Inhibitory Effect of Lactobacilli Filtrate on Klebsiella Pneumoniae Biofilm

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ABSTRACT:

BACKGROUND:

The formation of biofilms by K. pneumoniae is thought to protect the bacteria from antibiotics since these entities are highly resistant to such antimicrobial agents.

OBJECTIVE:

Investigating the role of *Lactobacillus* spp. supernatant in eliminating *K. pneumoniae* biofilms.

METHODS:

Three hundred and twelve specimens of urine, wounds and sputum were collected from patients attending five hospitals in Baghdad. All specimens were identified using biochemical tests and Api 20 E system. The ability of *K. pneumoniae* isolates to produce biofilm were evaluated using crystal violet staining technique in pre-sterilized 96-well polystyrene microtiter plates. The effect of MIC of Amikacin and Gentamicin was assessed on biofilm. The ability of acid and alkaline supernatants of *Lactobacillus acidophilus* and *L. fermentum* to inhibit planktonic as well as biofilm of *K. pneumoniae* K14, K15, K17 and K19 isolates were separately tested.

RESULTS:

Twenty two (39.29%) *K. pneumoniae* isolates were considered as high biofilm producers. Moreover, *K. pnumoniae* isolates; K14, K15, K17 and K19 produced the thickest biofilm. All biofilms treated with Amikacin and Gentamicin developed a presence of live cells when cultured on plate count agar. Neither neutralized supernatant of *Lactobacillus acidophilus* (Lb1) and *L. fermentum* (Lb2) nor sterile MRS broth has an effect on planktonic *K. pneumoniae* K14, K15, K17 and K19 isolates. Yet, results showed that lactobacilli acid supernatant developed a marked inhibitory effect. Significant differences ($P<0.05$) were found in O.D. and viable count between pre and post treatment of *K. pneumoniae* K14, K15, K17 and K19 biofilms with acid supernatants of Lb1 or Lb2. Unlike the Amikacin and Gentamicin treatment which left live bacterial cells, *Lactobacillus* supernatant left no live cells except for two cases; Lb1 against K17 and Lb2 against K15.

CONCLUSION:

Lactobacillus supernatant is a potent antimicrobial agent against *K. pneumoniae* biofilms.

***KEYWORDS*:** Lactobacillus**,** Klebsiella pneumoniae, biofilm

INTRODUCTION:

Genus *Klebsiella* commonly cause human infections, being the gastrointestinal tract the major reservoir of *Klebsiella* [(1)]. The most regular area of bacterial colonization of this pathogen is the urinary tract: in the population setting is reported to cause from 2 to 15% of cystitis cases. Moreover, the incidence of *Klebsiella pneumoniae* increases in the nosocomial infections [(2)].

The establishment of biofilms by *K. pneumoniae* on the tissues of susceptible hosts is believed to protect the pathogen against host defense mechanisms, facilitate bacterial communication leading to expression of virulence determinants [(3)].

Biofilm resistance to antibiotics necessitates new measures for the management of the infections produced by the responsible biofilms [(4)].

Lactobacilli have been extensively studied due to their remarkable ability to inhibit the growth of other organisms through bactericidal activity and by producing lactic acid as a byproduct of its metabolism [(5)].

Hence the present work aimed to investigate alternative therapeutic protocols that include probiotic products on biofilms formation by *K. pneumoniae*.

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MATERIALS AND METHODS:

Specimens collections

Three hundred and twelve specimens included: urine, wounds and sputum, were collected in sterilized containers from five hospitals in Baghdad including Educational Laboratories, Educational Baghdad Hospital, Gazi Al-Harirri Martyr Hospital, Al-Wasity teaching Hospital for Reconstructive surgery and Educational Ibn Al-Nafees Hospital.

Isolation and identification

In the laboratory within aseptic conditions, the collected specimens were streaked directly on MacConkey agar (BD, France) and incubated for 24h at 37ºC. Pink mucoid colonies were picked and recultured on another MacConkey and Uri-select 4 (Bio-Rad Laboratories Ltd, France) plates in order to obtain pure well isolated colonies. Further identification tests included the morphological characteristics and biochemical tests were carried out depending on Forbes *et al*. [6].

Antibiotic Susceptibility Test

Antibiotic susceptibility test towards Ampicillin, Amikacin, Cefotaxime, Ceftriaxon, Ciprofloxacin, Gentamicin, Nalidixic acid, Nitrofurantoin, Tetracycline, Tobramycin and Trimoxazole was done according to the method of Bauer *et al*. [7]. A standard *E. coli* strain (*E. coli* ATCC 25922) was used as the quality control strain.

Minimum inhibitory concentration and Minimum bactericidal concentration were estimated for Amikacin and Gentamicin since they developed the highest sensitivity

Lactobacilli isolation and identification

A sample was taken from yoghurt, cultured by spreading method on MRS agar (Hi-Media, India). Another sample taken from a healthy reproductive age women (33 year) by rolling the swab over high vaginal wall and placed in sterile screw cap tubes containing MRS broth (Hi-Media, India) and also cultured on MRS agar plate, the plates were incubated at 37ºC in anaerobic jar for 24-48 hour. After incubation period smooth convex whitish to creamy colonies were isolated and sub-cultured on MRS agar medium incubated for 24-48 hour [8]. Bacterial colonies phenotypic characteristics were identified for the colonies that grown on the agar media depending on the colony shape, color, viscosity, colonies margin shape and other characteristic mentioned by Forbes *et al.* [6].

Biofilm assay

Klebsiella pneumoniae biofilm formation

Method described by Maldonado *et al.*[2] was followed to achieve biofilm formation:

Studied *K. pneumoniae* isolates cultured in Brain Heart Infusion (BHI) broth (Rashmi, India) incubated at 37ºC for 18 hour, after that bacterial culture was diluted in BHI broth and adjusted in comparison to MacFarland tube no. 0.5. Two hundred microliters of this bacterial culture were used to inoculate pre-sterilized 96-well polystyrene microtiter plates and later incubated for 24 hours at 37ºC. After incubation, all wells were washed with phosphate buffer saline for the elimination of unattached cells. Afterward, 25 µl 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 µl sterile phosphate buffer saline (PBS). This process was repeated three times. The crystal violet bound to the biofilm was extracted later with 200µl of ethyl alcohol, and then absorbance was determined at 540 nm in an ELISA reader (Beckman coulter, Austria). Controls were performed with crystal violet binding to the wells exposed only to the culture medium without bacteria. All the assays were performed in triplicates. The data obtained were used to classify the strains as high producers (OD_{540} higher than 0.500), good producers (OD_{540} between 0.500 and 0.100) or poor producers (OD_{540} lower than 0.100). At the same time viable count depending on the procedure described by Harley and Prescott (9) was performed to determine the viability of bacterial cells within the biofilm.

Inhibitory effect of antibiotic on biofilm

For the inhibition of biofilm assay, the highest biofilm producing isolates of *K. pneumoniae* (K14, K15, K17, and K19) were selected to be assayed.

Same protocol described earlier was followed to produce a biofilm. Then, before the staining step, the previously prepared Amikacin or Gentamicin containing media with minimum inhibitory concentration (MIC) were added to the biofilm containing wells:

Subsequently, the tray was incubated for another 24 hours at 37ºC, after incubation period all wells were washed and stained as the same procedure described above. Also viable count was carried

out depending on the procedure described by Harley and Prescott [9].

In vitro inhibitory activity of *Lactobacillus* supernatant on planktonic *K. pneumoniae*

Overnight *Lactobacillus* cultures contained 1.5 × 10^8 colony-forming units/ml (in accordance to McFarland tube no. 0.5) were grown in MRS broth at 37°C for 24 hr under anaerobic conditions using an anaerobic jar. These cultures were centrifuged at 6000 rpm/min for 10 min at 4°C. The resulting supernatants were filtered through a 0.2-µm membrane filter. All supernatants were cultured on MRS agar in order to confirm the absence of lactobacilli cells. Thereafter, they were stored at 4°C until the assay. Aliquots of supernatants were neutralized with 1N NaOH were prepared as well [10]. Well diffusion method described by Ikeagwu *et al*. [11] was followed to detect *Lactobacillus* supernatants inhibitory effect.

In vitro inhibitory activity of *Lactobacillus* supernatant on biofilm of *K. pneumoniae*

Acid supernatant in addition to MRS broth were investigated to evaluate their inhibition activity on *K. pneumoniae* (K14, K15, K17, and K19) biofilm.

Same protocol described earlier was followed to produce a biofilm. Then, just before the staining step, two hundred microliters of acid supernatants were added to wells containing *K. Pneumoniae* biofilm. The plates were incubated for 24 hours at 37ºC. The bacterial biofilm was evaluated by using crystal violet as described earlier. All the assays were performed in triplicates. At the same time viable count depending on the procedure described by Harley and Prescott [9] was performed to determine the viability of bacterial cells within the biofilm.

Statistical analysis

All data were compared using one-way analysis of variance (ANOVA) and the Tukey Least significant difference (LSD) test. Differences were considered significant when $P<0.05$.

Results and Discussion

According to figure 1, *K. pneumoniae* was isolated in high percentages 36.13% (n= 56) out of *Klebsiella* isolates, while, *K. oxytoca* constituted 20.64% (n= 32), *K. terrigena* achieved 18.06% (n= 28), *K. mobilis* formed 16.13% (n= 25) and *K. ornithinolytica* accomplished 9.03 % (n= 14) out of *Klebsiella* isolates.

Many authors mentioned that *Klebsiella* spp. form the most biggest and important ratio among clinically isolated pathogens that prevailing in hospitals (Nosocomial pathogens) (12-15). Darweesh [16], in respect of contamination of local hospitals, mentioned that *Klebsiella* were isolated from one of these hospitals in a percentage of 31.42% from different kinds of clinical specimens. Rasool [17] mentioned that *Klebsiella* spp. accounted in an average 42% of nosocomial bacteria among number of local Iraqi hospitals.

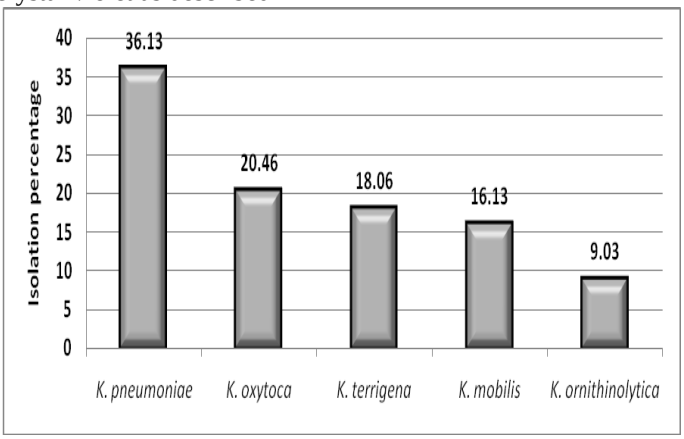

Figure 1: Isolation percentages (total number of *Klebsiella* isolates is 155)

It is very noteworthy that all local studies done by Al-Shukr [18], Al-Charrakh, [19], Najmadeen [20], Al-Sa'doun [21], Al-Qafaji [14], Makya, [22] and Omar-Zahid [15] indicated the relative high percent of nosocomial as well as the outpatient's infections in Iraq, a fact that we should stop at it and pay more attention.

Antibiotics resistance

Data presented in figure 2 showed a marked variation in the susceptibility pattern toward different antibiotics. The highest resistance percentages were found to ampicillin (81.5%), tetracycline (74.1%) and trimoxazole (74.1%). Moreover, 59.3%, 51.9%, 48.1%, 40.7%, 40.7%, 33.3% and 22.2% of the *K. pneumoniae* isolates were resistant to nitrofurantoin, cefotaxime, Tobramycin, nalidixic acid, ciprofloxacin, ceftriaxon and Gentamicin, respectively. On the other hand, Amikacin was the most effective antibiotics since it recorded the lowest resistance percentage; 7.41%. Furthermore, all the 56 isolates of *K. pneumoniae* developed multidrug resistance.

Klebsiella pneumoniae has been found to be the most common species to produce extended-spectrum β-lactamases (ESBLs)، and in some countries the prevalence of ESBL production approaches 50% [23,24].

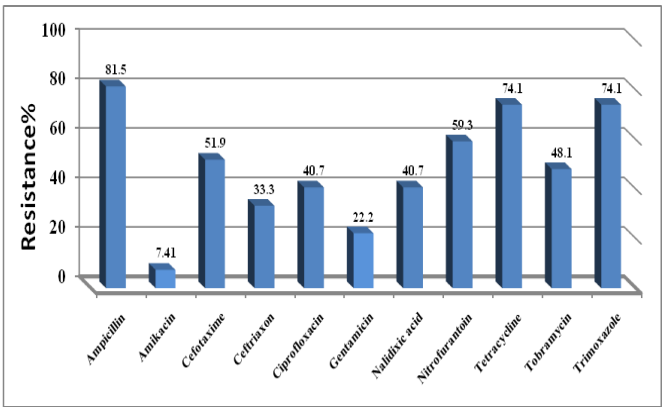

Figure 2: K. pnuemoniae isolates resistance towards Antibiotics

Klebsiella pneumoniae biofilm formation

All *K. pnumoniae* isolates assayed for the production of biofilm, and the results obtained are presented in table 1. The results indicated that each isolate showed a different potential to form biofilm under the same conditions of experimentation.

Twenty two (39.29%) isolates were high producers while 34 (60.71%) isolates were good producers and none of the testes isolates were poor producers. What's more, *K. pnumoniae* isolates; K14, K15, K17 and K19 produced the thickest biofilm; 0.935, 1.281, 1.227, and 1.698, respectively. Obviously, *K. pnumoniae* K19 achieved the highest biofilm thickness.

The differences in biofilm thickness resulted from different reasons such as differences in isolates capacity to form biofilm [25,26]. Furthermore, perhaps the primary number of cells that succeeded in adherence and the differences of quality and quantity of autoinducers (quorum sensing signaling molecules) that produced from each isolate play an essential as well as important role.

Al- Qafaji, [14] mentioned that all *K. pneumoniae* isolates were producing biofilm but with differences in. In other local study done by Makia [22], biofilm was evaluated by using test tube method, which found that pathogens isolated from urine specimen of catheterized patients, include yeasts, *Proteus* spp., *Klebsiella* spp, *Pseudomonas* spp. and *Candida* spp., formed biofilm but in various thicknesses.

Table 1: Absorbance for *Klebsiella pneumoniae* biofilm at 540 nm*

Isolate code	Absorbance ± SD	Isolate code	Absorbance ± SD
K1	0.601 ±0.079	K29	0.468 ±0.163
K2	0.362 ±0.185	K30	0.263 ±0.031
K3	0.742 ± 0.124	K31	0.203 ±0.052
K4	0.657 ±0.158	K32	0.451 ±0.145
K5	0.481 ±0.113	K33	0.502 ±0.167
K6	0.480 ±0.160	K34	0.414±0.133
K7	0.447 ±0.169	K35	0.384 ±0.112
K8	0.476 ±0.056	K36	0.427 ±0.137
K9	0.555 ±0.184	K37	0.554 ±0.166
K10	0.336 ±0.167	K38	0.570 ±0.071
K11	0.283 ±0.045	K39	0.602 ±0.181
K12	0.535 ±0.118	K40	0.232 ±0.110
K13	0.669 ±0.006	K41	0.461 ±0.041
K14	0.935±0.205	K42	0.183 ±0.058
K15	1.281±0.355	K43	0.507 ±0.094
K16	0.286 ±0.051	K44	0.392 ±0.175
K17	1.227±0.335	K45	0.568 ±0.151
K18	0.307 ±0.115	K46	0.133 ±0.091
K19	1.698±0.322	K47	0.250 ±0.046
K20	0.451 ±0.098	K48	0.351 ±0.069
K21	0.502 ±0.056	K49	0.544 ±0.162
K22	0.481 ±0.081	K50	0.214 ±0.048
K23	0.484 ±0.183	K51	0.304 ±0.131
K24	0.422 ±0.114	K52	0.437 ±0.159
K25	0.454 ±0.167	K53	0.254 ±0.031
K26	0.520 ±0.051	K54	0.270 ±0.057
K27	0.712 ±0.251	K55	0.606 ±0.281
K28	0.342 ±0.105	K56	0.532 ±0.137

*Each datum is a mean of triplicate. SD= standard deviation. LSD=0.267. P= 5.07×10^{-7}

Minimum Inhibitory Concentrations (MICs)

Results of MIC and MBC are shown in table 2. For Amikacin the lowest MIC was for K14 and K17 isolates, while the highest MIC was for K15 isolate. While Gentamicin showed the lowest MIC for K15 and K17 isolates, while the highest MIC was for K19 isolate. While the MBC for Amikacin the lowest MBC was for K14 and K17 isolates, while the highest MBC was for K15 isolate. For Gentamicin the lowest MBC was for K15 isolate, while the highest MBC was for K19 isolate.

Table 2: Values of MICs of elected antibiotics for *Klebsiella pneumoniae* isolates

Klebsiella pneumoniae isolate	Amikacin (Ak)		Gentamicin (Gn)	
	MIC (µg/ml)	MBC (µg/ml)	MIC (µg/ml)	MBC (µg/ml)
K14	10	30	10	20
K15	70	100	5	10
K17	10	30	5	30
K19	30	50	70	90

Inhibitory effect of antibiotic on biofilm in microtitration plates

The effect of MIC of Amikacin and Gentamicin, which achieved the highest sensitivity percentage among several antibiotics, were assessed on biofilm for the isolates K14 and K15 and the results revealed that the absorbance and viable count for these isolates declined in comparison to pretreatment data($P<0.05$). These isolates showed significant differences ($P<0.05$) after treatment with Amikacin and gentamicn. K17, according to OD parameter, showed significant

reduction (P<0.05) in comparison to control when treated with Amikacin while in case of Gentamicin there was an insignificant reduction; however, viable count showed that Gentamicin as well as Amikacin had a significant effect (P<0.05) on biofilm of *K. pneumoniae* K17 (table 3).

Regarding the isolate K19 a significant differences were observed when treated with Amikacin while insignificant decrease in viable count was found when the treatment involved Gentamicin.

Despite all these results, all tested biofilms even those which showed a decline in absorbance or in viable count, when cultured on plate count agar the result assured a presence of live cells, as shown in table 3. These finding strongly suggest that most efficient antibiotics which succeeded in killing the planktonic cells of *K. pneumonia*e, failed to effectively kill all bacterial cells within the biofilm which will be able to establish a new biofilm. This result agreed with many other authors since they reported that bacterial biofilm is significantly less responsive to antibiotics and antimicrobial stressors than planktonic organisms of the same species (27-30).

Table 3: O.D. and viable count (CFU/ml) for *Klebsiella pneumoniae* biofilm after treatment with Amikacin and Gentamicin[1, 2].

Klebsiella pneumoniae isolate	parameter	Before treatment	After treatment with		P value	LSD
			Amikacin	Gentamicin		
K14	OD	0.903 ± 0.238 a	0.484 ± 0.040 b	0.34 ± 0.017 c	0.006498	0.126
	VC	658667 ± 7767.45 a	260667 ± 6027.71 b	197667 ± 4509.25 c	2.00E-10	5644
K15	OD	2.225 ± 0.88 a	0.594 ± 0.075 b	1.057 ± 0.076 c	0.019823	0.462
	VC	8466667 ± 208166 a	373667 ± 3511.88 b	945400 ± 14640.12 c	4.00E-10	108909.21
K17	OD	0.532 ± 0.193 a	0.163 ± 0.027 b	0.465 ± 0.106 a	0.026	0.27
	VC	348333 ± 3055.05 a	5567 ± 208.16 b	284000 ± 2645.75 c	4.00E-12	2112
K19	OD	0.614 ± 0.126 a	0.436 ± 0.076 b	0.71 ± 0.180 c	0.111	0.096
	VC	446667 ± 4141.45 a	219333 ± 2516.61 b	464000 ± 5686.24 a	9.00E-10	3870

[1]O.D.= optical density, VC= viable count, LSD= least significant difference. Each datum is the mean of triplicate.[2]Similar letters in the same raw refer to insignificant differences.

Anderl *et al.* [31] attributed such failure to the ability of an antibiotic to penetrate a biofilm. There is also evidence for gradients of physiological activity in response to anti microbial treatment. For example, the pattern of respiratory activity of a *K. pneumoniae* biofilm in response to monochloramine (an oxidatively active biocide) treatment showed that cells closest to the biofilm–bulk-liquid interface lost activity first. Similarly, when biofilm cells were treated with the antibiotic fleroxocin, cell elongation was observed and was most extreme in cells located close to the exposed side of the biofilm. Consequently, the response to antimicrobial agents can greatly vary, depending on the location of a particular cell within a biofilm community [32].

Drenkard [33] reported that expression and over production of multidrug-resistant (MDR) pumps may play an important role in increasing biofilm resistance to antimicrobial agents. Moreover, results showing that expression of genes encoding the AcrAB efflux pump was enhanced upon *E. coli* entry into stationary phase suggested that efflux pumps may be up-regulated in biofilms.

Even in planktonic cultures of *K. pneumonia*, deprivation of oxygen or nutrients, respectively, has resulted in slow growth and antibiotic resistance innate and induced resistance mechanisms of bacterial biofilms [31,34]. Because

antibiotics typically act upon rapidly growing bacteria, slow or non growing microorganisms would be protected from killing [35].Yet the drugs only affected the biofilm edge [31,36]. Thus, limited metabolic activity within these biofilms, created by oxygen and nutrient gradients, protects the constituent bacteria from antibiotic killing. The slow growth and altered metabolic activity apparent in biofilms have led some researchers to suggest that the biofilm bacteria are in a stationary-phase state [31].

A further complication is that the spread of antibiotic resistance genes borne on plasmid DNA (pDNA), within and between species, is greatly exacerbated in biofilm communities [30].

As a consequence to this increase in resistance, researchers have turned to a number of alternatives to synthetic antibiotics, including: bacteriophage [37] and bacteriophage lytic enzymes [38], probiotics [39,40] and human antimicrobial peptides (defensins, cathelicidins, and histatins) [41]. The success of these alternatives awaits much development and optimization. Unfortunately, most of these alternatives are still based upon some mechanism of killing or terminating the target bacteria; an approach some feel pre-ordains the development of resistance in bacteria [30].

Additionally, table 3 demonstrates that the isolate *K. pneumoniae* K19 showed an increase in biofilm thickness and viable count after treatment with Gentamicin. Hoffman *et al.* [42] reported that sub inhibitory concentrations of amino glycoside antibiotics induced biofilm formation in *P. aeruginosa* and *E. coli*. Hoffman's results along with those of Bagge *et al.* [43] suggest enhanced biofilm formation in the presence of antibiotics may be one universal defense mechanism of bacteria in avoiding the lethal effects of antibiotics. Delivery of sub-lethal dosages of antibiotics can lead to accelerated biofilm formation and induced virulence factor expression.

The more radical hypotheses for biofilm resistance to antibiotics is that an "altruistic" majority of sub lethally damaged cells in a population commit suicide (apoptosis), thereby providing some protection to the survivors (persisters) [44].

The presence of persister cells and small-colony variants (SCVs) has been associated with enhanced antibiotic resistance of many organisms in biofilms as shown in figure 2.

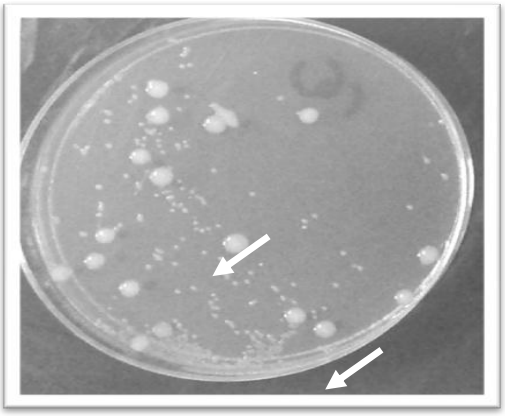

Figure 2: Small-colony variants (arrows) in Gentamicin resistant *Klebsiella pneumoniae* K14 biofilm.

The killing of biofilm cells by all of the antibiotics showed the presence of persister cells– most cells in the population died; leaving a fraction that persisted, even at higher concentrations of the antibiotics. These persisters represented a transient resistant phenotype and reverted to a killing curve resembling that of the wild-type parent upon re-exposure to the antibiotics [45].

The treatment of planktonic cells with Amikacin yielded a small subpopulation of survivors that included persisters (at numbers significantly lower than for the biofilms) and highly resistant, stable SCVs with an increased biofilm-forming capacity in comparison with the wild-type parent. Biofilms harbour a large number of persisters in comparison with planktonic cultures, which either do not harbor persisters or harbour only a small number, SCVs have an increased biofilm-

forming capacity and this may explain the frequent isolation of SCVs from biofilm-associated infections. The intrinsic resistance of these variants may in turn contribute to the enhanced antibiotic resistance of the biofilms thus formed [46].

While the specific mechanism(s) of SCVs formation still remains largely unresolved, it is suggested that regulatory components may be required for the emergence of this phenotype upon exposure to aminoglycosides. The association was found between the stress-associated sigma factor B of *S. aureus* and the SCVs phenotype raises the possibility that this phenotype confers a selective advantage to the bacterium in difficult environments. In this regard, the slower metabolism of SCVs may decrease their susceptibility to antibiotics and other harmful molecules, thus enabling the survival of the bacterium in stringent conditions and the establishment of chronic and difficult to-treat infections [47].

Results of Cerca *et al*. [29] demonstrated that antibiotics that target cell wall synthesis have a reduced activity in biofilms, independent of the size of the antibiotic molecule, but antibiotics that target RNA and protein synthesis have similar activities on planktonic cells as they do on cells in biofilms, suggesting that the phenotypic resistance of cells in biofilms to antibiotics is affected primarily by the mechanism of action of the antibiotic.

Noticeably, table 3 showed a reduction in the absorbency of biofilm than it depicted in table 1 and that could be partly ascribed to the age of biofilm has an effect on biofilm thickness (i.e. absorbency).On contrast, a remarkable exception accredited to the isolate *K. pneumonia* K15 since it revealed an increase in thickness after 48 hr of incubation.

As the biofilm grows, the nutrient diffusion limitation increases, resulting in a decrease of the growth rate in the active region. As a consequence, in a thick biofilm the base will be highly aerobic but the interface with the wastewater will be fully anaerobic. These changing growth conditions within the biofilm may influence EPS production. This in turn affects the cohesive energy, resulting in surface layers of the biofilm having low polysaccharide concentrations and low levels of cohesive energy per unit volume [48]. Moreover, Hurtley [49] mentioned that as biofilms aged, nutrients become limiting and waste products accumulate, and biofilm disassembly is triggered.

Lactobacillus spp. Isolation and Identification

Based on the criteria mentioned by Holt and Krieg [50] and Hammes and Hertel, [51], tow *Lactobacillus* isolates were identified as *L. acidophilus* and *L. fermentum* and designated Lb1 and Lb2, respectively.

Inhibitory activity of *Lactobacillus* supernatant on planktonic *K.* ***pneumoniae***

Neither *Lactobacillus* neutralized supernatant nor did MRS broth have an effect on planktonic *K. pneumoniae* isolates. However, results showed that acid supernatant developed an inhibitory effect observed by formation of inhibition zones around the acidic supernatant-containing wells (figure 2).

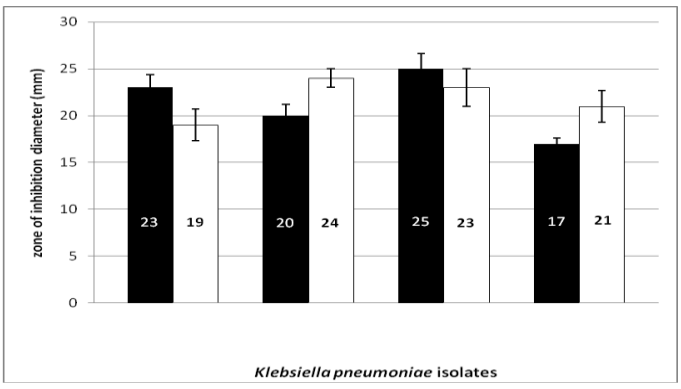

Figure 3: Inhibitory effect of acidic supernatant of *L. acidophilus* (white bars) and *L. fermentum* (black bars) against *Klebsiella pneumoniae*

Kenreigh and Wagner [52] pointed out to lactic acid bacteria produces specific natural antibiotics that inhibit and eliminate pathogenic bacterium. For example, *L. acidophilus* produces acidophilin, hydrogen peroxide, bacterial peptides; these are all anti-septic to pathogenic bacterium. Also the same authors indicated that *Lactobacillus fermentum* is a well-characterized probiotic strains with efficacy in the prevention and treatment of urogenital infections in women, it also produces hydrogen peroxide and inhibited the growth of intestinal and urogenital pathogens. Affection mechanism of bacteriocins explained by affection on cellular membranes instability and changing its aspiration by formation of complex or ionic canals by binding itself receiving particles such as lipids or proteins, lead to dispersion and lose ability to formation of protons propelling force [53]. In spite of the sensitivity of gram negative bacteria to bacteriocins produced by lactic acid bacteria is not common, Riaz *et al*. [54] suggested that bacteriocin produced by *L. fermentum* and *L. acidophilus* can be used for the control of infection caused by cephalosporin resistant *E. coli*. Westbroek *et al*. [5] mentioned that abundant of researches involved the remarkable ability of *Lactobacilli* in inhibiting pathogens growth through its bactericidal activity (such as production of bacteriocins and the hydrogen peroxide and by producing lactic acid as a byproduct of metabolism) and allow the body's immune system to overcome the infection without the use of antimicrobials.

Inhibitory activity of *Lactobacillus* supernatant on biofilm of ***K. pneumoniae*** Treating biofilms of *K. pneumoniae* K14, K15, K17 and K19 with supernatants of Lb1 and Lb2, significant differences ($P<0.05$) were found in O.D. and viable count between pre and post treatment. The obtained results are presented in table 4.

Table 4: O.D. and viable count (CFU/ml) for *Klebsiella pneumoniae* biofilm after treatment with acidic supernatant of *Lactobacillus acidophilus* (Lb1) and *L. fermentum* (Lb2)[1, 2] and statistical analysis.

Klebsiella pneumoniae isolate	parameter	Before treatment	After treatment with supernatant of		P value	LSD
			Lb1	Lb2		
K14	OD	0.758 ± 0.107 a	0.384 ± 0.040 b	0.399 ± 0.017 b	0.166	0.115
	VC	45666.67 ± 3511.88	ND	ND	2.03E-07	1832.57
K15	OD	0.982 ± 0.133 a	0.393 ± 0.177 b	0.408 ± 0.076 b	0.164	0.106
	VC	906666.7 ± 24944.38	ND	446.666 ± 30.912	1.46E-09	15941.87
K17	OD	0. 906 ± 0.149 a	0.454 ± 0.148 b	0.435 ± 0.112 b	0.193	0.109
	VC	703333.3 ± 20548.05	1833 ± 169.96	ND	1.24E-09	23190.37
K19	OD	1.28 ± 0.271 a	0.543 ± 0.125 b	0.507 ± 0.179 b	0.171	0.120
	VC	90666.7 ± 44969.13	ND	ND	1.59E-08	26090.99

[1]OD= optical density, VC= viable count, LSD= least significant difference. Each datum is the mean of triplicate.[2]Similar letters in the same raw refer to insignificant differences.

Unlike the Amikacin and Gentamicin treatment which left live bacterial cells, *Lactobacillus* supernatant left no live cells except for two cases; Lb1 with K17 and Lb2 with K15. Furthermore, the cognate O.D. reading perhaps referred to the remaining exopolysaccharids.

Although the new biofilm control strategies based on the use of biological-based solutions with high antimicrobial activity and specificity seem to be a step ahead in overcoming the biofilm resistance issue [55]. We perceived a lack of information regarding the role of

Lactobacillus spp. in the inhibition of *Klebsiella* biofilms.

Maldonado *et al*. (2) studied the inhibitory effect of *L. fementum* acid supernatant on both the growth and the formation of biofilm. Because the strain used produces high levels of lactic acid, and hydrogen peroxide, it was able to inhibit the *Klebsiella* proliferation in associative cultures. The neutralized supernatant inhibited the biofilm formation in a lower degree than the other fractions evaluated. One of the possible explanations could be the release of different metabolites to the culture media, as for example, biosurfactants, or other substances.

More recently, deconvolution microscopy technique was employed to investigate the role of *L. rhamnosus* GR-1 (non H_2O_2 producer) and *L. reuteri* RC-14 (low H_2O_2 producer) in inhibiting *Gardnerella vaginalis* biofilm, Saunders *et al.* [56] showed that pH and hydrogen peroxide alone cannot be deemed responsible for displacement and loss of viability. The authors also stated that it is possible that biosurfactants known to be produced by *L. reuteri* RC-14 and *L. rhamnosus* GR-1 may have played a role in displacement, while production of anti infective bacteriocins and signaling molecules may have affected viability and pathogen growth.

CONCLUSION:

Neither neutralized supernatant of *Lactobacillus acidophilus* (yogurt isolate) and *L. fermentum* (vaginal isolate) have an effect on *K. pnumoniae* planktonic cells. Yet, results showed that acid supernatant developed an inhibitory effect. Treating the biofilms of *K. pnumoniae* with Amikacin and Gentamicin developed presence of live cells when cultured on plate count agar. Unlike Amikacin and Gentamicin treatment which left live bacterial cells, *Lactobacillus* supernatant left no live cells in the *K. pnumoniae* biofilms. Such finding highly suggested this supernatant as a potent antimicrobial agent against *K. pneumoniae* biofilms.

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