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CORRELATION OF BLOOD PROTEIN LEVELS AND VAGINAL MICROBIOTA ABUNDANCE IN PARTICIPANTS OF A 5-DAY DRY IMMERSION EXPERIMENT

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Introduction. Recently, women have increasingly participated both in ground-based model experiments simulating specific spaceflight factors and in actual space missions. During spaceflight, the human body is exposed to various factors that may pose health risks. When modeling various spaceflight factors, the human microbiota undergoes adverse changes: the proportion of opportunistic microorganisms increases, while the abundance of protective microorganisms decreases. Pronounced changes also occur in the blood protein profile. The relevance of this study is due to the close relationship between the blood protein profile and the composition of the vaginal microbiota; disruptions in the microbiota can affect protein metabolism and biochemical parameters of the body, provoking the development of pathologies.

Objective. To identify the relationship between the blood proteome and the bacteria of the vaginal biotope in participants of a 5-day dry immersion experiment.

Materials and methods. Seven female volunteers of reproductive age (mean age 29 ± 1 years) participated in the dry immersion study. The participants did not receive antibacterial treatment during the experiment. The subjects were divided into two groups: the L + P group ($n = 3$) consumed a lactoferrin-based drink (200 mg/day) together with intravaginal glucose-based placebo suppositories; the P + P group ($n = 4$) consumed a placebo preparation (200 mg glucose) and intravaginal maltodextrin placebo suppositories (200 mg/day). Vaginal and endocervical swab samples were collected twice: before the start of the experiment and 5–7 days after its completion, with smear collection synchronized to days 19–22 of the menstrual cycle. For each participant, vaginal smear microscopy was performed, and microorganisms were identified to species level using MALDI-TOF MS analysis. Blood samples for proteomic analysis were examined by liquid chromatography and mass spectrometry. Statistical analysis was performed using Statistica 12.0. The STRING.DB platform was used to identify clusters among the proteins extracted from factor analysis.

Results. Proteomic analysis of dried blood spot extracts allowed identification of 818 distinct proteins. Using factor analysis before the start of dry immersion, seven factors were extracted (according to the Kaiser–Meyer–Olkin criterion, only data with KMO ≥ 0.7 , indicating acceptable, good, or excellent suitability for factor analysis, were included). Analysis of the extracted factors showed that before dry immersion, the association between blood proteins and bacteria was determined by the following processes: ubiquitination (37.9% of variance), immune responses (12.3% of variance), and hormonal status (13.7% of variance). After completion of the experiment, the key processes in the protein–bacterium relationship became ubiquitination (37.1% of variance in the P + P group), immune mechanisms (43.88% of variance in the L + P group), and estradiol synthesis (56.12% and 33.5% of variance in the L + P and P + P groups, respectively). The following microorganisms showed the greatest sensitivity to proteins associated with estradiol: *Lactobacillus* spp., *Corynebacterium* spp., *Staphylococcus* spp., and *Cutibacterium* spp.

Conclusions. Before and after dry immersion, the interaction between blood proteins and bacteria was determined by ubiquitination and immune processes. After the experiment, the relationship within the protein–bacterium system became stronger, and the microorganisms most sensitive to proteins involved in estradiol processing were representatives of *Lactobacillus* spp., *Corynebacterium* spp., *Staphylococcus* spp., and *Cutibacterium* spp. This suggests the possibility of strengthening the colonization resistance of the vaginal microbiota through targeted modulation of specific biological processes in the body.

Keywords: dry immersion; vaginal microbiota; proteomics; proteins; ubiquitination; prolactin; estradiol

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Compliance with ethical principles: the study was conducted in accordance with the principles of biomedical ethics, approved by the Bioethics Commission of the IBMP RAS (minutes No. 645 dated 07.09.2023), and fully complies with the principles of the Declaration of Helsinki. All study participants voluntarily signed informed consent after being informed about the potential risks, benefits, and the nature of the upcoming study.

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ВЗАИМОСВЯЗЬ УРОВНЕЙ БЕЛКОВ КРОВИ И ЧИСЛЕННОСТИ МИКРООРГАНИЗМОВ ВАГИНАЛЬНОГО БИОТОПА У УЧАСТНИЦ 5-СУТОЧНОЙ «СУХОЙ» ИММЕРСИИД.В. Комиссарова[✉], Д.Н. Каширина, Л.Х. Пастушкова, И.М. Ларина, В.К. Ильин

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Введение. В последнее время женщины все чаще участвуют как в наземных модельных экспериментах, имитирующих отдельные факторы космического полета, так и в реальных космических миссиях. Во время космических полетов организм человека подвергается воздействию разнообразных факторов, способных нести угрозу здоровью. Микрофлора человека при моделировании ряда факторов космического полета претерпевает негативные изменения: увеличивается доля условно-патогенной составляющей и снижается численность протективных микроорганизмов. Также происходят выраженные изменения в белковом профиле крови. Актуальность исследования обусловлена тесной взаимосвязью белкового профиля крови и состава вагинальной микробиоты: ее нарушения могут влиять на белковый обмен и биохимические показатели организма, провоцируя развитие патологий.

Цель. Выявление взаимосвязи между протеомом крови и бактериями вагинального биотопа у участниц эксперимента с 5-суточной «сухой» иммерсией.

Материалы и методы. В исследовании с «сухой» иммерсией принимали участие 7 женщин-добровольцев репродуктивного возраста (средний возраст 29 ± 1 год), которые на протяжении эксперимента не использовали антибактериальные препараты. Участницы были разделены на две группы: группа «Л + П» ($n = 3$) применяла напиток на основе лактоферрина (200 мг/сут) совместно с интравагинальными плацебо-свечами на основе глюкозы; группа «П + П» ($n = 4$) использовала плацебо-препарат (200 мг глюкозы) и интравагинальные плацебо-свечи (200 мг/сут мальтодекстрима). Образцы отделяемого влагалища и цервикального канала отбирали дважды: до начала эксперимента и через 5-7 дней после его завершения с синхронизацией мазков на 19–22-й дни менструального цикла. Каждой участнице выполнили микроскопию вагинального отделяемого и видовую идентификацию микроорганизмов методом MALDI-TOF MS-анализа. Исследование проб крови для проведения протеомного анализа проводили методами жидкостной хроматографии и масс-спектрометрии. Статистическая обработка данных выполнена с помощью программы Statistica 12.0. Для выявления кластеров среди белков, выделенных в результате факторного анализа, использовали платформу STRING.DB.

Результаты. Протеомный анализ экстрактов сухих пятен крови позволил идентифицировать 818 различных белков. В результате факторного анализа до начала СИ выделено 7 факторов (согласно критерию Кайзера – Мейера – Олкина (КМО) включались данные с $\text{КМО} \geq 0,7$, т.е. соответствующие приемлемой, высокой и безусловной адекватности данных для факторного анализа). При анализе полученных факторов было выявлено, что до начала СИ взаимосвязь между белками крови и бактериями определялась следующими процессами: убиквитинированием (37,9% дисперсии), иммунными реакциями (12,3% дисперсии) и гормональным фоном (13,7% дисперсии). После завершения эксперимента ключевую роль в системе «белок–бактерия» стали играть процессы убиквитинирования (37,1% дисперсии в группе «П + П»), иммунные механизмы (43,88% в группе «Л + П») и синтез эстрадиола (56,12 и 33,5% дисперсии в группах «Л + П» и «П + П» соответственно). Наибольшую чувствительность к белкам, ассоциированным с эстрадиолом, продемонстрировали следующие микроорганизмы: *Lactobacillus* spp., *Corynebacterium* spp., *Staphylococcus* spp. и *Cutibacterium* spp.

Выводы. До и после «сухой» иммерсии взаимодействие белков крови и бактерий определялось процессами убиквитинирования и иммунитетом; при этом после эксперимента связь в системе «белок–бактерия» усилилась, а наиболее чувствительными к белкам, вовлеченным в процессинг эстрадиола, оказались представители *Lactobacillus* spp., *Corynebacterium* spp., *Staphylococcus* spp. и *Cutibacterium* spp., что позволяет предполагать возможность укрепления колонизационной резистентности вагинальной микрофлоры через точечное воздействие на ряд биологических процессов в организме.

Ключевые слова: «сухая» иммерсия; микрофлора влагалища; протеомика; белки; убиквитинирование; пролактин; эстрадиол

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Соответствие принципам этики: исследование проведено в соответствии с принципами биомедицинской этики, одобрено биоэтической комиссией ГНЦ РФ — ИМБП РАН (протокол № 645 от 07.09.2023) и полностью соответствует принципам Хельсинкской декларации. Все участницы исследования добровольно подписали информированное согласие после объяснения им потенциальных рисков, преимуществ и характера предстоящего исследования.

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INTRODUCTION

During spaceflight, the human body is exposed to various factors that may pose a threat to health. The human body is a complex system of interconnected elements. In addition to its own cells, a significantly larger number of diverse microbial cells coexist in symbiosis with the body, forming biotopes of the skin, mucous membranes, and intestines. All these components actively interact with each other, ensuring the normal functioning of the host [1].

Proteins play a key functional role in the body: they perform a wide range of tasks, from substance transport and mechanical functions to the regulation of processes. Under normal conditions, the protein composition of biological fluids is dynamic, with only a small fraction of proteins being involved in regulatory molecular pathways (e.g., peptide hormones). However, deviations in the levels of non-regulatory proteins can also signal the development of pathologies, such as infections, autoimmune disorders, or metabolic disturbances. Moreover, proteins serve as a link between organs and systems, coordinating their functions, which is clearly evident in interorgan metabolic cycles [3].

The microbiota of the vaginal mucosa in women is essential for maintaining health: it protects against infections and supports local immunity. Disruption of the microbial balance in this area can lead to various diseases, including bacterial vaginosis and candidiasis [4]. Notably, a close relationship exists between blood proteins and the vaginal microbiota; specifically, changes in the composition of the microbiota can provoke an increase in the concentration of certain proteins in the blood [5]. This may affect the functioning of the immune system and the overall condition of the body. Therefore, investigating the associations between the blood protein profile and the composition of the vaginal microbiota is important for understanding the mechanisms of health maintenance and disease prevention. In particular, it is relevant to determine which shifts in the microbiota could potentially disrupt protein metabolism or influence biochemical parameters of the body.

Under spaceflight conditions, the body encounters multiple factors that can adversely affect health and exert a direct or indirect impact on the microbiome. Ground-based analog studies, such as isolation and dry immersion experiments, have shown that the vaginal microbiota undergoes adverse changes under conditions simulating specific aspects of spaceflight; in particular, an increase in the proportion of facultative anaerobes and a decrease in the abundance of protective *Lactobacillus* spp. have been observed [6, 7].

The aim of the study was to identify the relationship between the blood proteome and bacteria of the vaginal biotope in participants of a 5-day dry immersion experiment.

MATERIALS AND METHODS

Experiment design

Seven female volunteers of reproductive age (mean age 29 ± 1 years), who did not take antibacterial drugs during the experiment, participated in the dry immersion study. Simulation of spaceflight conditions was conducted using the dry immersion model. Dry immersion is an experimental model for simulating the effects of microgravity on Earth. The subject is placed in an immersion bath filled with water at a comfortable temperature. The body does not come into direct contact with the liquid, as it is isolated by a special waterproof, highly elastic film that conforms to the body contours and creates a feeling of floating without direct water contact. Dry immersion reproduces key factors of spaceflight: redistribution of body fluids (increased central blood volume and decreased peripheral blood volume), reduced mechanical loading on the musculoskeletal system, hypokinesia (limited physical activity), and partial sensory deprivation due to reduced tactile and proprioceptive stimuli [8].

The participants were divided into two groups based on the combination of local and oral preparations used for the correction of the vaginal and endocervical microbiocenosis:

- Placebo + Placebo group (P + P) ($n = 4$): Women used an intravaginal placebo preparation (capsules containing 200 mg glucose, once daily) during their 5-day stay in the immersion bath. Additionally, they received an oral placebo (200 mg/day maltodextrin) for 30 days, starting from the first day of immersion.
- Lactoferrin + Placebo group (L + P) ($n = 3$): Participants used an intravaginal placebo preparation (glucose-based) and simultaneously received oral lactoferrin (200 mg/day).

Vaginal and endocervical discharge samples were collected twice: before the start of the experiment and 5–7 days after its completion. Sample collection was performed in all participants on days 19–22 of the menstrual cycle (luteal phase).

Collection of vaginal and endocervical discharge samples, cultivation, and identification of microorganisms

For each participant, vaginal smear microscopy with Gram staining and a culture-based examination were performed according to the medical technology “Integral assessment of the vaginal microbiota status. Diagnosis of opportunistic vaginitis” [9]. Material was collected using a sterile Dacron swab from the posterior vaginal fornix; after cleaning the cervix with a sterile cotton swab, a sample was taken from the endocervical canal. Samples were placed into tubes containing Amies transport medium (Medical Wire, UK). Vaginal and endocervical discharge samples were inoculated onto selective and non-selective solid agar culture media:

- For isolation of facultative anaerobic microorganisms: Columbia agar, Brilliance chromogenic medium (Oxoid, UK), mannitol salt agar (Himedia, India), enterococcal agar, Endo agar, and Sabouraud agar (Federal Scientific Center for Preventive Medicine and Public Health Protection, Obolensk, Russia).
- Lactobacilli were cultured on Lactobacagar medium (Federal Scientific Center for Preventive Medicine and Public Health Protection, Obolensk, Russia).
- Strict anaerobes were grown on pre-reduced Schaedler agar (Oxoid, UK) with appropriate supplements in an anaerobic chamber (Whitley DG 250 Anaerobic Workstation, UK) under an atmosphere of a three-component gas mixture (N_2 — 80%, CO_2 — 10%, H_2 — 10%).

Cultures were incubated in a CO_2 incubator (Jouan, France). Species identification of the microorganisms was performed by MALDI-TOF MS analysis using an Autoflex III time-of-flight mass spectrometer with MALDI BioTyper v3.0 software (Bruker Daltonics, Germany).

Microbiological analysis of the vaginal and endocervical microbiota was performed at the Research Center for Obstetrics, Gynecology and Perinatology.

Collection of dried blood spot samples

The timing of capillary blood collection coincided with the time of vaginal and endocervical sampling. The samples were dried at room temperature for 2 h and then stored at $-20^\circ C$.

Preparation of dried blood spots for liquid chromatography-mass spectrometry analysis

The sample preparation included the following steps:

1. Protein extraction in a buffer containing 25 mM ammonium bicarbonate, 1% sodium deoxycholate, and 5 mM TCEP (tris(2-carboxyethyl)phosphine hydrochloride, Thermo Scientific).
2. Incubation at $60^\circ C$ with stirring at 1000 rpm in a thermomixer (Eppendorf) for 1 h.
3. Reduction, alkylation, precipitation, and trypsin digestion according to the method described in [10].

Liquid chromatography-mass spectrometry analysis of dried blood spot extracts

The analysis was performed in two stages: separation of tryptic peptide mixtures by liquid chromatography using a Dionex Ultimate3000 nano-LC system (Thermo Fisher Scientific, USA); mass spectrometry analysis using a TimsTOF Pro instrument (Bruker Daltonics, USA) with parallel accumulation–serial fragmentation (PASEF) technology [11].

Statistical analysis was performed using STATISTICA 12.0. Factor analysis was used to assess the relationship between proteins and bacteria, with the critical significance level set at $p \leq 0.05$. Additionally, the STRING.

DB platform was used to identify clusters among the proteins extracted from factor analysis [12, 13]. The Kaiser–Meyer–Olkin criterion was used to determine the suitability of the data for factor analysis. Only data with KMO values ≥ 0.7 , indicating acceptable, good, or excellent adequacy for factor analysis, were included. To assess the significance of each factor, the percentage of variance was used, which reflects the degree of data variability and the contribution of each factor to the description of the overall dataset. Values of 20–30% of variance indicate that the contribution reflects a large-scale systematic pattern in data variability.

RESULTS

Proteomic analysis of dried blood spot extracts from the study participants identified 818 distinct blood proteins. These proteins regulate processes such as ubiquitination, proteasome complex function, lipid transport, metabolic pathways (including carbohydrate metabolism), platelet degranulation; they are also components of chaperone and hemoglobin complexes, and regulate citrullination, secretion of sex hormones (specifically, estradiol and prolactin), immune system function (high-density lipoproteins), cellular response to stress, complement system activation, and complement cascades.

The following bacteria were identified in the vaginal microbiome: *Escherichia coli*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Staphylococcus hominis*, *Enterococcus faecalis*, *Enterococcus avium*, *Streptococcus agalactiae*, *Streptococcus anginosus*, *Corynebacterium glucuronolyticum*, *Gardnerella vaginalis*, *Veillonella dispar*, *Veillonella parvula*, *Veillonella denticariosa*, *Anaerococcus murdochii*, *Fingoldia magna*, *Peptoniphilus harei*, *Prevotella bivia*, *Cutibacterium acnes*, *Bifidobacterium bifidum*, *Lactobacillus crispatus*, *Lactobacillus iners*, *Lactobacillus jensenii*, *Lactobacillus gasseri*, *Limosilactobacillus vaginalis*.

After factor analysis of the correlation matrix using principal component analysis, it was found that a number of proteins correlated with bacteria of the vaginal biotope; the proteins were grouped with the bacteria according to the factor extracted by statistical analysis. For convenience, factor analysis was performed at the baseline period (time point “Before”) for both groups together (P + P and L + P). Separate calculations were performed for each group after the 5-day dry immersion at the “After” time point, because the combinations of preparations used differed between the groups.

During the study, factor analysis at the baseline time point (“Before”) extracted seven factors influencing proteins and bacteria in all subjects across the groups. The greatest contribution to changes in proteins and bacteria grouped by factor analysis was made by the first factor, which reflected the involvement of the ubiquitination/deubiquitination system in cellular metabolism, accounting for 37.9% of the variance. The first factor included 260 proteins (Fig. 1). Using the STRING.DB platform,

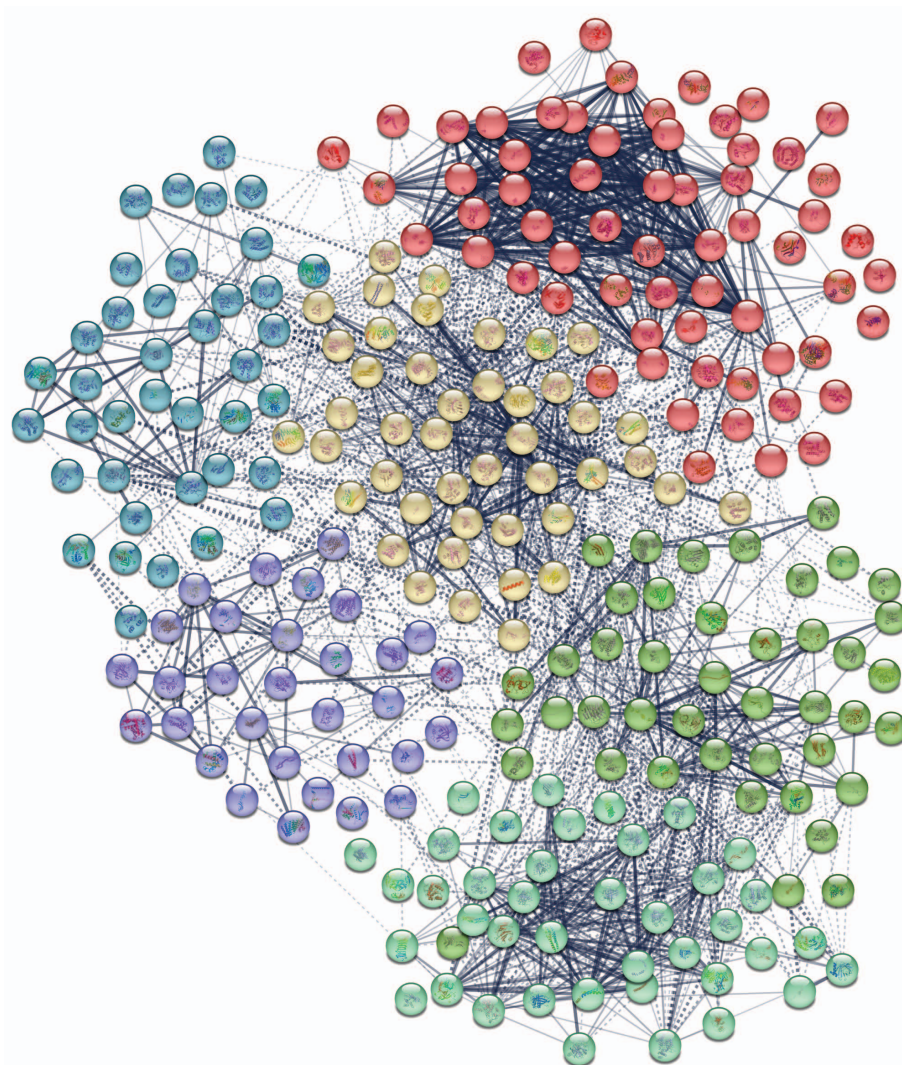


Figure prepared by the authors using their own data

Fig. 1. Protein clusters identified in Factor 1 for all participants before the start of dry immersion. Red — proteins associated with ubiquitination and proteasome complex function; beige — proteins associated with substance transport and metabolic processes; green — proteins associated with platelet degranulation; light blue — proteins associated with the chaperone complex; blue — proteins associated with the hemoglobin complex; purple — proteins associated with citrullination

the resulting list of proteins was clustered, and six main clusters, each containing more than three proteins, were identified.

The deubiquitination process plays an important role in the regulation and activation of several hormones, including thyroid hormones [14, 15]. Molecular chaperones, in turn, influence the maturation of newly synthesized proteins as well as their ubiquitination and deubiquitination [16]. Furthermore, ubiquitination is also associated with specific metabolic processes [17]. Based on this, it can be concluded that Factor 1 reflects the involvement of the ubiquitination/deubiquitination system in cellular metabolism.

Factor 2 contained 43 proteins and 4 bacterial species (*C. glucuronolyticum*, *V. parvula*, *F. magna*, and *B. bifidum*) and accounted for 13.7% of the total variance. Among the identified proteins, 31 are involved in the regulation of reproductive system function. Analysis

of the association between the hormonal status of the participants and the species diversity of the vaginal microbiota revealed a relationship between *B. bifidum* and prolactin: according to regression analysis results, this bacterium influenced prolactin levels. Within Factor 2, a relationship between proteins and prolactin was identified, specifically involving elongin B and peptidylprolyl cis-isomerase. Elongin B likely suppresses JAK/STAT signaling by inhibiting JAK2 kinase. JAK2 plays a key role in signal transduction, interacting with type I receptors, including those for growth hormone, prolactin, leptin, and others [18]. Peptidylprolyl cis-isomerase has been shown to suppress prolactin-induced activation of the Rac gene system [19].

Establishing direct relationships between bacteria and prolactin presents certain difficulties. Nevertheless, there are data indicating that some species of *Corynebacterium* provoke inflammatory processes in women with

hyperprolactinemia and granulomatous mastitis [20]. Furthermore, a hypothesis has previously been proposed that *Bifidobacterium Lactis* V9 can influence sex hormone synthesis via the gut-brain axis [21]. Thus, the second factor likely reflects the complex interaction between prolactin, blood proteins, and *B. bifidum*.

Factor 3 accounted for 12.3% of the variance and included 27 proteins: 16 were associated with immune system function (specifically, 67 kDa matrix metalloproteinase-9, complement C4-A alpha chain, leukocyte surface antigen CD47, and others); 14 were associated with acetylation processes (vesicle-fusing ATPase, enoyl-CoA hydratase/3,2-trans-enoyl-CoA isomerase, triosephosphate isomerase, and others; some proteins were included in both of the above groups); and a single bacterial species — *S. epidermidis*.

Several studies have shown that certain members of the genus *Staphylococcus* (e.g., *S. aureus*) can provoke epigenetic changes in leukocytes — specifically, influence histone acetylation [22]. Based on these data, it can be hypothesized that Factor 3 reflects the interaction of the opportunistic microorganism *S. epidermidis* with immune system cells.

Factor 4 accounted for 10.3% of the variance and included 23 proteins (LIM and SH3 domain protein 1, methanethiol oxidase, nucleosome assembly protein 1-like 4, etc.) and 3 bacterial species: *V. dispar*, *V. dentocariosa*, and *L. iners*. Analysis of the relationships among the proteins revealed predominantly weak correlations at the level of co-expression. It should be noted that 12 of the 23 proteins were also related to acetylation processes. However, no previous literature has documented any

association between these bacteria (or their closely related species) and intracellular acetylation mechanisms.

Factor 5 included only 12 proteins, and no relationships among them were found, even at the level of co-expression (these included DDI1 homolog 2, dipeptidyl peptidase 3, Src substrate cortactin, and others). Factor 5 accounted for 9.1% of the variance.

Factor 6 included 13 proteins (e.g., transaldolase, serum paraoxonase/lactonase 3, glycogenin-1, and others), most of which are associated with metabolic processes in the liver. This factor accounted for 8.5% of the total variance.

Factor 7 contained 8.2% of the variance and included 15 largely unrelated proteins (cytosolic acyl-CoA thioester hydrolase, XRP2 protein, flotillin-1, phosphoribosylformylglycinamide synthase, and others) and a pool of bacteria: *E. coli*, *S. haemolyticus*, *E. faecalis*, *S. agalactiae*, *S. anginosus*, *C. tuberculostrictum*, *L. gasseri*, *L. vaginalis*, *L. rhamnosus*.

Analysis of the data in the P+P group at the “After” time point identified three factors, each with a different set of microorganisms and proteins. Factor 1 accounted for 37.1% of the total variance, Factor 2 for 33.5%, and Factor 3 for 29.4%.

Factor 1 included 238 proteins and 2 bacterial species: *S. lugdinensis* and *F. magna*. An association between certain proteins and *S. lugdinensis* was also observed in a 3-day immersion study after the end of the experiment [23]. Using DBSCAN clustering on the STRING.DB platform, the resulting protein pool was grouped into six main clusters, each containing more than three proteins (Fig. 2).

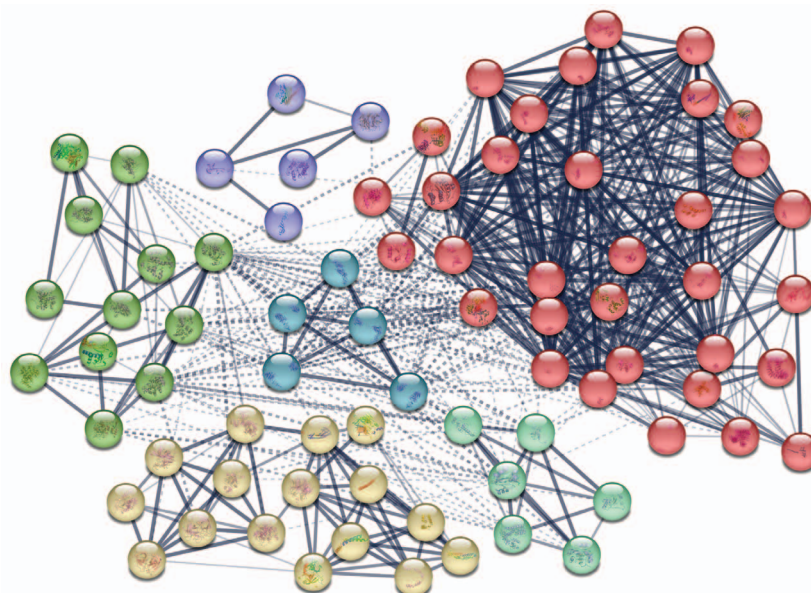


Figure prepared by the authors using their own data

Fig. 2. Protein clusters identified in Factor 1 in the Placebo + Placebo group after 5-day dry immersion. Red — proteins associated with ubiquitination and the proteasome complex; beige — high-density lipoproteins and proteins involved in immune processes; green — proteins associated with cellular response to stress and antioxidant activity; light blue — proteins associated with carbohydrate metabolism; blue — proteins associated with the chaperone complex; purple — proteins associated with COPI-independent retrograde transport from the Golgi apparatus to the endoplasmic reticulum (ER)

After 5-day dry immersion, the first cluster in the Placebo + Placebo group contained the largest number of proteins — 29 proteins, all associated with the organization and function of the proteasome complex, as well as with ubiquitination and deubiquitination systems. Similar findings were also obtained in a 3-day dry immersion experiment [23]. The second cluster included high-density lipoproteins and a group of proteins involved in immune processes (LTF, CLU, PRTNS, and others). The third cluster comprised proteins whose main function is cellular response to stress. The fourth cluster consisted of proteins involved in carbohydrate metabolism. The fifth cluster consisted almost entirely of chaperone complex proteins. The sixth cluster contained proteins involved in retrograde transport to the endoplasmic reticulum.

Based on the analysis of the obtained results, the following key observations can be highlighted:

- Before the immersion exposure, the relationships between proteins and bacteria were predominantly weak. This is supported by the fact that the dominant factor (exerting the greatest influence, with the largest share of variance) before the experiment contained no bacterial components. However, after the experiment, the situation changed: the factor with the maximum variance included not only proteins but also two bacterial species.
- Under the influence of the stressful immersion factor, activation of the ubiquitination and deubiquitination

systems likely occurred. At the same time, the nature of the relationships changed substantially: before the experiment, the interactions within these systems were primarily determined by metabolic processes, whereas after the study, an additional group of immune proteins became part of the protein pool of the ubiquitination system. These include, in particular: proteins involved in neutrophil degranulation; antimicrobial peptides; proteins involved in antigen processing; and other immunologically active components.

Thus, the immersion exposure not only strengthened the links between protein and microbial components but also broadened the functional aspects of the ubiquitination system by incorporating immune mechanisms.

In the P + P group after 5-day dry immersion, Factor 2 included 190 proteins associated with regulation of the complement system, coagulation cascades, proteins expressed in the female reproductive system and gonads, proteins involved in estradiol metabolism, carbohydrate metabolism, the proteasome complex, as well as proteins involved in cellular antioxidant processes, cellular detoxification, and ubiquitination. The factor also included six bacterial species: *S. haemolyticus*, *C. glucuronolyticum*, *C. tuberculostearicum*, *K. schroeteri*, *C. acnes*, and *L. gasseri*. Of the 190 proteins, 166 were involved in endocrine system functioning. Clustering of this protein pool yielded several groups based on their functions. The most significant clusters, containing four or more proteins, are discussed below (Fig. 3).

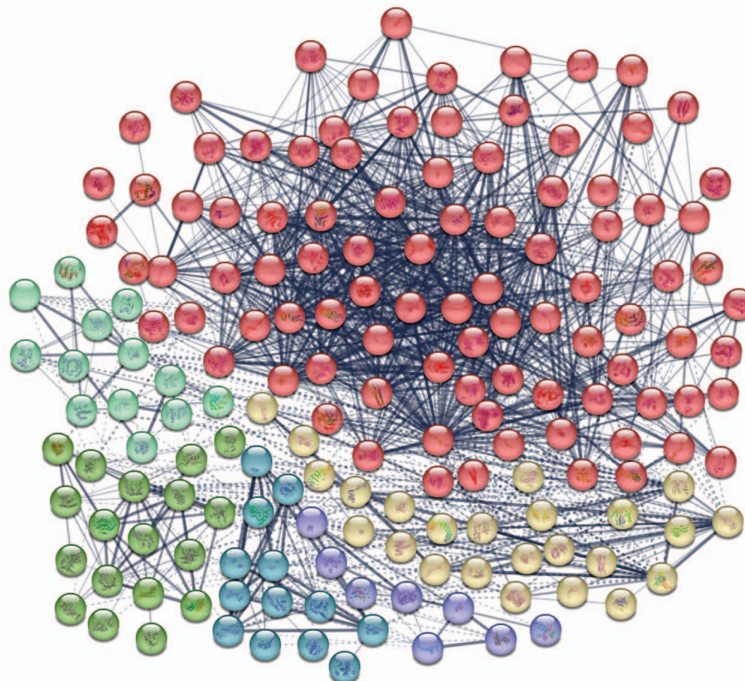


Figure prepared by the authors using their own data

Fig. 3. Protein clusters identified in Factor 2 in the Placebo + Placebo group after 5-day dry immersion. Red — proteins associated with the complement system and coagulation cascades; beige — cytosolic proteins involved in cellular response to stress, expressed in the female reproductive system and endocrine glands, as well as proteins involved in estradiol metabolism; green — proteins associated with carbohydrate metabolism; light blue — proteins associated with the proteasome complex; blue — proteins involved in cellular antioxidant processes and cellular detoxification; purple — proteins associated with ubiquitination

The first group of proteins was associated with hemostasis, specifically the function of the complement system and coagulation cascades. The second group was associated with the female reproductive system, endocrine glands, and estradiol. It is known that estradiol influences certain blood parameters, such as the coagulation index [24]. Estradiol can also provoke hypercoagulation and more active thrombin generation [25]. The third protein cluster was associated with carbohydrate metabolism. The fourth protein cluster was associated with the organization and function of the proteasome complex. It is known that the proteasome system also participates in the regulation of estrogen receptor activity [26]. The fifth protein pool was associated with antioxidant processes and cellular detoxification. The sixth protein pool comprised proteins associated with ubiquitination.

Studies investigating the relationship between hormones and vaginal microbiota have noted the effect of *S. haemolyticus* on estradiol levels after the end of immersion. Before the experiment, this microorganism correlated negatively with estradiol levels, whereas after immersion it correlated positively [27]. Several publications have shown that the abundance of certain bacteria is indeed related to sex hormone levels: for example, the viability of *S. aureus* decreased with increasing estradiol levels [28]. There are also studies on the influence of some *Corynebacterium* species on estradiol levels, although these were performed in model organisms [29–30]. Regarding the relationship between lactobacilli and steroid hormones, it has been reported that estradiol inhibited the growth of *L. paracasei* and activated the growth of *M. luteus* [31]. Another study noted a positive effect of estradiol on *L. crispatus*, which resulted in

enhanced adhesion of bacterial cells to the vaginal epithelium [32].

Considering the obtained results and data from previous studies, it can be concluded that the hormonal and protein profiles of blood are closely related and influence the vaginal microbiota. Conversely, there appears to be a reciprocal influence of bacteria on hormones and the proteomic composition of blood. It is likely that bacterial metabolites exert a broad influence on various processes in the body at the molecular level, such as ubiquitination, the complement system, hemostasis, and chaperone proteins, which in turn alter the synthesis and function of certain hormones. Under conditions of stress after the end of the immersion exposure, the relationships between proteins, hormones, and bacteria become more significant. This suggests that by influencing one component of this chain, it may be possible to regulate processes occurring in the body, including mitigating the negative effects of dry immersion.

Factor 3 included 200 proteins and 4 bacterial species: *S. epidermidis*, *S. hominis*, *L. iners*, and *L. jensenii*. When clustering the obtained proteins, the only cluster that was functionally homogeneous was the one associated with the proteasome complex. The remaining proteins were mainly grouped according to their expression site and cellular localization. This factor partially overlaps with Factor 2 in terms of the protein list, but includes a different set of bacterial species and, consequently, different mechanisms of protein-bacteria interaction, primarily due to the distinct metabolism of this group of bacteria.

In the L + P group of participants after the end of immersion (at the “After” time point), only two factors were extracted, each contributing approximately equally to the variance. Factor 1 accounted for 56.12% of the total variance and included 388 proteins and 5 bacterial species: *S. hominis*, *F. magna*, *C. acnes*, *L. crispatus*, *L. Jensenii* (Fig. 4).

When clustering the obtained proteins, the first (most numerous) cluster primarily included proteins involved in the proteasome system and associated with the immune response. The second cluster grouped proteins associated with estradiol metabolism. The third cluster consisted of proteins related to carbohydrate metabolism. Additionally, smaller clusters of 5–7 proteins each organized complement activation system proteins (complement factors B, H, D, I, complement C4-A alpha chain, and others), proteins associated with hemostasis (fibronectin, fibulin-1, talin-1, and others), and proteins related to purine metabolism (purine nucleoside phosphorylase, adenylosuccinate lyase, and others). The protein LTF (lactotransferrin), a biological analog of lactoferrin, was not included in either Factor 1 or Factor 2. It should be noted, as indicated above, that the composition of the vaginal microbiota at the “After” time point did not differ statistically between the P + P and L + P groups, suggesting that lactoferrin is likely not involved in

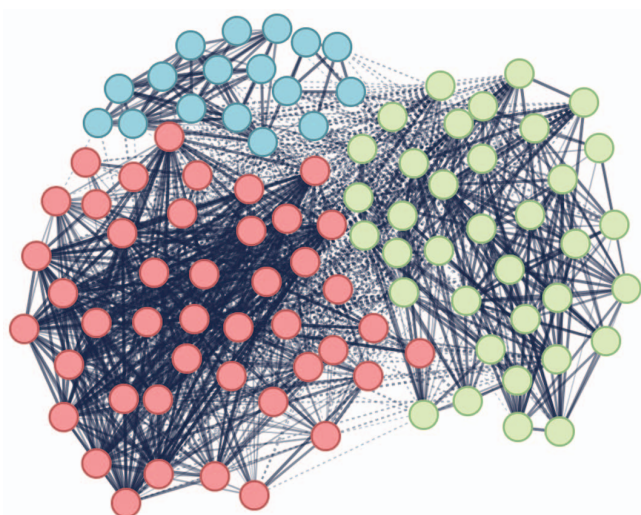


Figure prepared by the authors using their own data

Fig. 4. Protein clusters identified in Factor 1 in the Lactoferrin + Placebo group after 5-day dry immersion.

Red — proteins associated with the proteasome complex; green — proteins associated with estradiol metabolism; blue — proteins associated with carbohydrate metabolism

the interaction processes between the vaginal bacterial flora and blood proteins.

Factor 2, with a variance share of 43.88%, included 270 proteins and 6 bacterial species: *E. coli*, *S. aureus*, *E. faecalis*, *S. agalactiae*, *S. anginosus*, *L. gasseri*. Upon clustering the protein pool, the largest cluster, containing 67 proteins, primarily comprised proteins responsible for the cellular response to stress and proteins involved in the immune response (von Willebrand factor antigen 2, fibrinogen gamma chain, integrin-linked protein kinase, and others). A considerably smaller second cluster included 16 proteins of the proteasome complex (proteasome subunits PSMD7, PSMF1, PSMD1, PSMD9, and others). Proteins involved in neddylation and immune system proteins were assigned to separate clusters. The remaining clusters comprised 2–3 proteins each.

Several studies have shown that certain lactobacilli can block inflammatory reactions in the intestinal epithelium *in vitro* and induce the production of reactive oxygen

species via deneddylation of cullin-1 [33]. However, the presence of an entire group of proteins involved in neddylation (i.e., the process by which the ubiquitin-like protein NEDD8 conjugates to target proteins and facilitates the activation of cullin-RING ligases, which then transfer ubiquitin) in Factor 2 raises the inevitable question of whether such a process can occur in the vaginal biotope. There is also evidence that neddylation is enhanced in tissues following *S. aureus* infection [34]. It is likely that neddylation may be regulated by the use of probiotic preparations. For the vaginal biotope, preparations based on *Lactobacillus* spp. are more appropriate. The use of such agents may strengthen the colonization resistance of the vaginal microbiota by influencing a range of biological processes in the body, including the aforementioned neddylation. Summary data regarding the factors influencing the “bacterium-protein” system before and after the end of dry immersion in both groups of participants are presented in Table.

Table. Correlation between blood protein levels and vaginal biotope bacteria before and after dry immersion in the study groups

Factor	Variance share, %	Microorganism names	Proteins, <i>n</i>	Protein functions and processes defining this factor
Before 5-day dry immersion				
1	37.9	–	260	Ubiquitination and metabolism
2	13.7	<i>C. glucuronolyticum</i> , <i>V. parvula</i> , <i>F. magna</i> , <i>B. bifidum</i>	43	Prolactin metabolism
3	12.3	<i>S. epidermidis</i>	27	Immune system
4	10.3	<i>V. dispar</i> , <i>V. denticariosa</i> , <i>L. iners</i>	23	Unknown
5	9.1	–	12	Unknown
6	8.5	–	13	Metabolic processes in the liver
7	8.2	<i>E. coli</i> , <i>S. haemolyticus</i> , <i>E. faecalis</i> , <i>S. agalactiae</i> , <i>S. anginosus</i> , <i>C. tuberculostrictum</i> , <i>L. gasseri</i> , <i>L. vaginalis</i> , <i>L. rhamnosus</i>	15	Unknown
After 5-day dry immersion, Placebo + Placebo group				
1	37.1	<i>S. lugdinensis</i> and <i>F. magna</i>	238	Ubiquitination and deubiquitination, immune system
2	33.5	<i>S. haemolyticus</i> , <i>C. glucuronolyticum</i> , <i>C. tuberculostrictum</i> , <i>K. schroeteri</i> , <i>C. acnes</i> , <i>L. gasseri</i>	190	Complement system, estradiol metabolism
3	29.4	<i>S. epidermidis</i> , <i>S. hominis</i> , <i>L. iners</i> , <i>L. jensenii</i>	200	Proteasome complex
After 5-day dry immersion, Lactoferrin + Placebo group				
1	56.12	<i>S. hominis</i> , <i>F. magna</i> , <i>C. acnes</i> , <i>L. crispatus</i> , <i>L. jensenii</i>	388	Proteasome complex and estradiol metabolism
2	43.88	<i>E. coli</i> , <i>S. aureus</i> , <i>E. faecalis</i> , <i>S. agalactiae</i> , <i>S. anginosus</i> , <i>L. gasseri</i>	270	Immune system and cellular stress response

Table compiled by the authors based on their own data

Note. «—» — absent.

DISCUSSION

The vaginal mucosa represents one of the colonization barriers for opportunistic microflora in the female reproductive system. The immunity of the vaginal mucosa is formed both by the adaptive component and by innate immune cells, such as neutrophils, dendritic cells, and macrophages, which express PRRs on their surface [35]. PRRs interact with pathogen-associated molecular patterns, activating monocytes and macrophages in the tissues [36]. Macrophages, in turn, typically secrete pro-inflammatory cytokines and modulate the formation of various inflammasome complexes in response to different microbial stressors [37]. Furthermore, according to the literature, antimicrobial amphipathic peptides (i.e., partially membrane-embedded) are present on the surface of the vaginal mucosa; these are either constitutively expressed or their production is induced by immune cells. AMPs are influenced by hormones, whose levels vary during the menstrual cycle and depend on the composition of the vaginal microbiota [38]. It is also important to note that AMPs are relatively resistant to the resistance mechanisms employed by rapidly evolving opportunistic and pathogenic microorganisms.

Pro-inflammatory cytokines secreted by macrophages, as well as AMPs, represent a broad spectrum of substances, such as various interleukins, lactoferrin, human β -defensin-5, secretory leukocyte protease inhibitor, elastin, and others [39–41]. Thus, biochemical processes, particularly those related to immune regulation, are closely interconnected with the microbial community of the vaginal biotope and are, without doubt, influenced by external factors and stresses, such as dry immersion.

The data obtained in our study allow us to analyze how the emphasis in biochemical processes in the body changed depending on the experimental conditions experienced by the female participants of the dry immersion experiment. Before the start of the experiment, in the absence of the main stress factor — dry immersion — the relationships within the “bacterium-protein” system involved ubiquitination processes, the proteasomal apparatus, immune regulation, and hormonal status. All of these are closely interconnected and, as revealed by the study, are also linked to the vaginal bacterial flora. However, after dry immersion, the associations between proteins and the bacterial flora became stronger. For example, before the start of the dry immersion experiment, Factor 1, which was associated with ubiquitination and a number of metabolic processes (with the highest percentage contribution to the variance), included only proteins.

After 5-day dry immersion, associations between the vaginal microbiome and proteins were established in both groups of participants. The greatest contributions to the variance were made by ubiquitination and immune processes, as well as bacteria that apparently provoked an immune response or influenced the biochemical

processes of ubiquitination/deubiquitination, along with the mutual influence of estradiol and a number of bacteria. In the Lactoferrin + Placebo group, the influence of estradiol was more pronounced. Representatives of lactobacilli (*L. gasseri* in the Placebo + Placebo group and *L. crispatus*, *L. jensenii* in the Lactoferrin + Placebo group), corynebacteria (*C. glucuronolyticum*, *C. tuberculostearicum* in the Placebo + Placebo group), staphylococci (*S. haemolyticus* in the Placebo + Placebo group and *S. hominis* in the Lactoferrin + Placebo group), and cutibacteria (*C. acnes* in both groups) were found to be sensitive to estradiol.

The most important microorganisms for maintaining vaginal microbiota balance are, without doubt, *Lactobacillus* spp. The protective mechanism of lactobacilli is accomplished in several ways. First, lactobacilli actively compete with other microorganisms for nutrients and for attachment to the vaginal epithelium. Additionally, *Lactobacillus* spp. lowers the vaginal pH by producing organic acids through the degradation of glycogen released by vaginal cells. In addition, *Lactobacillus* spp. produces a range of antimicrobial substances, such as lactocins and hydrogen peroxide, which can suppress the growth of opportunistic microflora, and also participate in the modulation of local immunity. Currently, the molecular mechanisms by which lactobacilli attach to epithelial cells remain unknown. Some studies suggest that adhesion of *Lactobacillus* spp. is mediated by a number of proteins [42, 32], while others propose a role for lipoteichoic acid [43] and carbohydrates [44]. The relationship between estradiol and lactobacilli has also been investigated previously, and it has been shown that estradiol improves the adhesion of lactobacilli to the vaginal epithelium [45].

Corynebacteria are also considered by some authors as potentially probiotic strains; however, there is also evidence that they can cause vaginitis [46, 47]. The mutual influence of estradiol and corynebacteria has hardly been studied. There is only evidence of a decrease in the number of corynebacteria following an increase in estradiol and progesterone levels in cattle [28]; however, no studies have been conducted in humans. With regard to the relationship between estradiol and other bacteria, information is also insufficient. For example, the influence of progesterone and estradiol on the growth, adhesion, biofilm-forming ability, and antibiotic sensitivity of methicillin-resistant *Staphylococcus aureus* has been studied. It was found that both hormones reduced the viability of *S. aureus* [28]. There is evidence of an association between *C. acnes* and hormonal changes in the body. In combination with excessive sebaceous gland activity or a reduction in the number of protective groups of microorganisms, changes in hormonal status can lead to active proliferation of *C. acnes* and biofilm formation, which is one of the virulence factors of this bacterial species [48].

The study results confirm the presence of a close association between specific representatives of the vaginal

microbiota and the host's blood proteins. This relationship is realized through two pathways:

- direct mechanism — via the release of microbial metabolites that directly affect cellular processes and form a specific microenvironment within the biotope;
- indirect mechanism — via the influence of the same metabolites on general biochemical processes in the body, including at the level of protein metabolism and hormonal regulation.

In other words, microorganisms of the vaginal microflora are capable not only of locally changing the environmental conditions through their metabolites, but also of exerting a systemic effect on biochemical reactions, in particular influencing protein function and the hormonal balance of the host.

CONCLUSIONS

1. Before the start of dry immersion, the interaction between blood proteins and bacteria was determined

by ubiquitination processes, the proteasome apparatus, immunity, and hormonal status, with the “protein-bacterium” relationship being weaker than after dry immersion.

2. After the end of the experiment, ubiquitination and immune processes played a role in the “protein-bacterium” system; proteins involved in biochemical processes and estradiol synthesis also appeared to be important.

3. The microorganisms most sensitive to proteins involved in the biochemical processing of estradiol were representatives of *Lactobacillus* spp., *Corynebacterium* spp., *Staphylococcus* spp. и *Cutibacterium* spp.

4. The results of the analysis of the association between blood proteins and vaginal bacteria in participants of the 5-day dry immersion study indicate the potential for developing agents that strengthen the colonization resistance of the microbiota through targeted modulation of specific biochemical and proteomic processes in the body.

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