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Urinary metabolomic fingerprinting after consumption of a probiotic strain in women with mastitis

Rosa Vázquez-Fresno[a,b], Rafael Llorach[a,b,*], Jelena Marinic[c], Sara Tulipani[a,b,d], Mar Garcia-Aloy[a,b], Irene Espinosa-Martos[b,e], Esther Jiménez[b,e], Juan Miguel Rodríguez[b,e], Cristina Andres-Lacueva[a,b]

[a] Biomarkers and Nutrimetabolomic Lab., Nutrition and Food Science Department, XaRTA, INSA, Torribera Campus, Pharmacy Faculty, University of Barcelona, Av. Joan XXIII s/n, 08028 Barcelona, Spain
[b] INGENIO-CONSOLIDER Programme, Fun-c-food CSD2007-063, Ministry of Science and Innovation, Barcelona, Spain
[c] Department of Chemistry and Biochemistry, School of Medicine, University of Rijeka, Brace Brancetta 20, 51 000 Rijeka, Croatia
[d] Biomedical Research Institute (IBIMA), Service of Endocrinology and Nutrition, Málaga Hospital Complex (Virgen de la Victoria), Campus de Teatinos s/n, University of Málaga, Málaga 29010, Spain
[e] Department of Nutrition, Food Science and Technology, Complutense University of Madrid, Avda. Puerta de Hierro, s/n, 28040 Madrid, Spain

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ABSTRACT

Infectious mastitis is a common condition among lactating women, with staphylococci and streptococci being the main aetiological agents. In this context, some lactobacilli strains isolated from breast milk appear to be particularly effective for treating mastitis and, therefore, constitute an attractive alternative to antibiotherapy. A ^{1}H NMR-based metabolomic approach was applied to detect metabolomic differences after consuming a probiotic strain (Lactobacillus salivarius PS2) in women with mastitis. 24 h urine of women with lactational mastitis was collected at baseline and after 21 days of probiotic (PB) administration. Multivariate analysis (OSC-PLS-DA and hierarchical clustering) showed metabolome differences after PB treatment. The discriminant metabolites detected at baseline were lactose, and ibuprofen and acetaminophen (two pharmacological drugs commonly used for mastitis pain), while, after PB intake, creatine and the gut microbial co-metabolites hippurate and TMAO were detected. In addition, a voluntary desertion of the pharmacological drugs ibuprofen and acetaminophen was observed after probiotic administration. The application of NMR-based metabolomics enabled the identification of the overall effects of probiotic consumption among women suffering from mastitis and highlighted the potential of this approach in evaluating the outcomes of probiotics consumption. To our knowledge, this is the first time that this approach has been applied in women with mastitis during lactation.

Introduction

Mastitis is a common disease during lactation with an incidence of up to 33% of lactating mothers [1], and 74–95% of cases observed in the first 3 months post-partum [2]. This condition is usually defined as an inflammation of the mammary gland, characterized by a variety of local and, sometimes, systemic symptoms [3]. These symptoms of mastitis are accompanied by changes in the biochemical and immunological composition of milk, and in its sensorial properties.

The infectious aetiology of lactational mastitis is usually so high that some authors define the term "mastitis" as an infectious process of the mammary gland, involving staphylococci, streptococci and/or corynebacteria [2,4]. Traditionally, *Staphylococcus aureus* has been considered as the main aetiological agent of acute mastitis, although *Staphylococcus epidermidis* is emerging as the leading cause of both subacute and chronic mastitis in human medicine [5–7]. Multiresistance to antibiotics and/or formation of biofilms is very common among clinical isolates of these two staphylococcal species. This explains why this condition used to be elusive to

Abbreviations: A, acetaminophen; CFU, colony-forming unit; FID, free induction decay; HCA, hierarchical cluster analysis; IB, ibuprofen; NMR, nuclear magnetic resonance; OSC-PLS-DA, orthogonal signal correction partial least squares-discriminant analysis; PB, probiotic; TMA, trimethylamine; TMAO, trimethylamine-N-oxide; TSP, 3-(trimethylsilyl)-proprionate-2,2,3,3-d4.

* Corresponding author at: Department of Nutrition and Food Science, XaRTA, INSA, Campus Torribera, Pharmacy School, University of Barcelona, Barcelona, Spain. Tel.: +34 4033798; fax: +34 4035931.
E-mail address: rafallorach@ub.edu (R. Llorach).

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antibiotic therapy. In addition, maternal antibiotic therapy may disturb the normal microbiota of the respiratory and digestive tracts of the mother and breastfed infant pair, a fact that may itself further inhibit defence against infection [8]. Therefore, there is a need to develop strategies that represent an alternative to the use of antibiotics and, in this context; probiotics seem to be an appealing approach.

Dysbiosis is the change in the balance of microbiota composition such that it may become harmful to host health [9]. Mammary dysbiosis may lead to mastitis, a condition that represents the first medical cause for undesired weaning [3,10]. Probiotics which have been defined by the WHO/FAO as "live microorganisms that when administered in adequate amounts confer a health benefit on the consumer, can potentially influence systemic health with several mechanisms of action" [11]. In this regards, probiotics in several studies are focused of research [9].

Human milk is a source of bacteria to the infant gut. In addition, modulation of maternal gut microbiota during pregnancy and lactation could have a direct effect on infant health [10]. Recent studies indicate that the mammary gland contains its own microbiota during late pregnancy and lactation. Particularly, selected strains isolated from breast milk can be good candidates for use as probiotics [12,13]. Noticeably, probiotic bacteria that are originally isolated from human milk are specifically attractive organisms since they would fulfil some of the main criteria generally recommended for human probiotics, such as human origin, a history of safe prolonged intake by a particularly sensitive population (neonates, infants), and adaptation to mucosal and dairy substrates [14].

The advent of "-omics" approaches, particularly, metabolomics analysis promises to accelerate progress in our understanding to discern the molecular pathways and biochemical mechanisms under the influence of the microbiota [15]. ^{1}H nuclear magnetic resonance (^{1}H NMR) based on metabolomics, combined with multivariate statistical analysis, has proven to be a powerful technology for providing profiles of numerous components present in biological fluids, enabling non-discriminant, non-destructive, highly reproducible, high-throughput analysis [16]. In this regard, metabolite profiling strategies have shown a remarkable potential to differentiate and characterize infectious diseases caused by microorganisms of different strains, species and genera [17], to obtain a metabolic footprint of microorganisms [18], and to assess the effects of probiotics on the mammalian metabolism [19].

This potential prompted us to examine the applicability of NMR-based metabolomics for identifying the metabolomics changes occurring in biofluids (i.e. urine) of these women as a reflect of overall biochemical effects that could occur among women suffering from lactational mastitis after probiotic consumption, an approach that has not been documented so far. In this context, a metabolomic approach based on ^{1}H NMR spectroscopy coupled with multivariate statistical analysis was used in order to investigate the metabolite composition of urine samples collected from nursing women with mastitis, before and after the oral administration of the probiotic strain *Lactobacillus salivarius* PS2.

Materials and methods

Subjects and study design

Initially, a total of 30 women with mastitis symptoms were screened and, of these, seven dropped out during the study. The reasons for dropping out, after the first analysis, were as follows: two women were suffering from severe anaemia (as assessed by blood analysis), four were taking antibiotics, and the last one moved to another country (Denmark) and could not deliver the final sample. Finally, 23 women finished the study (characteristics of the participants are reported in Supplementary Material, Table S1). All met the following criteria: breast inflammation, painful breastfeeding, milk bacterial count >3 $\log_{10}$ colony-forming unit (CFU)/mL, and milk leucocyte count >6 $\log_{10}$ cells/mL. None of them ingested commercial probiotic foods or supplements during the study. Women with mammary abscesses, Raynaud's syndrome or any other mammary pathology were excluded. All volunteers gave written informed consent to the protocol, which was approved by the Ethical Committee of the Hospital Clínico of Madrid (Spain). The study was registered in the ClinicalTrials.gov database (NCT00716183). All volunteers were asked to follow a controlled diet for 48 h (see Supplementary Material, Table S2) before the collection of the samples in order to facilitate metabolite detection in the urine samples. Women were asked to fill in a record questionnaire related to the evolution of mastitis and the presence of any potential secondary effect of probiotic treatment. The records were used to monitor adherence to the study protocol. All the 23 women reported daily ingestion of a capsule over the 21 days with the exception of two women who reported ingestion over 20 days.

The study lasted 21 days and, during this period, women consumed daily a capsule with 200 mg of a freeze-dried probiotic (PB) containing ~9 $\log_{10}$ CFU of *L. Salivarius*PS2. The capsules were kept at 4 °C throughout the study. Urine (24 h) samples from all the women were collected at the beginning (day 0, baseline) and at the end of the study (day 21, after PB) (see in Supplementary Material: *Collection Procedures*).

Sample preparation

A total of 46 urine samples (from the beginning and end of the intervention) from the 23 women were analyzed. The urine samples were thawed, vortexed and centrifuged at 13,200 rpm for 5 min. The supernatant (600 μl) from each urine sample was mixed with an internal standard solution [120 μL, consisting of 0.1% TSP (3-(trimethylsilyl)-proprionate-2,2,3,3-d4, chemical shift reference), 2 mM of sodium azide (NaN3, bacteriostatic agent) and 1.5 M KH_2PO_4, in 99% deuterium water (D2O)]. The optimized pH of the buffer was set at 7.0, with a potassium deuteroxide solution, to minimize variations in the chemical shifts of the NMR resonances [20]. This mixture was transferred to a 5 mm NMR tube.

Urine ^{1}H NMR spectra acquisition and processing

^{1}H NMR spectra were acquired on a Varian Inova-500 MHz spectrometer (Varian Inc.) operating at a frequency of 500 MHz and a temperature of 298 K. A NOESY-presat pulse sequence was applied to suppress the residual water signal. Free induction decay (FID) were collected into 32 K data points (128 scans) with a spectral width of 14 ppm, an acquisition time of 2 s, relaxation delay of 5 s, and a mixing time of 100 ms [20]. A 0.3 Hz line broadening function was applied to raw FIDs prior to Fourier transformation. The resulting NMR spectra were manually phased, baseline corrected and calibrated (TSP, 0.0 ppm) using ACD Labs 1D NMR Processor 12.0 software (Advanced Chemistry Development Inc., Toronto, Canada). Prior to integration, each spectrum was segmented into 0.025 ppm chemical shift bins (buckets). The residual water resonance signal (δ 4.73–5.00) was excluded, and the resulting data set in the range from 0 to 10 ppm was then submitted to MetaboAnalyst 2.0, a web-based platform for comprehensive analysis of metabolomic data (www.metaboanalyst.ca). The matrix was row-wise normalized (rows were samples) by the sum of the intensities to reduce systematic bias during sample collection.