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REGULAR ARTICLE

PROBIOTIC PROPERTIES OF VAGINAL LACTIC ACID BACTERIA SELECTED FOR HARMONIZATION OF MICROENVIRONMENT OF REPRODUCTIVE APPARATUS

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ABSTRACT

The aim of this study was isolation and screening of probiotic properties of *Lactobacillus* strains isolated from the vagina of heifers and cows with healthy reproductive apparatus. Initially tested properties were low pH tolerance, growth at different temperature, autoaggregation, fast growth and acid production. Strains were further tested for adherence to vaginal mucus. Negative selection was used and strains that did not meet tested criterion were excluded. From 244 samples taken from 122 heifers and cows were selected six strains of different taxa. Selected and identified were strains *L. buchneri* 5/K, *L. buchneri* 24S8, *L. mucosae* 29S8, *L. mucosae* 9/K and *L. mucosae* Biocenol^{TM} 7697. These strains showed different biochemical properties also within a taxon and exhibited differences in the quantification of selection criteria. Further evaluation of the selected strain properties will be performed to consider their inclusion in a probiotic for local use in reproductive apparatus.

Keywords: selection, criteria, *Lactobacillus* spp., probiotic, reproductive apparatus, cow

INTRODUCTION

Uterine infections are one of the main causes of infertility in postpartum cows **(Rajala-Schultz and Gröhn, 1999)**. They reduce the reproductive efficiency of cows, increase herd health costs, reduce feed consumption and cause a reduction in milk production. Some treatments contaminate the milk **(Otero *et al.*, 2006)**. The use of probiotic products in the urogenital tract could prevent the undesirable consequences of antibiotics routinely used in the treatment of infections **(Reid and Bruce, 2001)**. *Lactobacilli* maintain health of the urogenital system by preventing the overgrowth of pathogenic bacteria **(Lepagneur and Rousseau, 2002)**. Inoculated *lactobacilli* act as a "bridge" during the spontaneous tuning of vaginal ecosystem to physiological state **(Maldonado Galdeano *et al.*, 2007)**. **Kummer *et al.* (1997)** reported the response to topical administration of selected strains of *lactobacilli.* This response is achieved by cells responsible for resistance in the bovine endometrium, such as lymphocytes and mononuclear cells, facts that would support the immunostimulatory effect of *lactobacilli*. *Lactobacillus* spp. and other microorganisms of the indigenous microflora can contribute to the therapeutic treatment of genital tract infections, specifically of those which occur with high frequency during the cow postpartum period, mainly in dairy cows **(Otero *et al.*, 1999)**.

The objective of our study was the isolation of bovine vaginal lactic acid bacteria from healthy vaginal ecosystem and the screening of their beneficial properties to select those that could be used as probiotics in prevention and treatment of diseases of reproductive apparatus.

MATERIAL AND METHODS

Bacterial strains

Strains used in the experiment were isolated from the vagina of healthy heifers and cows in four localities in Slovakia. The vulvar area was washed with povidone-iodine and water and a disposable speculum was inserted into the vagina to swab the posterior area. Vaginal swabs were placed into the Amies agar gel with charcoal (DispoLab, Copan Italia, Brescia, Italy). Samples were diluted with physiological solution (Imuna Pharm a.s., Šarišské Michaľany, Slovak Republic). Strains were grown in de Man, Rogosa and Sharpe agar (MRS; Carl Roth GmbH+Co. KG, Karlsruhe, Germany) for 48 h at 37ºC under anaerobic conditions

(Gas Pak Plus, BBL Microbiology systems, Cockeysville, USA). All isolates were initially tested for colony morphology, induction of hemolysis, Gram reaction and catalase activity.

Low pH tolerance

Growth was observed in MRS broth (Carl Roth GmbH+Co. KG) adjusted to different pH (3-5).

Growth at different temperature

Growth was observed in MRS agar (Carl Roth GmbH+Co. KG) after incubation under anaerobic conditions (BBL Microbiology systems) at 15°C and 45°C for 48 h.

Aggregation test

The method was performed according to **Renier *et al.* (1992)**. Aggregation of cells and formation of the clear supernatant observed within two hours was evaluated as positive reaction.

Determination of growth curves

Single colonies that were grown on MRS agar plates (Carl Roth GmbH + Co. KG) were picked into MRS broth (Carl Roth GmbH + Co. KG) and were incubated for 20 h at 37ºC under aerobic conditions. Growth rate was determined by turbidimetric method in Synergi^{TM} 4 Multi-Mode Microplate Reader (BioTek Instruments Inc., Vermont, USA). 96-well microtiter plates (Greiner ELISA 8 Well Strips, 350 µl, Flat Bottom, Medium Binding; Cruinn Diagnostics Ltd., Dublin, Ireland) were used. Shaking and incubation at 37°C were done automatically. Measurements were made during two 23-hour cycles every 15 minutes.

Acid production

The amount of acids produced by *lactobacilli* was indirectly determined by measuring the pH of the culture supernate with pH meter (pH 340i, WTW, Weilheim, Germany). Measurements were made every two hours during three 16-hour cycles.

Sampling and preparation of mucus for adherence testing

Mucus was collected from the vagina of slaughtered heifers and cows with healthy reproductive apparatus by gently scraping the mucosa with a rubber spatula. Before use, mucus was diluted with 0.15 M phosphate-buffered saline (PBS; pH 7.2) in a 1:1 ratio, filtered through a glass filter (Papírna Perštejn spol. s r.o. Keseg & Rathouský, Pernštejn, Czech Republic) using vacuum and then through the membrane filter (Millipore, 0.22 micron, 47 mm, Fisher Scientific Ltd., Dublin, Ireland). A vacuum filtration kit was used for large volumes of mucus and syringe filters and manifold connected to a vacuum were used for small volumes.

Microtiter plate binding assays for testing of adherence

The method was performed according to **Štyriak and Ljungh (2003)** with some modifications. Microtiter 96-well plates (Greiner ELISA 8 Well Strips, 350 µl, Flat Bottom, Medium Binding; Cruinn Diagnostics Ltd., Dublin, Ireland) were used. The absorbance values ($A_{580\ nm}$) were determined in a Synergy™ 4 Multi-Mode Microplate Reader (BioTek Instruments, USA). Deionized water was used as a blank. Three kinds of control wells were used: MRS broth (Carl Roth GmbH + Co. KG) without added strains, wells treated only with Bradford reagent (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) and citrate buffer replaced with Bradford reagent. Control (broth without added strains) was subtracted from the $A_{580\ nm}$ values measured for each strain. Bacterias were classified as strongly adherent ($A_{580\ nm} \geq 0.25$), weakly adherent ($A_{580\ nm}$ 0.15-0.24) or nonadherent ($A_{580\ nm} < 0.15$).

Identification of selected bacteria

Selected bacteria were initially identified by API 50CH system (BioMérieux, Marcy-l´Etoile, France). Final testing of selected vaginal *Lactobacillus* strains by rep-PCR method was performed in Czech Collection of Microorganisms of Masaryk University (CCM MUNI, Brno, Czech Republic).

RESULTS AND DISCUSSION

Bacterial strains

Non-hemolytic, gram-positive, catalase-negative strains that grew under anaerobic conditions were selected.

Low pH tolerance

Most of the strains isolated were found to grow well at pH between 4-5. An earlier study also reported that vaginal *lactobacilli* grew well at pH around 4.5 **(Aroutcheva *et al.*, 2001)**. At pH 3.2, lactic acid bacteria from many commercial products exhibited fair growth rates **(Lin *et al.*, 2006)**. Strains that grew at pH 3.6 were selected.

Aggregation test

In order to manifest beneficial effects, probiotic bacteria need to achieve an adequate mass through aggregation. In most cases, aggregation ability is related to cell adherence properties **(Del Re *et al.*, 2000)**. Bacterial aggregation between cells of the same strain (auto-aggregation) or between genetically different strains (co-aggregation) is of considerable importance in several ecological niches **(Jankovic *et al.*, 2003)**. Strains that showed auto-aggregating abilities were selected.

Growth rate

During the design process of probiotic products, two of the main characteristics studied are the growth conditions and the technological performance of the selected microorganisms **(Mäyra-Mäkinen and Bigret, 1998)**. The fastest growth of microorganisms was observed between 8^{th}-12^{th} h of incubation. Decrease in growth occurred after 16 hours of cultivation and was caused by decrease in pH and consumption of substrate (Figure 1). After twelve hours, some curves started to have sawtooth shape, which is probably caused by aggregation of bacteria into larger clusters. This implies greater optical inhomogeneity of the sample.

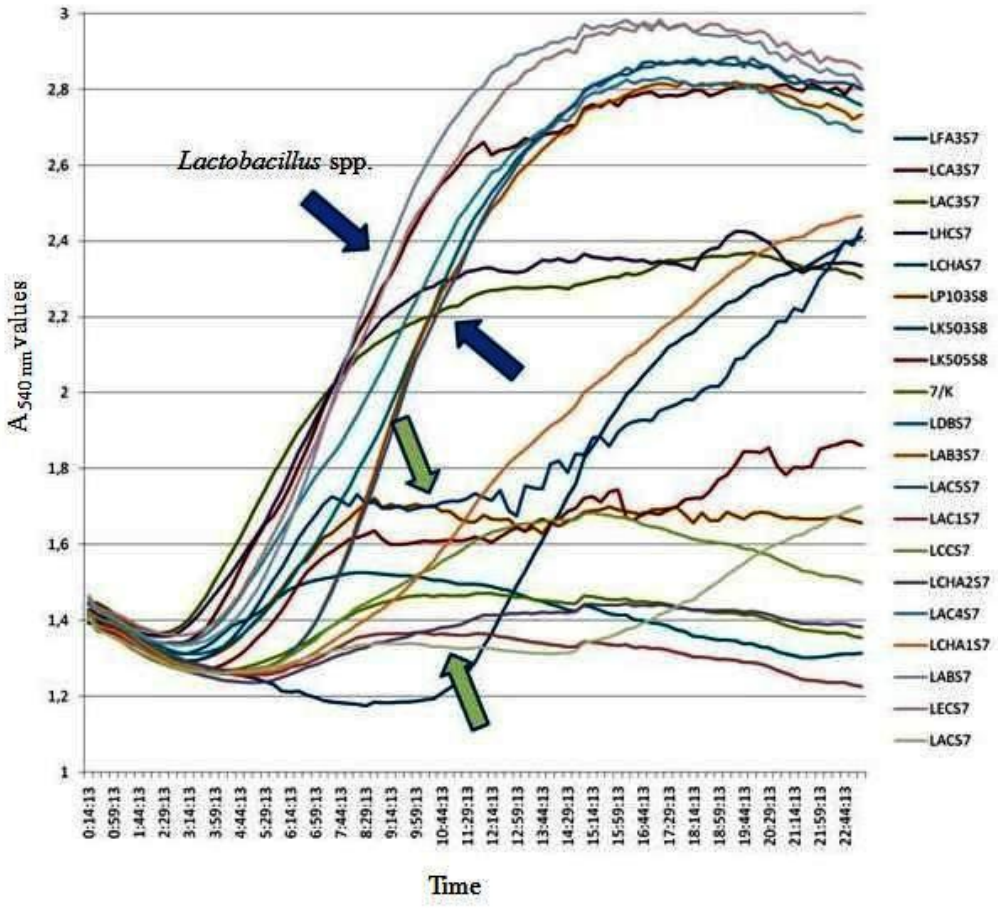


Figure 1 The average absorbance values of growth curves of indigenous vaginal microorganisms isolated from reproductive apparatus of healthy heifers and cows measured in Synergi^{TM} 4 Multi-Mode Microplate Reader.

Acid production

Relation between the increase in turbidity and decrease in pH was confirmed. pH measurement is not a reliable indicator of growth or growth inhibition of bacteria. pH can only give a partial idea of the organic acids´ concentration in the medium, because a large amount of organic acids is undissociated. Bacterial cell wall is non-permeable for dissociated acid fractions, while non-dissociated fractions passively diffuse through the cell membrane where they are ionized, depending on the intracellular pH. This leads to cytoplasmic acidification and production of inhibitors **(Nemcová, 1997)**. Decrease in pH was slower after six hours of the growth of microorganisms due to limitations in multiplication of microorganisms (Figure 2). Lactic acids and other fatty acids produced by *lactobacilli* may contribute to the maintenance of a low vaginal pH and a high redox potential, which can inhibit the growth of other bacterial species **(Holmes *et al.*, 1985)**.

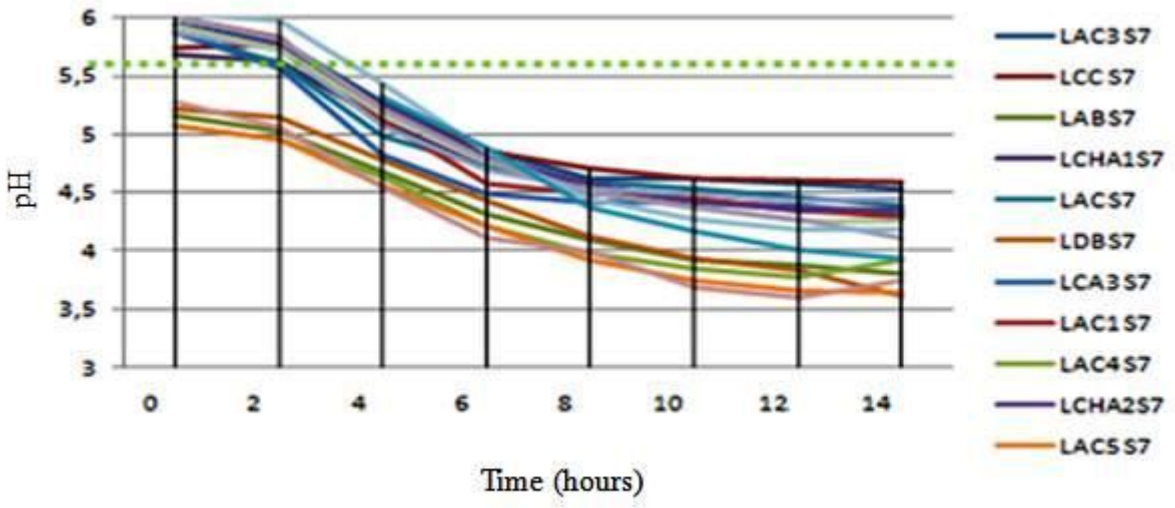
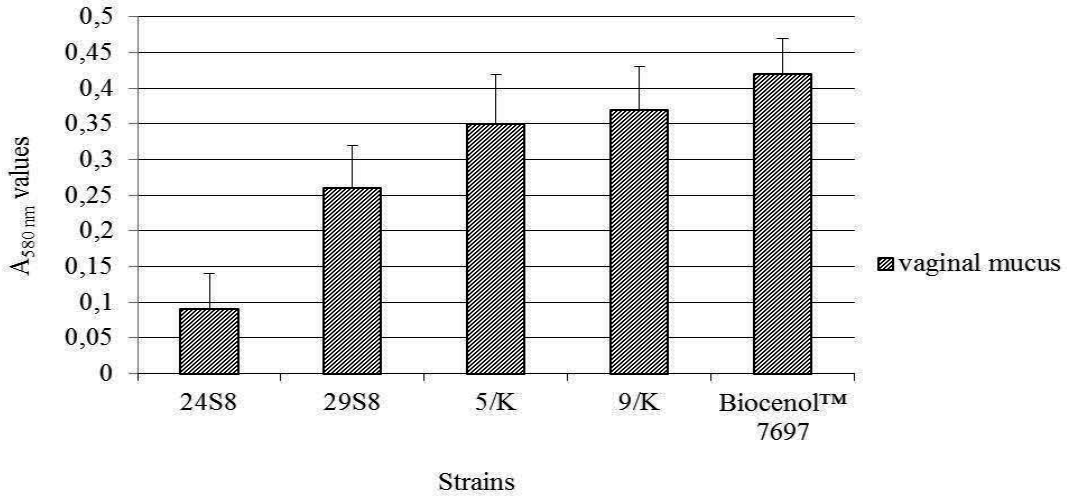


Figure 2 The average pH values measured during the growth of indigenous vaginal microorganisms isolated from healthy reproductive apparatus of heifers and cows.

Adherence to vaginal mucus

Adherence of *lactobacilli* to vaginal mucus is an important selective criterion. With the extrapolation of the criteria for intestinal probiotics it is supposed that indigenous strains of microorganisms that adhere to mucus will have significant effect on the stabilization and health of vaginal microbiocenosis **(Nemcová *et al.*, 2010)**. Strains *L. mucosae* Biocenol™ 7697 ($A_{580\ nm}$= 0.42), *L. mucosae* 9/K ($A_{580\ nm}$=0.37), *L. buchneri* 5/K ($A_{580\ nm}$=0.35) and *L. mucosae* 29 S8 ($A_{580\ nm}$ =0.26) were strongly adherent to vaginal mucus. Strain *L. buchneri* 24 S8 did not adhere to vaginal mucus ($A_{580\ nm}$=0.09). Results are shown in the figure 3.



Legend: *L. buchneri* 24S8, *L. mucosae* 29S8, *L. buchneri* 5/K, *L. mucosae* 9/K, *L. mucosae* Biocenol^{TM} 7697

Figure 3 Comparison of adherence of *lactobacilli* strains isolated from the vagina of healthy heifers and cows to the mucus collected from cows´ vagina

Identification of selected bacteria

Isolates of lactic acid bacteria 5/K and 24S8 were phenotypically identified in CCM MUNI (Brno, Czech Republic) as *L. buchneri* and this identification was confirmed by rep-PCR typization with primer GTG5. The remaining 3 isolates were further tested by sequencing of the gene pheS (phenylalanyl-tRNA synthase alpha subunit) in LMG Bacteria Collection, Ghent University, Belgium. Strains were identified as *L. mucosae.* Strain *L. mucosae* Biocenol was deposited in the CCM MUNI (Brno, Czech Republic) as CCM 7697 according to Budapest Convention.

During selection of the strains suitable for recolonization and harmonization of microflora of reproductive apparatus were used similar selection criteria that are used in the selection of intestinal probiotics **(Mudroňová *et al.*, 2010)**. Microorganisms with beneficial effects on health of vaginal biocenosis have to inhibit pathogens of reproductive apparatus, adhere to mucus of reproductive apparatus, produce inhibitory substances and survive in the reproductive apparatus **(Mastromarino *et al.*, 2002)**. Strains have to be considered as GRAS (Generally Regarded As Safe) and be sensitive to common antibiotics **(Havenaar *et al.*, 1992)**. Our findings are consistent with the findings of **Otero *et al.* (1999)** in terms of

occurrence of the strain *L. buchneri* in the vaginal microflora of healthy heifers and cows. The occurrence of *L. mucosae* in the mares´ vagina was described by **Fraga *et al.* (2008)**.

CONCLUSION

For further testing were selected strains according to their colony morphologies, non-hemolytic, gram-positive, catalase-negative, that grew under anaerobic conditions, grew in MRS broth adjusted to pH 3.6, grew under 15°C or 45°C, aggregated in MRS broth, with fast growth and acid production. In the preliminary selection were from 244 strains selected 20. These strains were tested for antibiotic sensitivity − resistant strains were excluded (unpublished data), for inhibition of pathogenic strains −strains which did not inhibit any tested pathogenic microorganism were excluded (unpublished data), for production of H_2O_2 − strains that did not produce H_2O_2 were excluded (unpublished data), for adherence to vaginal mucus − nonadherent strains were excluded. Negative selection was used and strains that did not meet the tested criterion were excluded. According to these criteria six strains of different taxa were selected. Further evaluation of the selected strain properties will be performed to consider their inclusion in a probiotic for prevention and treatment of infectious metritis.

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