

A simple technique to detect *Klebsiella* biofilm-forming-strains. Inhibitory potential of *Lactobacillus fermentum* CRL 1058 whole cells and products

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Klebsiella is one of the pathogens able to form biofilm and then to produce Catheter Associated Infections (CAI). The capability to form biofilm can be considered as a virulence factor. Lactobacilli are included into the potential protective microorganisms to be used for bacteriotherapy to prevent CAI and Urinary tract Infections (UTI). The aim of this work was to isolate *Klebsiella* strains from CAI and UTI, and to set up a simple technique to detect the biofilm-forming strains in polystyrene microplates by using a spectrophotometric assay. Also, to study the kinetics of biofilm formation, supported by standard microbiological assays. *Lactobacillus fermentum* CRL 1058 whole cells, acid and neutralized supernatants were tested for their inhibitory potential against one biofilm-forming *Klebsiella pneumoniae subsp pneumoniae* strain selected. The results show that the microplate technique is easy to perform, allows the rapid screening of many strains, and also that lactobacilli whole cells and the acid supernatant inhibit in higher degree that the neutralized supernatant to the biofilm produced by *Klebsiella*

Keywords: Biofilm; inhibition; *Klebsiella*; *Lactobacillus*

1.Introduction

Bacteria belonging to the genus *Klebsiella* frequently cause human infections, being the gastrointestinal tract the main reservoir of *Klebsiella* [1]. The most common area of bacterial colonization of this pathogen is the urinary tract: in the community setting is reported to cause from 2 to 15% of cystitis cases [2]. Also, the incidence of *Klebsiella pneumoniae* increases in the hospital infections [1]. While different typing methods are useful epidemiological tools for infection control, recent findings about *Klebsiella* virulence factors have provided new insights into the pathogenic strategies of these bacteria [2]. *Klebsiella* produces fimbriae that mediate attachment to host mucosal surface, a capsule that protect against phagocytosis and other immune responses, and similar to all Gram negative organisms immunosuppressive lipopolysaccharide (LPS) [2]. *Klebsiella pneumoniae* is able to scavenge iron and to enhance its own survive in acidic environment of the urinary tract by producing urease [2]. The role of type 1 and type 3 pili in mediating *Klebsiella pneumoniae* colonization of inert surface has been recently demonstrated [3, 4]. In addition, the incidence of isolation of antibiotic-resistant *Klebsiella pneumoniae* has increased in recent years, thus complicating the therapy of this infections [5].

Biofilm are complex communities of microorganisms attached to a surface or interface enclosed in an exopolysaccharide matrix of microbial and host origin to produce a spatially organized three dimensional structure [6,7]. Biofilms are universal, occurring in aquatic and industrial water systems as well as a large number of environments and medical devices relevant for public health. Using sophisticated analytical tools, biofilm researchers now understand that biofilms are not unstructured, homogeneous deposits of cells and accumulated slime, but complex communities of surface-associated cells enclosed in a polymer matrix containing open water channels. Microorganisms growing in a biofilm are highly resistant to antimicrobial agents by one or more mechanisms. Biofilm-associated

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microorganisms have been shown to be associated with several human diseases, and to colonize a wide variety of medical devices [5].

The role of biofilm formation and development by bacteria has been suggested to be an important stage in the pathogenesis of *Klebsiella* [8, 9]. When the formation of biofilm is conceived in the urinary tract, always the catheter related infections (CAI), one of the first recognized and most studied biofilm diseases, must be included. It is clear that biofilms are associated with urinary tract infection (UTI) where indwelling device are not the cause: bacteria show to form biofilm on bladder mucosal tissue and on the mucosal surface of the acini of prostate tissue in a rat model of bacterial prostatitis [3].

The formation of biofilms within the urinary tract is one of the best explanation for the recurrent and chronic infections [3]. The urinary tract is protected from pathogen colonization by the flushing action of sterile urine, the slaughting of uroepithelial cells and a glycosaminoglycan layer. The resulting limitations on the therapeutic options demand new measures for the management of the infections produced by the responsible pathogens. Then, research on alternatives therapies are increasing, that include probiotic products at different tracts or mucosa. More specifically, in the lower urinary tract, organisms as *Lactobacillus sp* prevent pathogens from attaching and establishing a tissue infection [10].

The aim of this work was to isolate *Klebsiella* strains from CAI and UTI, and to modify and set up a simple technique to detect the biofilm-forming strains in microplates by using a spectrophometric assay [11]. Also, to study the kinetics of biofilm formation, supported by standard microbiological assays. Different fractions of *L. fermentum* CRL 1058 was used to study the capability of this genus to inhibit the formation of biofilms.

2-Materials

2.1. Microorganisms and culture media

Twenty three strains of uropathogenic *Klebsiella sp* were randomly isolated from clinical sources and included in the present study. Nineteen strains were first isolated from urinary catheters and processed according to standard methods by culturing the device in Brain Heart Infusion BHI (Britania, Argentina). The other strains were isolated from mid-stream urine specimens obtained from ambulatory patients. All the strains were identified by standard biochemical tests used for clinical diagnosis. The phenotypic tests used were described in The Bergey's Manual of Determinative Bacteriology [12]. They were subcultured in BHI broth.

Lactobacillus fermentum CRL 1058 was isolated from the vaginal tract of BALB/c mice [13] and included into the culture collection of the Centro de Referencia para Lactobacillus (CERELA-CONICET). They were grown in MRS agar [14] (Difco Laboratories Inc., Detroit, MI).

2.2. Storage of microorganisms

Gram negative strains were stored in BHI broth-5% glycerol, while lactobacilli were stored in milk yeast extract (10% skim milk, 15% glucose, 0.5% yeast extract) with 5% glycerol at -20°C.

2.3. Screening of biofilm production in *Klebsiella* strains

Klebsiella strains were sub cultured three times in Luria Bertoni broth (Gibson Laboratories, USA) for 18 h at 37°C. Before the experiments, all the strains were vortexed for 5 minutes and the optical density adjusted to 0.56 to 0.64 (2×10^7 to 8×10^8 CFU/ml) at 540 nm in a spectrophotometer (Bausch and Lomb, USA).

Lactobacilli were subcultured in MRS broth for 16 h at 37°C, and the third culture used for the experiments with whole cells and supernatants. Aliquots of the supernatant (pH 3.8) obtained after centrifugation for 15 minutes (10000xg) were neutralized in sterile conditions with 8N NaOH (Anhydrous, Anedra, Argentina). All the supernatants were stored at 4°C until the assay.

All the *Klebsiella* strains were studied for their capability to produce biofilm in a modified microplate quantitative assay. For the inhibition of biofilm, one *Klebsiella* strain was selected to be assayed with *Lactobacillus fermentum* CRL 1058. This strain was previously isolated from urinary catheter and identified as *Klebsiella pneumoniae subsp pneumoniae*. The OD of *Klebsiella* grown in LB were adjusted, and aliquots of 200 μ l transferred to pre-sterilized, 96-well polystyrene microtiter plates commercially available (Deltalab S.L., Spain), and later incubated for 6 hours at 37°C. After incubation, 25 μ l of 1% Crystal Violet was added to each well, shaking the plates three times to help the colorant to get the bottom of the well. After 15 minutes at room temperature, each well was washed with 200 μ l sterile PBS to remove the planktonic cells and stain not adhered to the well. This process was repeated three times. Only the adhered bacteria forming the biofilm were kept on the surface of the well. The Crystal violet bound to the biofilm was extracted later with two washes of 200 μ l of ethyl alcohol. The liquid washing alcohol was transferred to a glass tube containing 1.2 ml of alcohol and agitated. To determine the degree of biofilm formation, the absorbance was determined at 540 nm in an UV spectrophotometer (Cecil 2000-Series, England). Controls were performed with Crystal Violet binding to the wells exposed only to the culture medium without bacteria. All the assays were performed by triplicate. The data obtained were used to classify the strains as high producers (OD higher than 0.500), producers (OD between 0.500 and 0.100) or poor producers (OD lower than 0.100).

2.4. Kinetic of Biofilm formation by *Klebsiella*

Aliquots containing different number of microorganisms ($1,2 \times 10^8$ CFU and 8×10^8 CFU) of an overnight culture of one *Klebsiella* strain selected as biofilm producer, was transferred to a sterile microplate to evaluate the kinetics of biofilm formation. The plates were incubated for 24 hours at 37°C. At different times (from the first 6 hours and later every 4 hours) the formation of the biofilm was detected, by the method described before.

2.5. Inhibition of biofilm formation by different lactobacilli fractions

Different fractions obtained from *Lactobacillus* were used to evaluate the inhibition of biofilm formation: acid supernatant, neutralized supernatant and whole cells. The KB was transferred to microplates as described before, and the different *Lactobacillus fermentum* fractions were added to each well to complete 200 μ l volume. The plates were incubated for 24 hours at 37°C and every 4 hours periods, the bacteria biofilm was evaluated by using Crystal Violet as described earlier. *Klebsiella* or *Lactobacillus* were included as control. All the assays were performed by triplicate.

2.6. Quantification of *Klebsiella* and *Lactobacillus* during the biofilm assays

The bacterial growth was determined by the plate dilution method. Dilutions were made in Peptone Water and plated onto Mc Conkey (Britania, Argentina) and MRS broth (Difco Laboratories Inc., Detroit, MI) incubated for 24-48 h at 37°C. The number of microorganisms was determined in aliquots obtained from the wells of plates.

3. Results

3.1. Isolation and Identification of *Klebsiella* from clinical sources.

The biochemical tests performed to identify the 23 isolated strains of *Klebsiella* indicate that most of the strains belong to *Klebsiella pneumoniae subsp pneumoniae* specie. On the other hand, only two strains were identified as *Klebsiella oxytoca* and one as *Klebsiella planticola*, as shown in Table 1.

Table 1 Phenotypic identification and origin of *Klebsiella* strains isolated from clinical samples,

<i>Klebsiella</i> sp Internal Identification	Phenotypic Identification	Clinical source	<i>Klebsiella</i> sp Internal Identification	Phenotypic Identification	Clinical source
Kper	<i>Kleb pneumoniae</i>	mid-stream urine	K15	<i>Kleb pneumoniae</i>	catheter
Kcar	<i>Kleb pneumoniae</i>	mid-stream urine	K11	<i>Kleb pneumoniae</i>	catheter
Kleo	<i>Kleb pneumoniae</i>	mid-stream urine	K7	<i>Kleb pneumoniae</i>	catheter
K4358	<i>Kleb planticola</i>	catheter	Khem	<i>Kleb pneumoniae</i>	catheter
K5	<i>Kleb pneumoniae</i>	catheter	K3	<i>Kleb pneumoniae</i>	catheter
Kchel	<i>Kleb pneumoniae</i>	mid-stream urine	K6	<i>Kleb oxytoca</i>	catheter
K4304	<i>Kleb pneumoniae</i>	catheter	K10	<i>Kleb oxytoca</i>	catheter
K4	<i>Kleb planticola</i>	catheter	K17	<i>Kleb pneumoniae</i>	catheter
Kdan	<i>Kleb pneumoniae</i>	catheter	K20	<i>Kleb pneumoniae</i>	catheter
Ksor	<i>Kleb pneumoniae</i>	mid-stream urine	K2002	<i>Kleb pneumoniae</i>	catheter
K4323	<i>Kleb pneumoniae</i>	catheter	K2004	<i>Kleb pneumoniae</i>	catheter
Ksan	<i>Kleb oxytoca</i>	mid-stream urine			

Table 1. All the strains were identified by standard biochemical phenotypic methods.

3.2. Screening of *Klebsiella* strains that produce biofilm

The strains assayed for production of biofilm, and the results obtained are summarized in Fig 1, where the capability of different *Klebsiella* strains from clinical source to produce biofilm is summarized. The results indicate that each strain shows a different potential to form biofilm under the same conditions of experimentation. The highest biofilm strains were *Klebsiella pneumoniae subsp pneumoniae* isolated from an urinary catheter. Those strains able to form a good biofilm are consider those that produce OD above 0.500, being five different strains belonging to different species: *Klebsiella planticola* (one strain) and *Klebsiella pneumoniae subsp pneumoniae* (four strains). The strains able to produce a medium degree of biofilm were nine strains of *Klebsiella pneumoniae* and two *Klebsiella oxytoca*, and those classified as poor forming biofilm, are three *Klebsiella pneumoniae* strains. Is important to notice that all the studies were performed after four hours of incubation at 37°C by using very high concentration of microorganism.

One strain of *Klebsiella pneumoniae subsp pneumoniae* (internal identification Kper) isolated from an urinary sample was selected by the capability to produce an extracellular mucous substance, and also classified as a good biofilm former, for further studies with *Lactobacillus fermentum*.

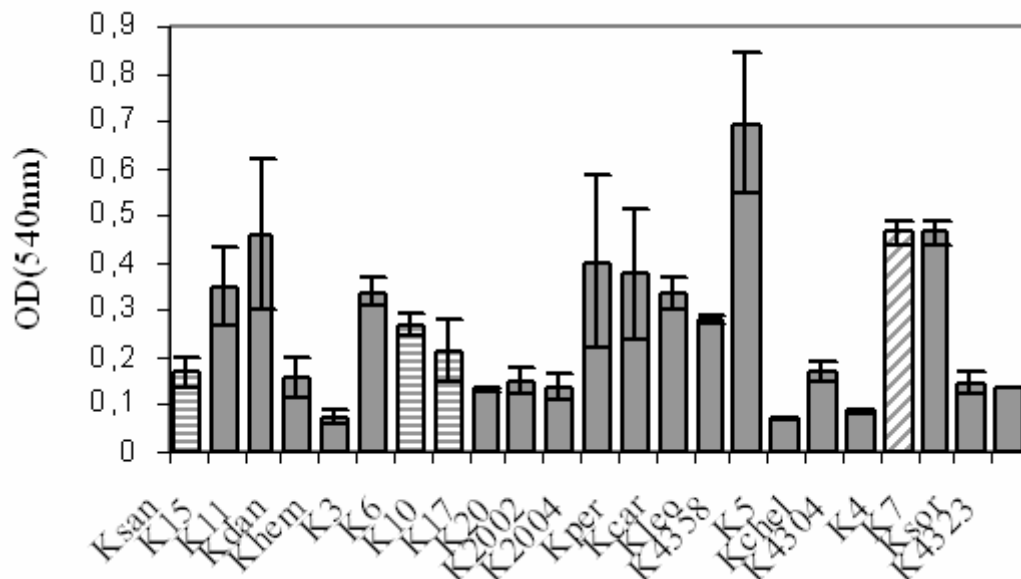


Figure 1. Biofilm formation of *Klebsiella sp* isolated from catheter and urinary tract determined by using the microtiter plate assay after incubation for 6 hrs at 37°C. *Klebsiella* were identified as ■ *Klebsiella pneumoniae subsp. pneumoniae*, ■ *Klebsiella oxytoca*, and ▨ *Klebsiella planticola*. The biofilm formation was determined as described in the text. The error bars represent the standard deviation for three replicated assays.

3.3. Kinetics of biofilm formation

To evaluate the kinetics of biofilm formation, one strain of *Klebsiella* was assayed at two different concentrations. The number of viable *Klebsiella* was also evaluated during the assays. The quantification of the biofilm formation was determined at different times during 24 hours. Bacteria were able to attach to the plates within the first six hours of incubation, and after that, the biofilm formation increased over the time until 24 h of the assay, as shown in Figure 2. The number of CFU were constant during all the assay, maintaining levels between 10^8 to 10^9 CFU/ml.

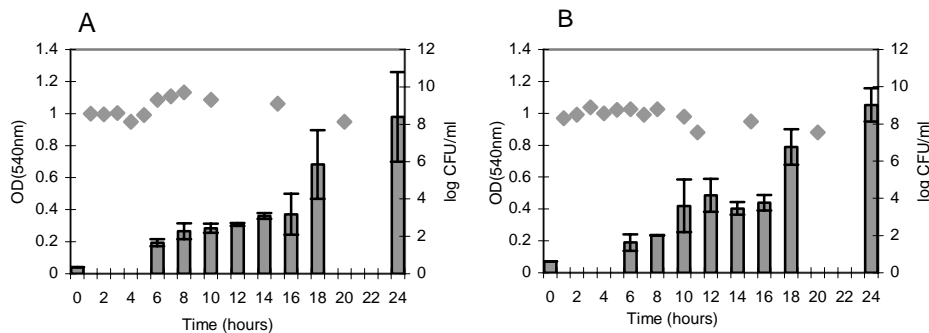


Figure 2. Kinetic of formation of biofilm by *Klebsiella pneumoniae subsp pneumoniae* at two different concentrations. A was performed with 1.2×10^8 CFU/ml and B with 8×10^8 CFU/ml. The number of *Klebsiella*, and the biofilm formation was determined as described in the text. ◆ CFU/ml *Klebsiella* into the wells. ■ Degree of biofilm formation by *Klebsiella*. The bars represent the SD of three experiments

When the evaluation of different fractions of lactobacilli on the formation of biofilm was performed, the results obtained are summarized in Figure 3. Whole cells of *L. fermentum*, and the cell-free acid

supernatant inhibited the formation of *Klebsiella* biofilm by the assayed methodology. Moreover, *L. fermentum* and the acid supernatant inhibited *K. pneumoniae* after 8 h of incubation, that decreased their numbers to 0 at 24 hours culture. The neutralized supernatant inhibited the formation the biofilm up to 8 h, but later, the pathogen began to grow and to form biofilm on the plates. Neutralized supernatant did not affect *Klebsiella* growth that reached similar numbers than the control. The formation of the biofilm in presence of neutralized supernatant, did not reach the same level then the control biofilm, as shown in Figure 3B. The viable cells of *L. fermentum* were not affected by *K. pneumoniae*, showing similar numbers in both pure or mixed cultures, as show in control in Figure 3D.

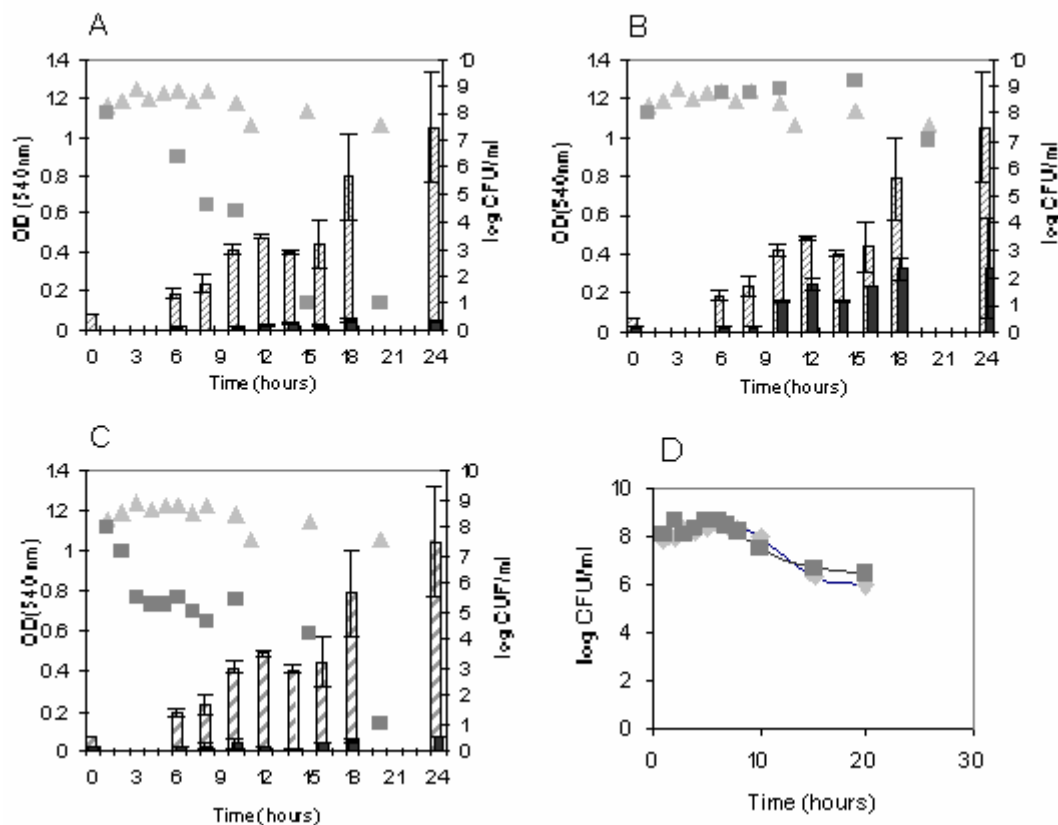


Figure 3. Inhibition of the formation of *Klebsiella* biofilm by different fractions of *L. fermentum*. **A: Acid Lactobacilli supernatant.** ▲ Control *Klebsiella* in CFU/ml. ■ *Klebsiella* in CFU in contact with acid supernatant. ▨ Control formation of *Klebsiella* ■ *Klebsiella* biofilm when acid supernatant was added. **B: Neutralized Lactobacilli supernatant.** ▲ Control *Klebsiella* in CFU/ml. ■ *Klebsiella* in CFU in contact with neutralized supernatant. ▨ Control formation of *Klebsiella* ■ *Klebsiella* biofilm when neutralized supernatant was added. **C: *L. fermentum* whole cells.** ▲ Control *Klebsiella* in CFU/ml. ■ *Klebsiella* in CFU in contact with whole cells ▨ Control formation of *Klebsiella* ■ *Klebsiella* biofilm when whole cells were added. **D. Growth control of *L. fermentum*:** ▲ *L. fermentum* single culture. ■ *L. fermentum* in associative cultures with *Klebsiella*.

4. Discussion

Implanted foreign polymer bodies become a common practice of modern medical care. The use of foreign material has led to associated complications because the insertion or implantation of medical devices is often related with microbial infections. The morbidity and mortality of device-associated infections contribute significantly to the increasing problem of nosocomial infections [15].

K. pneumoniae is a common pathogen of the urinary tract in both community and hospital settings. Infections are more often in hospitalized, chronically catheterized. This infection can be severe with high incidence of mortality by the potential capability of urosepsis. *K. pneumoniae* has a high number of virulence factors, including fimbriae, capsules, iron-scavenging systems, and urease. In UTI producing strains, type 3-fimbriae, enterochelin and biofilm producing capability were detected [3, 4].

The ability to adhere to materials and to form biofilm is an important feature in the pathogenesis of *Klebsiella* associated CAI due to the colonization of the polymeric surface by forming multi-layered cell clusters, embedded in extra cellular material. In this work, a modification of a rapid technique was set up to detect the strains able to form biofilm isolated from clinical sources [11]. The detection of the strains with a high capability to form biofilm is remarkable, basically by the potential modification of the therapy applied to patients and also to avoid the removal of the implanted device.

Biofilm formation proceed in two stages: a rapid attachment of the bacteria to the polymeric surface is followed by a more prolonged accumulation phase that involves cell proliferation and intercellular adhesion [16, 17]. The biofilm is formed at the interphase between the inert support of the microplate and the liquid media, and is quantified with an spectrophotometric detection, based in the technique published by O'toole et col [11]. The amount of bacteria assayed in the experimental protocol was used based on previous experiments (non-published results). At 4 hours incubation, the detection of biofilm is possible because the level attached to the plate was enough to be stained with crystal violet. This screening allowed to detect those *Klebsiella* strains that form different levels of biofilm by the technique applied, and help to select one strain of *Klebsiella pneumoniae subsp pneumoniae* isolated from an urinary sample to further study the kinetics of inhibition produced by a *Lactobacillus fermentum* strain.

One main problem associated with infections related with medical devices is the low sensitivity of the bacteria to the antibiotics used. The antibiotic concentrations applied to control and eradicate infections must increase in concentrations up to 1000 folds compared with planktonic bacteria [18]. New alternatives were proposed to control such type of infections. Probiotics, mainly of the genus *Lactobacillus* has been shown to be successful for the prevention of biofilm formation [19, 20, 21]. *L. fermentum* RC 14 produced biosurfactants, that participate in the adhesion of these strains to the device surface inhibiting then the uropathogen [22, 23]. In this paper, different fractions of *L. fermentum* CRL 1058 were used to study its inhibitory potential against the biofilm formation by *Klebsiella*. Whole cells of lactobacilli, and also the acid supernatant were able to inhibit both, the biofilm formation and the growth of *Klebsiella*. The effect of the whole cells could be explained by the adhesive characteristic of the lactobacilli strain used, because this is an auto-aggregating strain, with a high degree of hydrophobicity [24, 25, 26, 27]. Other authors have suggested the existence of an S-layer on the surface of specific *Lactobacillus* strains, that are involved in adhesion phenomenon [28, 29, 30]. This hypothesis was not studied in the *L. fermentum* strain, but they will be further studied.

The acid supernatant inhibits both the growth and the formation of biofilm, because the *L. fermentum* strain used produces high levels of lactic acid, and hydrogen peroxide, both able to inhibit the *Klebsiella* proliferation in associative cultures (not published results). The neutralized supernatant inhibits the biofilm formation in a lower degree than the other fractions evaluated, but not the pathogen growth. One of the possible explanations could be the release of different metabolites to the culture media, as for example, biosurfactants, or other substances that are not produced or released in enough amounts to inhibit the replication of the bacteria. Many scientist have published some alternatives to prevent the formation of biofilm by *Klebsiella* as chemical antibiofilm inhibitors [31], clorhexidine [32], antiseptics [33, 34] or metallic titanium/silver hard coating [35]. Further studies are being performed to understand the mechanisms involved in the described inhibitory effect.

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References

- [1] R. Podschun and U. Ullmann, *Clinical Microbiological Review* **11**, 589 (1998).
- [2] C. M. Collins and S. E. F. D'Orazio in H. L. T. Mobley and J. W. Warren, *Urinary Tract Infection* Edited by American Society for Microbiology. Washington DC 1996, Chapter 11, [299,312].
- [3] P. Di Martino, N. Cafferini, B. Joly and A. Darfeuille-Michaud, *Research in Microbiology* **154**, 9 (2003).
- [4] A. V. Suescún, J. R. Cubillos and M. M. Zambrano, *Biomedica* **26**, 528 (2006).
- [5] R. M. Dolan and J. W. Costerton, *Clinical Microbiology Review* **15**, 167 (2002).
- [6] J. W. Costerton, Z. Lewandowski, D. Cadwell, *Annual Reviews in Microbiology* **49**, 711 (1995).
- [7] J. Jass, S. Surman and J. T. Walker, *Medical Biofilms Detection, Prevention and Control*. Edited by J. Jass, S. Surman and J. Walker. England 2003, Chapter 1, [1,28].
- [8] T. Goto, Y. Nakame, M. Nishida and Y. Ohi, *International Journal of Antimicrobial Agents* **11**, 227 (1999).
- [9] H. Kumon. *Journal Infectious Chemotherapy* **2**, 18 (1996).
- [10] G. Reid and A. W. Bruce in *Microbial Biofilms*, Eds H. M. Lappin-Scott, J.W. Costerton, Cambridge University Press, Cambridge 1995. pp [274-281].
- [11] G. A. O'Toole and R. Kolter, *Molecular Microbiology* **28**, 449 (1998).
- [12] J. G. Holt, N. R. Krieg, P. H. A. Sneath, J. T. Staley and S. T. Williams. Ed W. R. Hensyl. *Bergey's Manual of Determinative Bacteriology*. Ninth Edition (1994).
- [13] C. Silva de Ruiz, M. E Lopez de Bocanera, M. E. Nader de Macias and A. Pesce de Ruiz Holgado, *Biological and Pharmaceutical Bulletin* **19**, 88 (1996).
- [14] M. Rogosa and M E Sharpe, *Journal of General Microbiology* **23**, (1960).
- [15] Nacional Nosocomial Surveillance System report (2000) URL. www.cdc.gov/ncidod/hid/surveill/NNIS.htm.
- [16] D. Mack, W. Fischer, A. Krokotsch, K. Leopold, R. Hartmann, H. Egge and R. Laufs, *Journal of Bacteriology* **178**, 175 (1996).
- [17] M. Herrmann, J. Hartleib, B. Kehrel, R.R. Montgomery, J. J. Sixma and G. Peter, *Journal of Infectious Diseases* **176**, 984 (1997).
- [18] J. W. Costerton, A. E. Khoury, K. H. Ward, H. Anwar, *International Journal of Artificial Organs* **16**,765 (1993)
- [19] L.A. Hawthorn and G. Reid. *Journal of Biomedical Materials Research* **24**, 39 (1990).
- [20] G. Reid and C. Tieszer, *Cells and Materials* **3**,171 (1993).
- [21] G. Reid and C. Tieszer, *International Biodeterioration and Biodegradation* **34**, 73 (1995).
- [22] G. Reid, C. Tieszer and D. Lam, *Journal of Industrial Microbiology* **15**, 248 (1995).
- [23] J.C. Howard, C. Heinemann, B.J. Thatcher, B. Martin, Bing Siang Gan, and G. Reid, *Technology Applied and Environmental Microbiology* **66**, 4396 (2000).
- [24] M. E. Nader-Macias, M. E. Lopez-Bocanera, C. Silva-Ruiz, A. Pesce-Ruiz Holgado, *Microbiology, Aliments, Nutrition* **10**, 43 (1992).
- [25] C. Silva, M. del R. Rey, M. E. Nader-Macías, *British Journal of Urology*, **91**, 878 (2003).
- [26] C. Silva-Ruiz, M. E. Lopez-Bocanera., M. E Nader-Macias, A. Pesce-Ruiz Holgado. *Biological and Pharmaceutical Bulletin*, **19**, 88 (1996).
- [27] C. Silva de Ruiz, M. del R. Rey, A P. de Ruiz Holgado and M. E. Nader-Macías, *Biological and Pharmaceutical Bulletin*, **24** (2) 127 (2001).
- [28] X. Chen, J. Xu, J. Chen, Z. Zhang and W. Fang, *International Journal of Food Microbiology* **20**, 115 (2007).
- [29] P. Schär-Zammaretti and J. Ubbink, *Ultramicroscopy* **1-4**, 199 (2003).
- [30] J. Frece, B. Koss, I. K. Svetec, Z. Zgaga, V. Mrsa and J. Suskovic, *Journal of Applied Microbiology* **98**, 285 (2005).
- [31] E. Burton, P. V. Gawande, N. Yakandawala, K. LoVetri, G. G. Zhanel, T. Romeo, A. D. Friesen and S. Madhyastha. *Antimicrobial Agents and Chemotherapy* **50**, 1835 (2006).
- [32] P. Bahna, H. A. Hanna, T. Dvorak, A. Vaporciyan, M. Chambers and I. Raad, *Oral Oncology* **43**,159 (2007).
- [33] G. Chaiban, H. Hanna, T. Dvorak and I. Raad, *Journal of Antimicrobial Chemotherapy* **55**,51 (2005).
- [34] A. Ewald, S. K. Glückermann, R. Thull and U. Gbureck. *BioMedical Engineering OnLine* **24**, 5 (2006).