Comparative effect of viable, heat- killed or sonicated *Lactobacillus fermentum* CRI 1058 in the protection of uropathogenic *E. coli* in the urinary tract of a murine experimental model

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Lactobacilli play a protective role against pathogens in the urogenital tract by a combination of multiple mechanisms, not fully understood until now. The purpose of the present paper was to study whether live, heat killed or sonnicated cells of *Lactobacillus fermentum* are able to protect against the challenge of uropathogenic *Escherichia coli* in the urinary tract of mice as experimental model. Sonicated, heat treated and live cells of *L. fermentum* CRL 1058 attached to agarose beads were inoculated intra-urethrally to different groups of 2-months-old female BALB/c mice in three doses of 10^8 CFU/dose. Thereafter, these mice were challenged with an uropathogenic strain, *Escherichia coli*, also inoculated in the urethra of the animals. A reinoculation of Lactobacilli at the same dose was performed on day 6 and after. Treated cells of *L. fermentum* exert a protective effect against *E. coli* colonization at different degree, being lower that that produced by viable cells. Our results suggest that *L. fermentum* can be used as a probiotic in those infections caused by certain pathogens such as *E. coli*, based in the results obtained in the *in vivo* experimental model.

KEYWORDS: *Escherichia coli*, *Lactobacillus fermentum*, preventive effect, probiotics, urinary tract, heat-killed, sonicated, viable

1. INTRODUCTION

Lactobacilli are the predominant microorganisms of the healthy urogenital tract, both from human and animal, being considered as beneficial and health promoters [1]. They have long been isolated as the main protective microflora in the vagina [2]. Historically lactic acid bacteria have been used as probiotic microorganisms at different hosts and tracts, mainly in the gastrointestinal area. During the last years, there has been an explosion of the number of publication in the subject and their application in many mucosas for different purposes. The mechanisms of action of probiotic products include the production of antagonistic substances, such as lactic and acetic acids, hydrogen peroxide [3] or bacteriocins [4], the formation of a protective biofilm by adhesion, autoaggregation or surface properties [5], the stimulation of the immune system [6], or the competition of nutrients [7]. Thus, they are able to restrict the growth of bacteria from different pathogenic genera, and to protect the host from infectious microorganisms. With regards to the urogenital tract infections, it was claimed in the pre-antibiotic era that a bladder infusion of lactobacilli could cure severe cystitis [8]. In the last few years, the modern medicine shows the tendency to use a special type of natural products, called probiotics, in preventive and therapeutic ways [9]. The need of alternative therapies promotes some studies as those published by Beerepoot et al, [10] on the NAPRUTI (Non-Antibiotic versus antibiotic prophylaxis for recurrent Urinary Tract infections in women). Some clinical assays in adult woman and some other experimental assays in animal models have shown the efficacy of probiotic microorganisms in the urogenital tract [11, 12, 13 to 20].

In a previous paper, the isolation of lactobacilli strains from the vagina of 2-months-old BALB/c mice [13] was reported, trying to set-up an experimental model which allowed studying the prevention and protection exerted by the lactobacilli as part of the indigenous microbiota. One *L. fermentum* CRL 1058 was se-

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lected for further studies because of the adhesive and inhibitory properties. Later this strain was embedded in microscopic agarose beads to inoculate the urethra of the mice [14]. The lower concentration required for the lactobacilli to remain in the urinary tract of the animals until the 7^{th} day post-inoculation was determined [14], showing there were not production of adverse or collateral effects [15]. The mice were protected against an uropathogenic *E. coli* strain by using the adequate dose of viable lactobacilli. This effect was not equally successful when the lactobacilli were used in therapeutic treatments [16]. Pathogen colonization was controlled with the co-administration of lactobacilli and low doses of ampicillin [17] and norfloxacin [18]. Later, the effect of estrogens and lactobacilli was demonstrated against Urinary Tract Infections (UTI) in mice [19, 20]. Other authors have shown that a single intravaginal application of capsules containing 10^8 *L crispatus* CTV-05 resulted in vaginal colonization in three of 10 animals 2 days after use [21]. The antimicrobial activity of the intraurethrally administered probiotic *Lactobacillus casei* strain Shirota against *Escherichia coli* in a murine UTI model was also examined [22].

Uropathogenic *E. coli* is frequently associated with human UTI. Since they are considered among the five most frequently pathogens of nosocomial UTI [23], the present study has been carried out in order to evaluate if the intra-urethral administration of sonnicated (cell walls), inactivated (heat-killed) or viable *L. fermentum* CRL 1058 to mice can protect the challenge with uropathogenic *E. coli*. The experiments were performed with the equivalent dose of lactobacilli (subjected to the three treatments) than those reported before, trying to obtain some type of preventive effect of protection against pathogenic microorganisms.

Eventhoug the definition of probiotics includes that the probiotic bacteria must be viable and administered in enough amounts to produce a health benefit in the host [9], there are many mechanisms suggested that could be responsible for the probiotic effect [24]. Then, not only studies with viable cells must be performed, but some other with bacterial fragments or heat killed microorganisms, to support the demonstrated effect or the mechanisms involved, which are the main objectives of the present paper.

2. MATERIALS AND METHODS

2.1. Microorganisms

L. fermentum CRL 1058 was isolated from the vagina of BALB/c mice. The *E. coli* strain was isolated from the infected urinary tract of adult women and identified by biochemical tests. This strain is also considered uropathogenic since presents the hemagglutination mannose resistant characteristic in the hemmaglutination test. It also produces hemolysins and shows a pyelonephrytogenic effect [25].

2.2. Agarose Beads preparation

Media, lactobacilli culture conditions and the preparation of microscopic agarose beads for intra-urethral administration were previously described [13, 14, 20], resumed as follows: *L. fermentum* was grown in LAPTg broth for 12 hours at 37ºC, harvested by centrifugation and washed twice with phosphate buffered saline (PBS – pH 7.0). They were resuspended in PBS. A suspension of 10^8 CFU/ml was mixed with the same volume of 1% agarose in PBS and maintained at 37-40ºC and three volumes of vaseline added at the same temperature.The mixture was gently vortexed for three minutes, and after standing at room temperature for 2 minutes, cooled in an ice bath and maintained at 0ºC for 7–10 min. The beads were washed by centrifugation at 1.000xg with peptone water (0.1%) to remove excess of vaseline. The supernatant was taken out with a Pasteur pipette

2.3. Heat killed bacteria

The microorganisms were heat treated for 1 hour at 100ºC. This suspension was cooled immediately, and used for the elaboration of agarose beads. The beads were stored at –70ºC for the seriated inoculation. The absence of live microorganisms was tested by culture in MRS agar.

2.4. Sonnicated microorganisms

The equivalent dose of *L. fermentum* was treated by sonication (Virtis Research Equipment, Gardiner, Nueva York) for 3 minutes. The viability of the microorganisms was tested as before.

2.5. Inoculation in mice

Different groups of 2-month-old female BALB/c mice from the breeding stock of our institute were used throughout the investigation. Animal inoculation procedure has been described before [13, 20], but basically mice were anaesthetized with sodium pentobarbital before intraurethral inoculation of *L. fermentum* included in agarose beads. A plastic catheter coupled to a syringe was used for this purpose. After each inoculation animals were returned to their cages.

2.6. Preventive assays

Intra-urethral administrations of a three-fold dose of 3×10^8 CFU of lactobacilli in agarose beads with 12 h. in between were given to the mice. With the same time-interval a suspension of 1×10^9 CFU *E. coli* was inoculated as a single dose. Six days later, three new lactobacilli inoculation were performed. Control mice were treated only with lactobacilli or challenged only with *E. coli*. Agarose beads obtained from the same amount of microorganisms, heat-treated or sonicated were administered following the same protocol to different groups of mice.

The scheme used for the inoculation of mice was as follows:

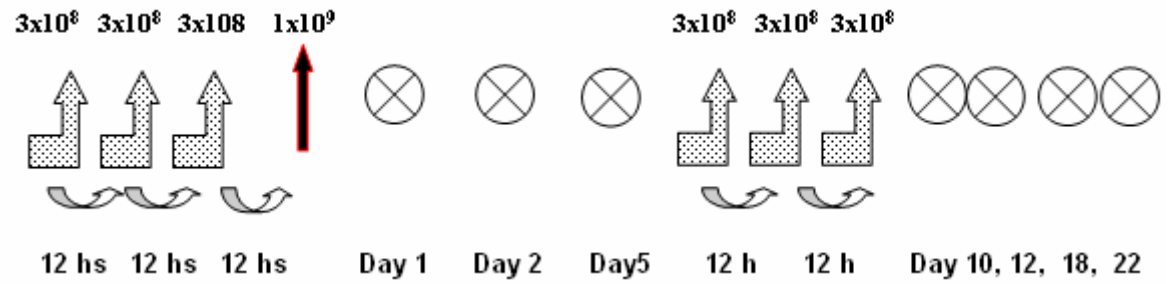

Figure 1. Inoculation scheme used for the different experimental assays: Black pointed arrows indicate lactobacilli or fraction inoculated. Black arrow indicates pathogen inoculation. Mice were sacrificed on different days, indicated with circles

2.7. Bacterial counts in tissue homogenates

The animals were sacrificed by cervical dislocation at different days post-pathogen challenge. Their urinary organs (urethra, bladder, ureters and kidneys) were removed aseptically, placed in 2 ml of 0.1% peptone-water and homogenized with a Teflon pestle. The method used for quantification of bacteria was previously described [13, 20]. The urethra and ureters were longitudinally cut previously with scissors to allow the release of the microorganisms. Sometimes, the homogeneization of the bladder was very difficult, because of the fibrous nature of the tissue. The bladder must be kept longer in the homogenization process. The samples were serially diluted (10-fold) in peptone water, from 1/10 to 1/1.000.000, using glass tubes with peptone water, automatic pipettes, and mixing vigorously each dilution tubes with vortex. A 0.5 ml aliquot of each sample dilutions was placed on the plate, 12-15 ml of melted culture medium, LBS agar (Lactobacillus selection agar) and MRS agar and on McConkey agar, a differential medium for Gram-negative bacilli. The plates were incubated in incubators at 37°C, for 48 h, and after this time, the number of CFU was determined, selecting the data of the plates which contains isolated colonies.

2.8. Statistical analysis

The results show the mean and the Standard Deviation of the data obtained from 3 to 5 mice. Each experimental group included 16 to 20 mice, and was performed at least twice. The Student's t-test was used to determine the differences statistically significant between the means.

3. RESULTS

3.1. Preventive assay in mice with viable *L. fermentum* cells and later inoculated with *E. coli*.

Animals treated with viable cells of *L. fermentum* showed a decrease in the number of pathogen recovered from the urethra, bladder, ureters and kidneys compared to control mice during all the days of the assay and up to the 22^{th} day as shown in Fig. 2, 3, 4 and 5. Lactobacilli were present in all the organs throughout the experiment (data not showed).

The control animals treated only with the pathogen showed very high numbers of *E. coli* (between 10^4 and 10^8 CFU/organ) in urethra, bladder and Kidneys during all the days of the experiment. In ureter the *E. coli* numbers were very low, around 10^1 during the first two days, reaching very high levels from day 12 on. During the last days of the experiment, mice challenged only with *E. coli* showed symptoms of sickness, supported also by the results obtained.

3.2. Preventive assay in mice with heat-killed or sonnicated *L. fermentum* and later inoculated with *E. coli*.

The assays performed with both, sonicated or heat-treated lactobacilli, showed a lower and different degree of protection than that obtained with live cells. A lower colonization and number of pathogens in treated mice was always observed when compared with control mice treated only with *E. coli.* The results obtained in each one of the organs are summarized in Fig 2, 3, 4 and 5.

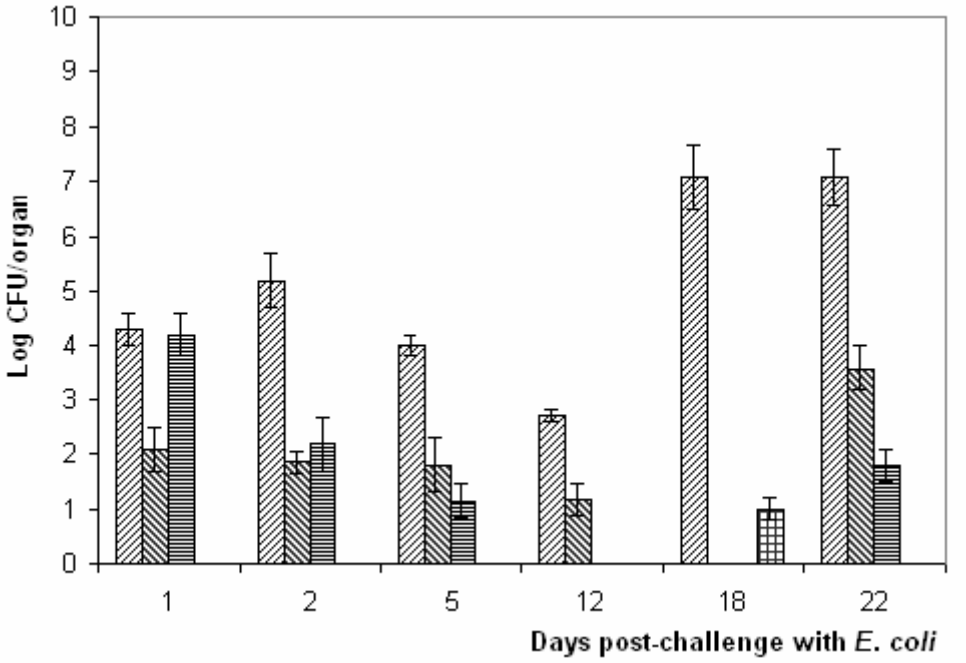

Figure 2. Colonization of uropathogenic *E. coli* in urethra of adult female BALB/c mice inoculated with agarose beads containing *L. fermentum* CRL 1058 subjected to different tretament. *L. fermentum* was administered previous to infection with *E. coli.* Mice were inoculated with a three dose of live, heat killed or sonni-

cated lactobacilli in agarose beads (10^8 CFU per dose) and challenged with *E. coli* (2 x 10^9 CFU). Control mice were inoculated with the same pathogenic dose as treated mice. The results are expressed as the mean ± S.D. of the log of CFU/urethra from three to four animals (∗p>0.05). (▨) Control (▨) Sonnicated (▨) Heat-treated (▨) Viable cells.

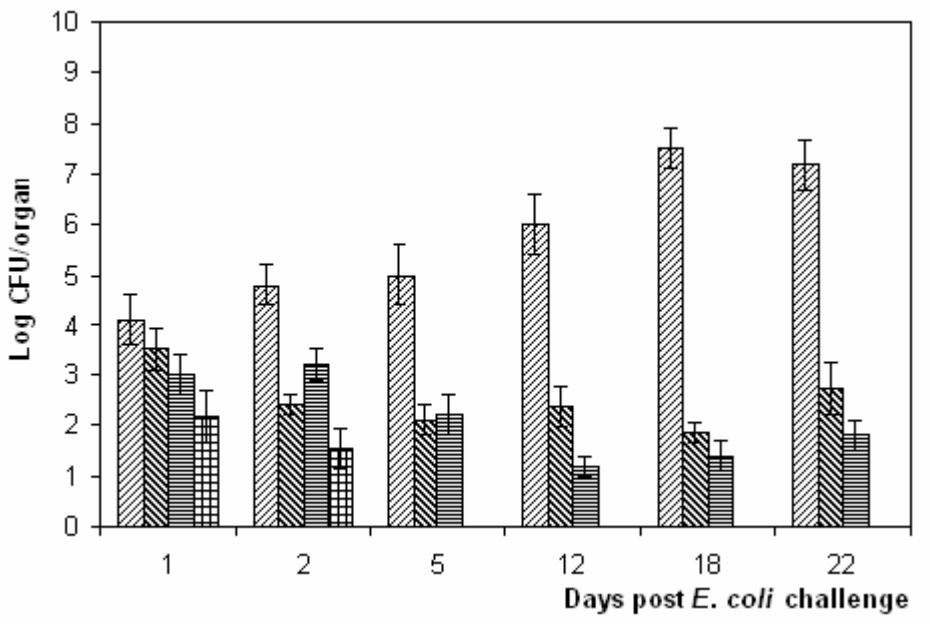

Figure 3. Colonization of uropathogenic *E. coli* in bladder of adult female BALB/c mice inoculated with *L. fermentum* CRL 1058 beads subjected to different treatments *L. fermentum* was administered previous to infection with *E. coli.* Mice were inoculated with a three dose of live, heat killed or sonnicated lactobacilli in agarose beads (10^8 CFU per dose) and challenged with *E. coli* (2 x 10^9 CFU). Control mice were inoculated with the same pathogenic dose as treated mice. The results are expressed as the mean ± S.D. of the log of CFU/bladder from three to four animals (∗p > 0.05). (▨) Control (▨) Sonnicated (▨) Heat-treated (▨) Viable cells.

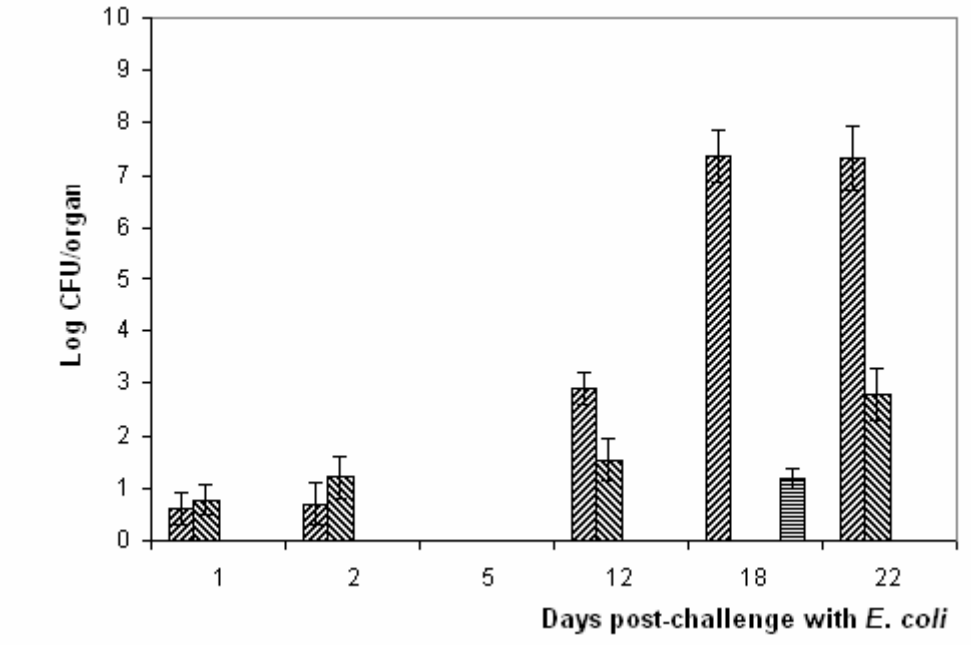

Figure 4. Colonization of uropathogenic *E. coli* in ureter of adult female BALB/c mice inoculated with *L. fermentum* CRL 1058 subjected to different treatments. *L. fermentum* was administered previous to infection with *E. coli.* Mice were inoculated with a three dose of live, heat killed or sonnicated lactobacilli in agarose beads (10^8 CFU per dose) and challenged with *E. coli* (2 x 10^9 CFU). Control mice were inoculated with the same pathogenic dose as treated mice. The results are expressed as the mean ± S.D. of the log of CFU/ureter from three to four animals (∗p > 0.05). (▨) Control (▨) Sonnicated (▨) Heat-treated (▨) Viable cells.

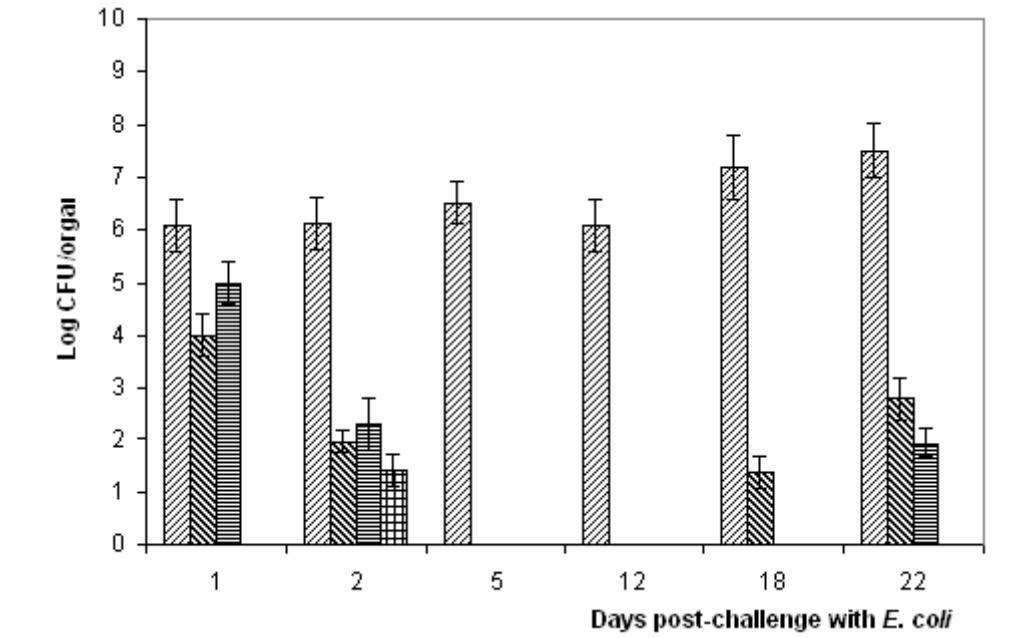

Figure 5. Colonization of uropathogenic *E. coli* in kidney of adult female BALB/c mice inoculated with agarose beads containing *L. fermentum* CRL 1058 subjected to different treatments. *L. fermentum* was administered previous to infection with *E. coli.* Mice were inoculated with a three dose of live, heat killed or sonnicated lactobacilli in agarose beads (10^8 CFU per dose) and challenged with *E. coli* (2 x 10^9 CFU). Control mice were inoculated with the same pathogenic dose as treated mice. The results are expressed as the mean ± S.D. of the log of CFU/kidney from three to four animals (∗p>0.05). (▨) Control (▨) Sonnicated (▨) Heat-treated (▨) Viable cells

4. DISCUSSION

The concept of probiotic implies that the beneficial microorganism must be administered in large numbers, enough to produce a health benefit in the host [9]. And also the inclusion of live microorganisms in conceived. But the mechanisms by which the probiotic products act in the host are not fully understood [24], and include from a very wide and different types of properties or activities displayed by the probiotic or beneficial microorganisms, to their participation in the target ecological niche or the consequences of their interaction with the host cells or systems [26].

Many scientists report the administration and effect of viable bacteria, study the optimal dose, the protective effect, but some others compare the differences between the effect produced by different bacterial species or genus, which is more and more related to the specific characteristics of very unique and particular strains [27]. Most of the studies were performed by administration of probiotics by oral way, trying to understand which are the type of cells involved in the protection or in the stimulation of the innate or specific immune response [6]. But some more recent studies were carried out trying to find out which are the bacte-

rial component responsible of each one of the different effects produced even in the host, in animals as experimental models, or through in vitro assays.

There are not many studies performed in the urogenital tract, showing the protective effect of probiotic lactobacilli. Previously, this probiotic effect was demonstrated by the administration of viable *L. fermentum* included in agarose beads, a strategy applied to increase the time of contact between bacteria-epithelia [13, 14, 28]. Then, our interest was focused in trying to understand if viable or non viable bacteria needs to be present, or which could be the contribution of the lactobacilli cell wall, or their components, by administering viable, non viable heat-killed or sonnicated bacteria in a preventive way, to compare between all the assays. The *L. fermentum* strain used was isolated from the urogenital tract of healthy mice, then the host-specificity required by some probiotic strains was achieved [29, 30, 31]. The results obtained show that the degree of protection of lactobacillus against *E. coli* is lower with non viable bacteria in both treatments; either agarose beads containing heat-killed *L. fermentum* or in those containing the cell walls obtained by sonnication. The effect obtained showed to be protective, because the number of *E. coli* obtained in all the assays was lower than in the control mice treated only with the pathogen. Mice were reinoculated 6 days later that the first set of lactobacilli administration. This scheme was performed, because previously was demonstrated that viable lactobacilli colonized the urogenital tract up tp 7^{th} day. The restimulation of the tract was produced, based on the fact the cells involved in the innate response could be some of the responsible of the protective effect. Other scientists have demonstrated the differential profile of cytokines stimulated by dead or alive cells [32, 33, 34, 35, 36, 37, 38, 39].

Then which are the mechanisms by which these strains, their cell wall or bacterial components are able to increase the resistance to pathogen infection and protect mice in the urogenital tract? Are the S-layer, peptidoglycan components, lipoteichoic acids, small peptides, or some other components released to the media while growing the molecules involved? Neither there are nor clear evidences at the urogenital level, but there are some approaches performed at different sites, areas, or experimental models, which could help in the understanding of such protective effect. Daily intake of heat killed *L. plantarum* increased the acquired immunity mainly in the $Thelper_1$ related immune functions in healthy adults, improving the health-related [33]. Laudano et al [40] have shown the anti-inflammatory effect of live or dead probiotic bacteria contained in a Bioflora pharmaceutical product, either orally or subcutaneouslly administered to rats. Also the antitumor activity of daily injections of heat-killed *L. plantarum* L 137 was demonstrated by Murosaki et al [32]. This same heat killed strain intraperitoneally injected showed to be useful for the prevention and treatment of food allergy [36] in mice, by stimulation of IL12 (p70) production, which turns shift the balance between the T helper type 2 to type T helper 1 type that have the potential to either prevent or ameliorate allergic disease. Other researchers [41] have shown the direct correlation of the amount of peptydoglican present in the cells, as a strain-dependent stimulatory activity

The S-layer that completely covers the surface of bacteria is determinant in the adhesion events in Lactobacilli. Schar- Zamanetti [42] have shown that the adhesion peaks is caused by the semi-crystalline characteristic of the protein layer, while high adhesion forces are related to a surface rich in polysaccharides. They also demonstrated that the external protein and lipoteichoic acid confers hydrophobic properties to specific strains, while polysaccharides confers hydrophibicity. Later, they [43] have compared the different characteristics of the S-layer of specific strains of Lactobacilli, showing the presence of a compact layer of globular proteins in the outer surface that determines the smooth property of the layer. In contrast, when the S-layer is covered by polymeric surface constituents, they confer a roughness property to the layer. On the other side, the removal of the S-layer, as a 45 Kda protein present in all the growth phases of L. acidophilus M 92 reduce the adhesion to mouse ileal epithelial cells [44]. This S-layer helps in the resistance to gastrointestinal conditions showing thus the functional role of the S-layer.

Other scientists have demonstrated [45] that a non-viable constituent, a protein extract of *L. helveticus* decreased *E. coli* O_{157} H_7 adherence and attaching-efficacy lesions in epithelial cells monolayers. Also the removal of S-layer in *L. crispatus* reduced autoaggregation and adhesion to Hela cells [38]. This S layer inhibited adhesion of *S. typhimurium* and *E. coli* by competitive exclusion [46].

The soluble polysaccharide–peptidoglycan complex released from the cell wall of *L. casei* strain Shirota did not induce IL 12, which plays a key role in activating the innate immunity. But the intact cell wall of lactobacilli strains having a rigid cell wall resistant to intracellular digestion effectively stimulates macrophages to induce IL12 [40]. However, the exopolysaccharide produced and released to the media induced a gut mucosal response in mice, both in small or large intestine [37].

Less precise mechanisms, as the presence of two small factors, less than 3Kda loosely associated with the cell walls, are determinant for the adhesion of *L. fermentum* to Caco-2 cells [46]. The non-bacterial fraction of milk fermented with *L. helveticus* for different time periods demonstrated the improvement of immunological defenses at the intestinal level, increasing also the host protection. This response was evaluated trough the production of IgA+cells and cytokines+cells (IL_2, IL_6, IL_{10}) at the mice gut lamina propria [35].

Heat-killed probiotic *L. gasseri* is able to prevent or ameliorate allergic diseases, by a strain-dependent stimulatory activity for IL_{12} (p70) production, due in part to the amount of peptydoglican present in the cells [36]. The mode of inactivation of cells also modifies the type of response, as supported by the results of Wong et al [34] that showed that heat-inactivated bacteria are able to stimulate the production of IL6 and IL8 in epithelial Caco-cells, when compared with cells inactivated by irradiation.

As the understanding of which are the mechanisms involved in the probiotic effect are not completely elucidated, we can think that there is an addition of effects, as the production of inhibitory substances, because *L. fermentum* CRL 1058 produces high levels of hydrogen peroxide, immune system stimulation, or competitive exclusion. Further studies are needed to demonstrate if different groups of cells at the mucosal level are involved in the different degree of protective effect produced by live or dead cells of the probiotic *L. fermentum*

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