The samples were centrifuged at 13,600 g for 15 min, and 50 µl of 98% sulfuric acid (Panreac, Germany) was added to 500 µl of the supernatant. The extraction of volatile fatty acids from the samples was conducted in 750 µL of isobutyl alcohol (Sigma-Aldrich, USA); the process was repeated twice. Acetate detection was conducted in a GC-2010 Plus gas-liquid chromatograph (Shimadzu, Japan) equipped with a flame ionization detector with an HP-FFAP capillary column (Agilent Technologies, USA), 0.32 mm in diameter, 50 meters long. The evaporator temperature was 240°C; temperature program for the capillary column: 0 min — 70°C, 10 min — 160 °C, 5 min — 180°C and 25 min — 240°C; detector temperature — 260°C. Helium was used as a carrier gas, the carrier gas velocity was 21 cm/sec. Concentrations were calculated by peak areas using the GCsolution software (Shimadzu, Japan).

Determination of the effect of acetate on the level of bacterial lysozyme resistance

Bacterial resistance to lysozyme was determined by cultivation in LB-Lennox broth with the addition of a series of dilutions of chicken egg white lysozyme (Sigma, Canada) at final concentrations from 5 to 40 µg/ml in triplicate.

Among gram-positive non-indigenous bacteria, we chose *Listeria monocytogenes* test culture (strain ICIS-280) as an indicator, since for this species the role of peptidoglycan de-N-acetylase in providing resistance to C-lysozyme has been most fully described [10]. Model incubation conditions of the test strain were set up: in LB-Lennox broth at 37 °C for 24 hours in a series of lysozyme dilutions identical to those indicated for bifidobacteria.

To determine the effect of acetate on the resistance of the studied bacteria to lysozyme, we used a neutral salt of acetic acid — ammonium acetate added to the culture medium in the range of molar concentrations of acetate produced by the studied strains of bifidobacteria. The presence of the growth of the test strain was determined photometrically on a Multiscan Ascent microplate photometer (Finland) at a wavelength of 450 nm and the optical density (OD) of the culture medium exceeded that of the control sterile broth by more than 0.01 units. Statistical analysis was performed using the Mann-Whitney test [12] in MINITAB 14.1 software.

Results

Characterization of acetate production of indigenous bifidobacteria

In bacteria of *B. bifidum* species, the release of acetic acid varied in the range from 6.4 mmol/L to 38.5 mmol/L, while in strains belonging to *B. longum subsp. longum*, the release of acetate was, on average, two times higher ($p < 0.01$), varied in the range from 11.2 mmol/L to 50.6 mmol/L (**Table 1**) and was quite consistent with the concentrations of acetic acid determined in the intestinal content [13].

At the same time, the maximum levels of acetate production in *B. bifidum* were 10 mmol/L higher than the average level determined for *B. longum* subsp. longum, but homologues of any of the seven genes of the two systems of active transport of fructose, mannose and ribose, described as key determinants of a high level of acetate production by bifidobacteria [4], were not identified in the previously sequenced genome of the strain with a record level of acetate production (*B. bifidum* ICIS-310; NCBI BioProject accession: PRJNA345151), in none of the other sequenced *B. bifidum* strains deposited in the NCBI (US National Institutes of Health) databases [14]. At the same time, in the genome of the strain with an average level of acetate production — 28.3 mmol/L (B. *longum subsp. longum* ICIS-505; NCBI BioProject accession: PRJNA379379) — all these determinants were present in the same form as in the genomes of type strains of *B. longum* MC-42 and *B. longum* NCC2705. These facts show that the high intensity of acetic acid production by bifidobacteria can be provided not only in the presence of the genetic determinants of membrane transport of carbohydrates indicated in the work [4].

Effect of acetate on lysozyme resistance of the indicator strain

To assess the effect of acetate on lysozyme resistance of bacteria deacetylating their peptidoglycan, the MIC of lysozyme in *Listeria monocytogenes* test strain was determined in media supplemented with ammonium acetate and lysozyme in three replicates. An increase in the optical density of the control strain was used as a criterion for the growth of the culture. In media with a combined addition of lysozyme and ammonium acetate in the range produced by indigenous bifidobacteria, a decrease in the MIC of lysozyme for the indicator strain more than two times was noted — from 40 to less than 20 μg/ml (**Fig. 1**). Listeria resistance to lysozyme in the control medium without the addition of ammonium acetate did not exceed 50 μg/ml (positive control). In the control medium without lysozyme (negative control), there was no inhibition of the growth of the indicator culture up to the maximum concentrations of added ammonium acetate (50 mmol/L).

Thus, the result obtained is consistent with the described effect of the reversibility of the carbohydrate deacetylation reaction [11]. An excess of acetate in the medium shifts the reaction of bacterial PG deacetylation towards the initial (native) state, which is sensitive to lysozyme. It was discovered that acetate produced by bifidobacteria indigenous to the biotope, under conditions of physiological acidity values, is also capable of achieving the described effect on a model system - a test culture of bacteria de-N-acetylating their peptidoglycan.

Characteristics of the acetate production of the studied bifidobacteria strains ($M \pm m$)

B. bifidum		*B. longum*	
strains	acetate, mM	strains	acetate, mM
ICIS-216	6,4 ± 1,4	ICIS-281	11,2 ± 1,7
ICIS-503	7 ± 2,3	ICIS-744	12 ± 1,8
ICIS-629	7,3 ± 1,7	ICIS-1112	14,3 ± 1,8
ICIS-040	7,4 ± 1,8	ICIS-347	18 ± 1,6
ICIS-745	8,4 ± 1,2	ICIS-953	20 ± 1,55
ICIS-600	9,2 ± 2,3	ICIS-609	20,5 ± 2
ICIS-059	9,4 ± 1,6	ICIS-1113	22 ± 2,25
ICIS-752	10 ± 2,8	ICIS-749	23 ± 2,2
ICIS-460	11 ± 2,3	***ICIS-505***	***28,3 ± 2,8***
ICIS-105	13 ± 2,6	ICIS-950	28,6 ± 4,3
ICIS-949	13 ± 2,4	ICIS-984	29,1 ± 2,7
ICIS-349	15 ± 2,5	ICIS-1122	35 ± 3,5
ICIS-1074	21 ± 3,5	ICIS-889	38 ± 2,9
ICIS-750	22,4 ± 1,7	ICIS-049	39,1 ± 3,4
ICIS-691	36,4 ± 2,7	ICIS-210	42,5 ± 3
ICIS-310	***38,5 ± 1,7***	ICIS-627	50,6 ± 3,1
Average	14,7 ± 2,5	Average	27 ± 2,9

Note. The strains with available complete genome sequences are emphazied.

Characteristics of lysozyme resistance of indigenous bifidobacteria

Cultivation of the studied strains of indigenous bifidobacteria (ICIS-216, ICIS-310, ICIS-505) in a me-

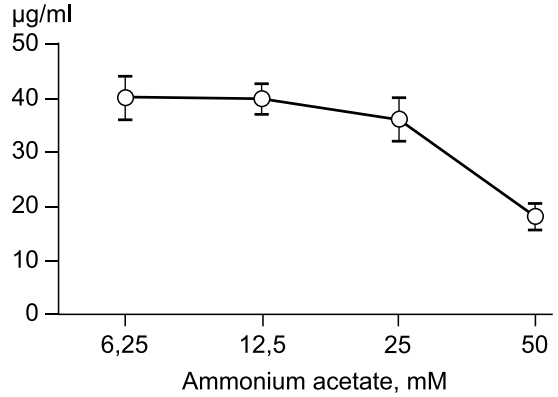

Fig. 1. Effect of the ammonium acetate on lysozyme minimum inhibitory concentration of the model strain *Listeria monocytogenes* ICIS-280.

DOI: https://doi.org/10.36233/0372-9311-86

dium with lysozyme and ammonium acetate did not show a significant difference in their growth parameters ($p > 0.05$) in the control broth from those in the medium with the addition of the maximum used concentrations of these substances (40 μg/ml lysozyme and 50 mmol/l ammonium acetate): 1.07 ± 0.05 and 1.01 ± 0.03 units. OD in strain ICIS-216; 0.72 ± 0.07 and 0.8 ± 0.05 units. OD in strain ICIS-310; 0.63 ± 0.2 and 0.63 ± 0.6 units. OD in strain ICIS-505, respectively. The data obtained indicate that resistance to lysozyme in indigenous bifidobacteria is not associated with the presence of acetate in the medium, which is consistent with the mechanism of ensuring their lysozyme resistance during O-acetylation of peptidoglycan — an excess of acetate in the medium does not suppress the acetylation reaction.

Discussion

The data demonstrates that the production of acetate by indigenous bifidobacteria provide not only the already known resistance of the biotope to some gram-negative pathogens but also discriminates non-indigenous gram-positive bacteria. At the same time, the required level of this production is provided not only by strains carrying the known determinants of the systems of active transport of fructose, mannose and ribose, but also observed in some strains of the species *B. bifidum*, widespread in the human intestine, which does not possess them.

The performed analysis allowed us to design a diagram of persistence mechanism of indigenous bifidobacteria (**Fig. 2**):

1. Once in the digestive tract, the peptidoglycan of gram-positive bacteria is in direct contact with the host lysozyme. Microorganisms with native, unmodified peptidoglycan are eliminated [15] — the "primary filter" of microsymbionts.

2. In the gut, bacteria that have passed lysozyme, acid and proteolytic filters, in addition to host lysozyme, meet significant amounts of acetate secreted by indigenous bifidobacteria and their metabolic associates (bacteroids) [3]. The presence of acetate in the medium shifts the equilibrium of the reversible deacetylation reaction [11] catalyzed by de-N-acetylase of the non-indigenous microbiota towards unmodified bacterial peptidoglycan, which leaves it sensitive to the action of lysozyme [10], returning to the elimination of its carriers — the "secondary filter" of microsymbionts.

In addition, as it demonstrated in our work, this effect was observed at physiological concentrations of acetate formed in the biotope.

3. O-acetylation of bacterial peptidoglycan retains its efficiency in the presence of acetate and ensures the persistence of microorganisms implementing it, including bifidobacteria [8], which, thus, form the pool of indigenous microbiota of a given biotope.

In this regard, the low level of antilysozyme activity, determined in bifidobacteria [2], turns out to be natural, enhancing their selective advantage in the gut. Thus, being released in the process of catabolism by bifidobacteria, acetate contributes to a change in the functioning of the mechanism of persistence of associate bacteria, performing, in fact, the function of a regulator of lysozyme resistance in the biotope.

Conclusion

The mechanism of persistence of indigenous bifidoflora was revealed through an alternative modification of peptidoglycan of microorganisms., Acetate emerged as a key regulator that determines the dominant role of bifidobacteria in the gut biotope of the host, providing both the primary discrimination of non-indigenous intestinal associates through blocking the de-N-acetylation of their peptidoglycans and the pres-

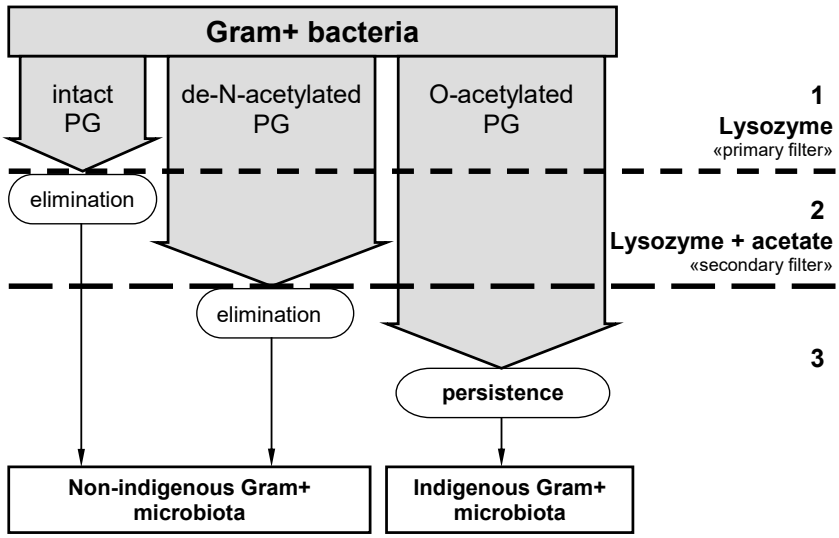

Fig. 2. Selection of gram-positive bacteria in the gut by the mechanism of their lysozyme resistance.

ervation of indigenous grampositive microbiota with O-acetylation of peptidoglycan. By performing the role of a biochemical regulator in the biocenosis it can enhance the effectiveness of measures to correct and prevent dysbiotic conditions.

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Author contribution. All authors made a substantial contribution to the conception of the work, acquisition, analysis, interpretation of data for the work, drafting and revising the work, final approval of the version to be published.

The article was submitted 10.09.2020;
accepted for publication 20.03.2021;
published 01.06.2021

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Участие авторов. Все авторы внесли существенный вклад в проведение поисково-аналитической работы и подготовку статьи, прочли и одобрили финальную версию до публикации.

Статья поступила в редакцию 10.09.2020;
принята к публикации 20.03.2021;
опубликована 01.06.2021