COMPARATIVE ANALYSIS OF THE CHARACTERS INVOLVED IN THE INFECTIVITY OF LACTOCOCCUS LACTIS STRAINS ISOLATED FROM DIFFERENT ENVIRONMENTS

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Abstract

Although most strains of Lactococcus (L.) lactis are considered Generally Recognized as Safe (GRAS), some data reported infectious diseases associated with this species. In order to identify virulence factors involved in pathogenicity of strains belonging to this species a comparative study was conducted on two L. lactis strains included, one isolated from a patient with endocarditis and another strain with probiotic potential, previously characterized. Main assessed tests were: capacity to auto-aggregate and co-aggregate with pathogenic strains (S. Typhimurium ATCC 14028, S. aureus subsp. aureus ATCC 25923 and E. coli with ESBL phenotype), biofilm formation, adherence to HT-29 cell line, hemolytic activity and serum resistance assay. Experimental results showed significant differences between the strains proving the virulence and pathogenicity potential of L. lactis strain isolated from patient with endocarditis. Yet, mechanisms involved in such traits remain still poorly understood because of lacking knowledge on the subject. In this concern, our study underlines the need for rigorous characterization in terms of virulence and pathogenicity traits of L. lactis strains before using them in biotechnological applications.

Key words: *GRAS, pathogen, endocarditis, food industry*

INTRODUCTION

L. lactis is one of the most intense studied lactococcal species as a consequence of its importance in the food industry and the biomedical field [1], having substantial economic importance. The majority of bacteria used in food industry are killed during digestion, but lactococcal cells remain viable during the transit of the gastrointestinal tract [2]. Ingestion of raw milk or unpasteurized dairy products can represent a predisposing factor to *L. lactis* infection, since this strain has the property of colonizing mucocutaneous surfaces [3] and replaces some microbial strains from colon microbiota [4]. *L. lactis* infection is not fully understood, but bacterial translocation from the gut has been shown to be a source of bacteremia in patients with short bowel syndrome [5]. Nevertheless, human infections with *L. lactis* have been described in immunocompromised patients, but also in immunocompetent adults and children [6]. Human infections with *L. lactis* are rare, but the confusion that may exist between different subspecies may influence their reporting rate [7], the actual number of cases being also influenced by the erroneous identification of lactococci as streptococci or enterococci [8].

The priority of the food industry is the prevention of diseases that can occur due to the consumption of food contaminated with pathogenic agents [9], but there is no improving in the selection methods of

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bacterial strains, which can more effectively solve this problem. Therefore, it is necessary to identify the mechanisms associated with the pathogenic potential of *L. lactis* strains, in order to optimize the selection methods and at the same time to understand the mechanisms involved in its virulence and pathogenicity.

MATERIAL AND METHOD

- Bacterial strains

In this study we included 4 *Lactococcus lactis* strains: *L. Lactis* 19.3 isolated from fermented milk product, *L. lactis* Bals isolated from a patient with endocarditis hospitalized at National Institute of Infectious Diseases "Prof. Dr. Matei Balș", *Lactococcus lactis* DSM 2079, *Lactococcus lactis subsp. cremoris* K10, *Salmonella* Typhimurium ATCC 14028, *Staphylococcus aureus* subsp. *aureus* ATCC 25923 and *E. coli* 956 strain with extended-spectrum β-lactamase (ESBL) phenotype. The *Lactococcus* strains were cultured in MRS broth at 37°C and the other strains were cultured in LB broth and TBX plates at 37°C.

- Auto-aggregation and co-aggregation

Auto-aggregation assay was conducted as described previously by Tuo et al. in 2013 with minor modification [10]. The bacterial cells were inoculated on liquid medium and the OD at 600nm was measured for each strain at the moment of inoculation. After an overnight incubation at 37°C, in static conditions, the OD_{600nm} from the upper layer was determined. For quantification the following formula was used:

$$\text{Auto-aggregation}\% = [1-(A_t/A_0)]\times 100$$

Where A_t represents the OD_{600} after the overnight incubation and A_0 is the initial absorbance.

For coaggregation assay we adapted the protocol from Ahmed et al in 2021 [11], very similar to auto-aggregation assay, except that we mixed each lactococcal strain (v/v) with *E. coli* 956. Formula used was:

$$\text{Coaggregation}\% = [(A_{mix0}-A_{mixt})/A_{mix0}]\times 100$$

Where A_{mixt} represents the OD_{600} of the mix after the overnight incubation and A_{mix0} is the initial measure of the mix at an OD of 600nm.

- Biofilm

Strains were centrifuged at 6000rpm for 5 minutes and we used the supernatant at an optic density of OD_{600}=0,1. In each well of a 96 well-plate, 80µl MRS broth with 20µl cell suspension was added. After 2, 5 and 7 days respectively, wells were washed 2 times with sterile water, fixed for 10 minutes with MeOH 100%, stained with crystal violet 1% for 15 minutes, washed and acetic acid 33% was added. OD_{560} was determined using Synergy™ HTX Multi-Mode Microplate Reader (BioTek) and Gen5™ software. We normalized the data by subtracting the absorbance read at time 0 and subsequently removing the background using a negative control consisting of liquid MRS medium only.

- Adherence to epithelial cells

To determine the bacterial strains capacity to adhere to epithelial cells HT-29 (ATCC HTB-38) cell line from human colorectal adenocarcinoma was used. Cell line was cultivated on 6-well plate in RPMI (Gibco™ RPMI 1640 Medium, Roswell Park Memorial Institute) media supplemented with 10% fetal bovine serum, incubated at 37°C in presence of 5% CO_2. When the confluence reached 80%, epithelial cells were co-incubated with the bacterial cells for 2h at 37°C. Bacterial cell suspension, obtained as described before, used in this assay was diluted to 0,5 McFarland turbidity in cell culture media. After 2h, the plates were washed 3 times and fixed with MeOH 100% for 5 minutes and Gram stained. The adherence was quantified measuring 5 different microscopic fields at the optic microscope (100X).

- Hemolysis assay

We tested lactococcal ability to lyse the cell membrane of red blood cells by plating them on blood agar medium for 48h at 37°C.

- Serum resistance assay

To determine the viability of the studied bacterial strains in the presence of immune cells we incubated bacterial cells, obtained as described before, adjusted at 0,5 McFarland, and supernatant, adjusted at pH=7 with NaOH 0.5N and filtered through a 0.2µm filter, in various combinations with whole human blood collected on heparin. The samples were incubated for 3h at 37°C. The viability of bacterial cells (CFU/ml) was determined at different time intervals by serial dilutions.

- Statistical analysis

We used GraphPad Prism 5 for statistical analysis, One-Way ANOVA for auto-aggregation and adherence to epithelial cells (Tukey post-test); Two-Way ANOVA, Bonferroni post-test for coaggregation, biofilm and serum resistance assay.

RESULTS AND DISCUSSIONS

- Auto-aggregation and co-aggregation

For all analyzed strains was observed a medium auto-aggregation capacity (Table 1), which can be correlated with the presence of exopolysaccharides or proteins from the bacterial cell surface [11]. For the analyzed bacterial strains (non-pathogenic or pathogenic bacteria) the results were similar (*p* value=0.993).

Other studies on *Lactobacillus sp.* demonstrated a positive correlation between auto-aggregation capacity and adherence to epithelial cells, influenced by proteins, glycoproteins, lipoteichoic and teichoic acids, which are found on the bacterial cell surface [10]. Furthermore, a high auto-aggregation supports and maintains the process of bacterial pathogenesis through the formation of biofilms and the host colonization [12].

Table 1. Auto-aggregation capacity of bacterial strains

Strain	Auto-aggregation (%)	Auto-aggregation (grade)
Lactococcus lactis Bals	58,125%	medium
Lactococcus lactis 19.3	61,285%	medium
Salmonella Typhimurium ATCC 14028	63,41%	medium
Staphylococcus aureus subsp. *aureus* ATCC 25923	60%	medium
Escherichia coli 956	57,95%	medium

Co-aggregation is another crucial factor involved in multi-species biofilm forming and supports colonization and expansion of pathogenic microbial populations [13]. Our lactococcal strains didn't show a high coaggregation capacity with the 3 pathogens tested (*p*=0.3548), even though indvidually they had a good auto-aggregation.

These assays are based on an indirect method to investigate aggregation, because the bacterial cells have a tendency to settle at the bottom of the tube [12].

- Biofilm development

After 5 days a significant difference (Figure 1) was observed between *L. lactis* Bals and *L. lactis* 19.3, the potential pathogenic strain presented also the highest capacity of forming biofilms.

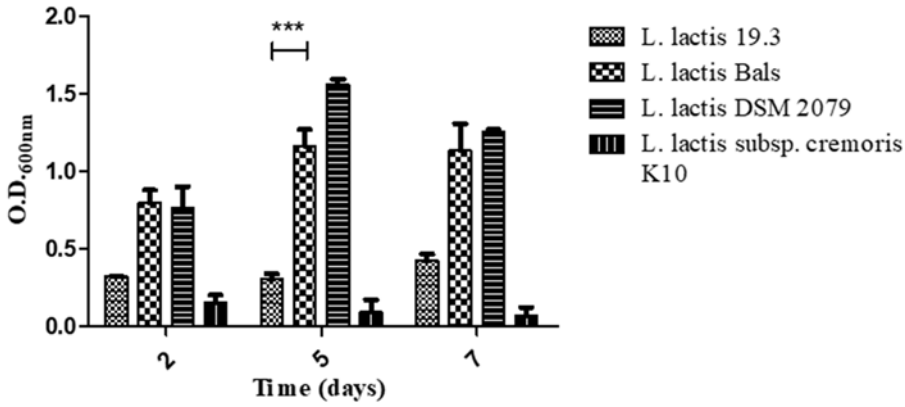


Fig. 1 *L. lactis* capacity to form biofilms within 24 to 168 hours; ***=p≤0.001.

Auto-aggregation, previously demonstrated, has an important role in biofilm forming, but it is not the only factor. Both probiotic and potentially pathogenic strains indicated a medium auto-aggregation capacity but the ability to develop biofilm is higher in the case of *L. lactis* Bals (OD_{600}=1,15925) than *L. lactis* 19.3 (OD_{600}=0,30825) ($p \leq 0.001$). Previously reported data correlated the *L. lactis* biofilm formation with some plasmidial genes [14]. According to Rabin et al. in 2015 [15], microorganisms that are forming biofilms have a higher infectious persistence and the data obtained for *Lactococcus lactis* Bals indicate the assumtion that this strain is able to resist in the host environment and support the infectious process.

- Adherence to epithelial cells

L. lactis Bals demonstrate a high capacity of adherence to human epithelial cells, even higher than the virulent strain *E. coli* 956 (Figure 2). Moreover, *L. lactis* 19.3 appear to have the lowest competence to stick to epithelial host cells. We can safely assume that our potentially pathogenic lactococcal strain is able to adhere and persist better in the host than a known pathogen.

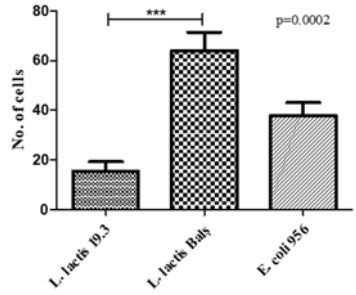


Fig. 2 *L. lactis* capacity to adhere to epithelial cells. ***=$p \leq 0.001$

As previously described by Sârbu and collaborators in 2013 [16], 3 types of attachment to eukaryotic cells are distinguished: localized (where microorganisms are forming micro colonies in distinct regions), diffuse (bacteria adhere uniformly over the entire surface of EK cells), aggregative (bacteria adhere arranged in the form of bricks). Thus, under the optical microscope, we predominantly observed a localized attachment for *L. lactis* strains (19.3 and Bals) and for *E. coli* 956 a diffuse type of attachment. When lactococcal strains were co-incubated with the pathogen, the attachment to epithelial cells was an aggregative one (Figure S3).

- Hemolysis assay

Of the 3 described types of hemolysis (α, β, γ), both *L. lactis* strains presented alpha-hemolysis, where they formed a bright yellow halo (Figure S4). The partial hemolysis can be caused by hydrogen peroxide produced by lactococcal strains.

- Serum resistance assay

The viability of lactococcal strains (CFU/ml) was quantified by serial dilutions assay on MRS agar plates. When co-incubated with *E. coli*, the lactococcal strain isolated from endocarditis presented a significant higher viability, after 3h, than the strain isolated from fermented food (Figure 3). Furthermore, *L. lactis* Bals had a significant better survival when co-incubated with pathogen compared with strain alone, as it has been pointed out after 3h of incubation.

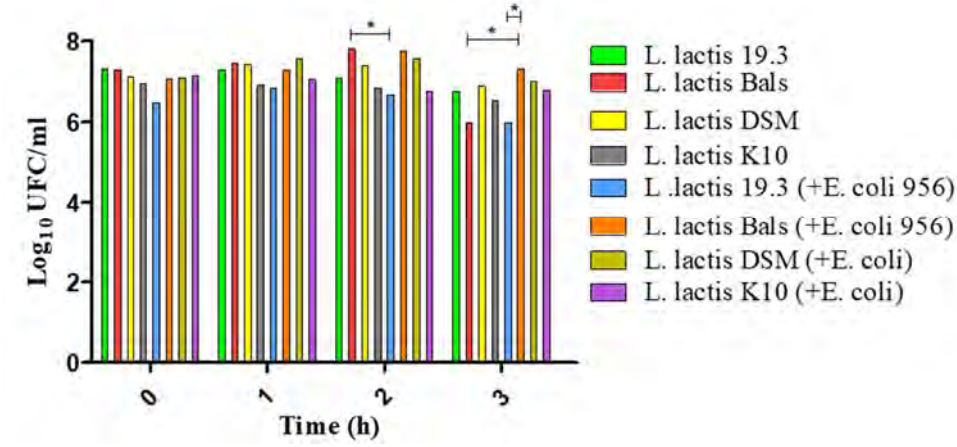


Fig. 3 *L. lactis* viability in presence of whole human blood, quantified with CFU/ml method on MRS agar. DW=distilled water. *=$p \leq 0.05$

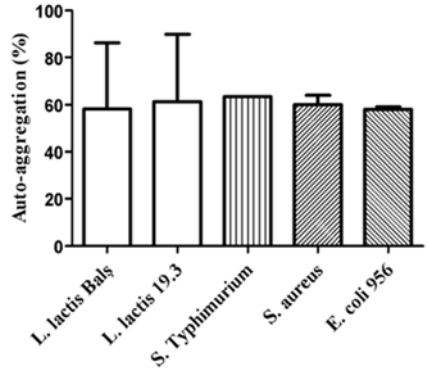
According with the obtained data *L. lactis* Bals can persist in the host and avoid the immune system better when associated with other pathogenic bacteria in our case *E. coli.* Concomitantly, the non-pathogenic lactococcal strain indicated a low rate of survival in the presence of host defense mechanisms.

CONCLUSIONS

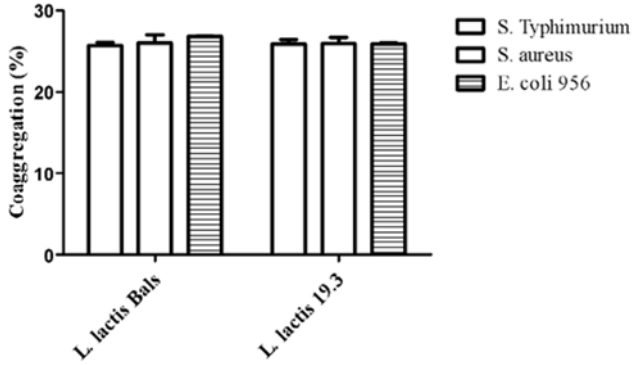
The strains studied possess a good ability to auto-aggregate, a trait beneficial for both environmental and pathogenic bacteria. Furthermore, this character can provide protection against various external stressors and from the host immune system, contributing to biofilm forming. *L. lactis* Bals presented the highest capacity of

forming biofilms, an important and crucial initial step in the process of infectivity. Moreover, according to obtained data *L. lactis* Bals has adherence ability to inert support and to eukaryotic cells (localized and aggregative attachment type) and number of bacterial cells attached to eukaryotic cells is higher compared to the strain isolated from fermented food. Due to the fact that auto-aggregation and adhesion to inert support and eukaryotic cells represent key event in pathogenesis [17], this trait can determine other carachteristics including resistance to host immune system and antimicrobial compounds and promote internalization [18]. The protection against host immune cells has been demonstrated for the strain isolated from endocarditis, yet more it has been stimulated by *E. coli* 956. All presented data suggest that *L. lactis* Bals strain presents various traits which are involved in virulence and pathogenicity.

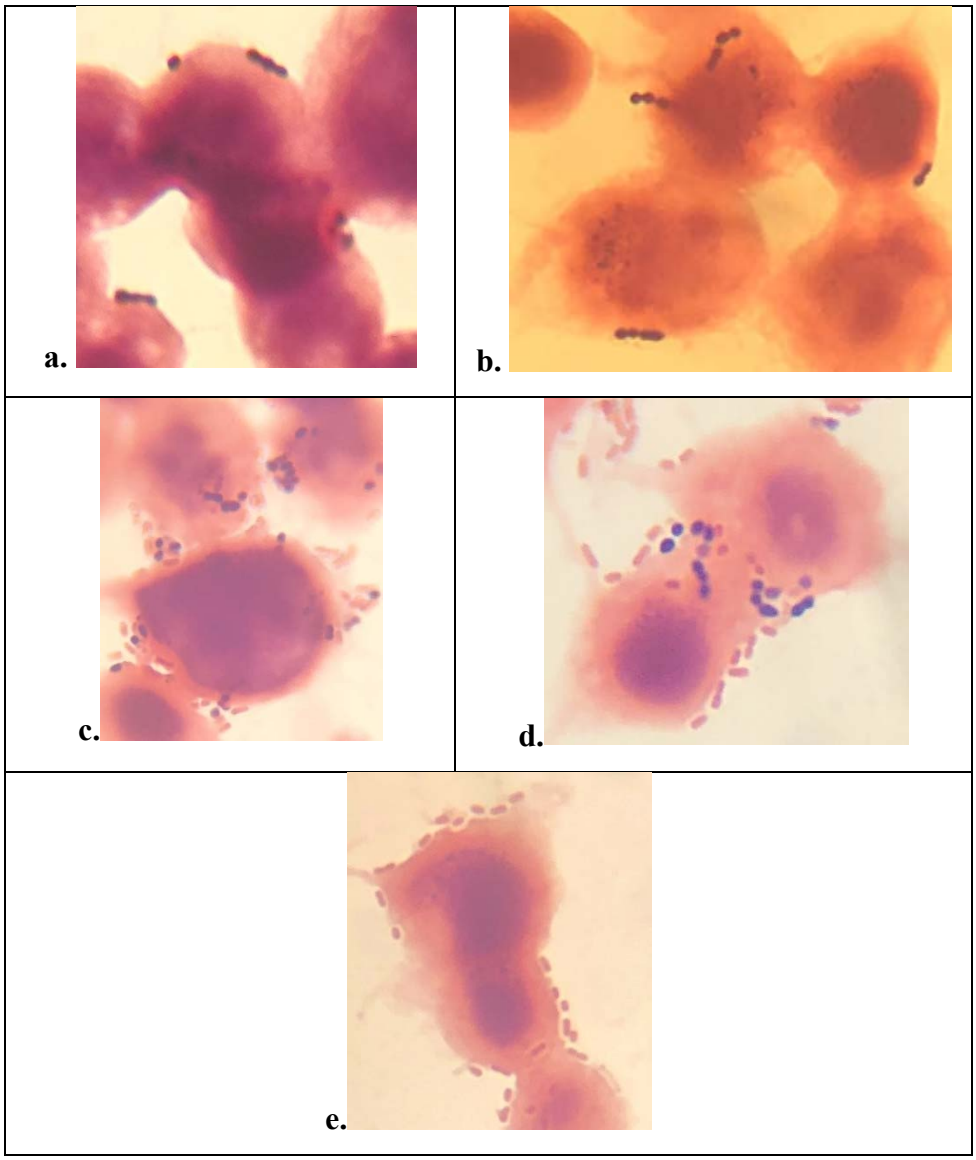
SUPPLEMENTARY



S. 1 Percentage of auto-aggregation of bacterial strains, One-Way ANOVA test, Bonferroni post-test.



S. 2 *Lactococcal* strain capacity to co-aggregate with pathogenic strains



S. 3 Visualization at optic microscope 100X, Gram staining, of attachment types of bacteria to HT-29 cell line. **a.** *L. lactis* 19.3; **b.** *L. lactis* Balș; **c.** *L. lactis* 19.3 + *E. coli* 956; **d.** *L. lactis* Balș + *E. coli* 956; **e.** *E. coli* 956.

S. 4 Hemolysis produced by *L. lactis* 19.3, *L. lactis* Balș and *Staphylococcus aureus* subsp. *aureus* ATCC 25923 on medium with blood agar.

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