

Antibiosis of Cefotaxime/Clindamycin and *Lactobacillus acidophilus* on Related Bacteria to Diabetic Foot Ulcer

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Abstract

Diabetic foot complications are very common and represent a serious health problem in Mexico because of their high frequency, high costs and difficulties in handling. The treatment of choice to inhibit bacteria related to diabetic foot ulcer consists mainly of the use of cefotaxime however the problem with this treatment (antibiotics) is not always effective due to the pathophysiological condition of the patient, together with the resistance bacteria develop to the drugs. OMS has suggested the use of probiotics for research directed to the development of microbial interference therapies. This project used the Lyophilized conditioned medium with probiotics, extracellulars of probiotics, because there are reports in which wound healing in mice is observed employing probiotics. The objective of this study was to evaluate the biological activity of cefotaxime, clindamycin and thelyophilized conditioned media *Lactobacillus acidophilus* (LCMLa) on bacterias isolated from diabetic foot ulcer, this bioassay was performed by the turbidimetric method. The macroscopic analysis of the colonies was carried out and the morphological analysis of the bacteria was carried out using the atomic force microscope; in addition, the type of Gram and oxygen requirements for its growth were determined. From the diabetic foot ulcers, three strains were isolated, of which strain 1 and 3 whose morphology corresponds to a bacillus, was susceptible to cefotaxime and to the lyophilized conditioned medium of *L. acidophilus*. The potential of microbial interference that exhibits *L. acidophilus* on bacteria related to diabetic foot ulcer is demonstrated.

Keywords

Cefotaxime, Clindamycin, *Lactobacillus acidophilus*

1. Introduction

Diabetes is an assimilation of carbohydrates disorder, carbohydrates are all possible forms of simple and complex sugars in our food chain. In order that the sugar obtained from food can enter the cell, the place where it is consumed, the human being needs a special hormone, insulin. In diabetes the production of enough insulin in the pancreas usually occurs, but at the cellular level sensitivity to the effect of insulin is low. It is known as a relative insulin deficiency. Diabetes can cause diabetic angiopathy, neuropathy and one of the late complications of diabetes is the diabetic foot ulcer which is the most devastating.

Worldwide, there are over 220 million people with diabetes. In Mexico, diabetes has become the leading cause of death by contributing 12% of total deaths. More than 80% of diabetes deaths occur in countries of low and middle income. Nearly half of those deaths are in people under 70, and 55% women [1].

The diabetic foot is the loss of subcutaneous barrier integrity. As a consequence of diabetic neuropathy, the protective sensation is lost, which favors the appearance of skin lesions, mainly traumatic whereby the skin barrier is broken and the penetration of microorganisms may occur. Beside neuropathy, there is often ischemic factor contributing to hinder wound healing.

For treatment of patients suffering from diabetic foot ulcer, drugs of choice are cefotaxime and clindamycin, however taking probiotics surprised us an important role also for this treatment, using for the first time this term probiotic in 1965 to name the products of gastric fermentation [2] 1989 and defining them as “those living microorganisms, mainly bacteria and yeast, which are added as a dietary supplement and that beneficially affect the development of the microbial flora in the gut” [3], which can be functional for the general public or to particular groups thereof [4].

A recent research shows evidence that suggests that the soluble polysaccharide fraction from *L. acidophilus* may constitute a novel anticancer agent, which manifests a high degree of selectivity for human cancer cells [5]. *Lactobacillus* Gorbach-Goldin known as *Lactobacillus* GG has a beneficial effect on human health [6]. Studies in children older than two years who received probiotic supplementation showed improvement in cases of diarrhea (7% to 31%) as in diarrhea associated with rotavirus (10% to 39%), these results showed significant difference [7].

However recently it reported a possible response mechanism about probiotics-epithelial cell interaction [8]. *In vitro* studies have shown that *Lactobacillus salivarius* inhibits the ability of *H. pylori* to colonize the mouse stomach mucosa [9], and *Lactobacillus acidophilus* also exert an inhibiting power in reported cases of diarrhea [10].

1.1. Cefotaxime and Clindamycin

For the treatment of bacterial infections in diabetic foot ulcer, a variety of therapeutic agents used for topical treatment, including products derived from

hydrogen peroxide, iodine, hypochlorous acid (HOCl), sodium hypochlorite (used NaOCl), and benzoyl peroxide chlorhexidine, nitrofurazone, benzalkonium chloride, among others. However, the traditional allopathic treatment for diabetic foot ulcer, currently used as drugs of choice cefotaxime and clindamycin. These drugs are the third generation of cephalosporins. For treatment of diabetic foot ulcer caused by gram-negative bacteria, ciprofloxacin and other quinolones and trimethoprim are chosen [11].

a) **Cefotaxime:** Is an antibiotic from the group of third-generation cephalosporins. It has a broad spectrum of activity against bacteria that cause many kinds of infections, including those affecting the lung, skin, bones, joints, stomach, urinary tract, gynecological and blood. It can be applied muscular and intravenously. Overall, cefotaxime is well tolerated and adverse reactions are rather local after administration intravenously or intramuscularly. The most common side effects are pain at the injection site, induration and phlebitis. Cefotaxime may trigger hypersensitivity reactions where rash, itching, fever and eosinophilia are included. Cefotaxime is active against *Streptococcus pneumoniae* and *Staphylococcus aureus*. These two bacteria are the most representative in the *Diabetes mellitus* disease and in the diabetic foot ulcers. However these drugs sometimes are not entirely effective because there are involved several intrinsic factors as the pathophysiology of the patient, sometimes their circulatory system is not efficient, causing liver and kidney damage [12].

b) **Clindamycin:** Is a semisynthetic antibiotic produced by the substitution of 7(R)-hydroxyl group by chlorine in position 7(S) of the parent compound, lincosamides and lincomycin derivative by substitution of a chlorine atom for a hydroxyl group (HO). It is most effective against infections that involve the following types of organisms: aerobic gram-positive cocci, including some staphylococci and streptococci and anaerobic gram-negative bacilli, including some members of the genera *Bacteroides* and *Fusobacterium*. Common side effects are mainly gastrointestinal disorders [12]. Clindamycin is active against most gram-positive bacteria. *Staphylococcus aureus*, *S. epidermidis*, *Streptococcus pyogenes*, *S. pneumoniae*, *S. viridans*, *S. durans*, *S. bovis*, *Clostridium tetani*, *C. perfringens* and *C. diphtheriae*. Most of which are present in the diabetic foot ulcer.

In various pathological alterations in the human, in which bacteria are involved, these develop resistance to the drugs of choice, due to factors such as the previously explained, as well as the failure to follow the treatment or also because of other factors such as patient hygiene, food and drug availability. In our gut millions of bacteria of many types live, forming what we call intestinal microbiota. Some of these bacteria (a small percentage) can harm us. Other bacteria are beneficial and are what we call probiotics.

1.2. Probiotics and Their Clinical Significance

Probiotics produce lactic acid and acetic acid which create a pH alteration that works as a digestive system antiseptic and at the same time it minimizes the spread of pathogenic microorganisms by competing for nutrients and lodging in

the intestinal walls [13]. Some mechanisms of action of probiotics for resistance to pathogens are as follows: Production of antimicrobial substances such as lactic acid, bacteriostatic substances, competition for adhesion receptors, competition for nutrients and immune stimulation, effects on cell membranes by altering their permeability pathogenic microorganisms, altering pH levels and oxygen making them unfavorable to pathogens [14].

In a recent paper, the improvement of skin sensitivity is reported when using lyophilized *Bifidobacterium longum* in both *in vitro* and *in vivo* investigations [15]. In other studies have demonstrated that probiotics are able to prevent *Candida* growth and colonisation in neonates, whereas their role in preventing invasive candidiasis in such patients is still unclear. Moreover, there are no published data on role of probiotics supplementation in the prevention of candidiasis in critically ill children beyond neonatal period [16].

Other researchers evaluated the biological activity of *Lactobacillus plantarum* and *Lactobacillus brevis* exopolysaccharides (EPS) on wounds of rats. In which it was shown that when using *L. plantarum* EPS completely healed of the wound in 21 days, the mice were subjected to this treatment showed a marked improvement compared to the negative control (untreated wound) and the positive control (wounds treated with Eucerin), in the experiment (eucerine + EPS-*L. brevis*). Also in this investigation, no infection is observed in the wounds in rats for 21 days of the trial [17]. Probably because probiotics prevent infection in the wound the antimicrobial mechanism apparently occurs by the secretion of antimicrobial peptides produced by probiotics preventing infection by pathogenic bacteria adhering to epithelial cells [18].

2. Material and Methods

2.1. Biological Material

Lactobacillus acidophilus (strain ATCC 4356).

2.2. Antibiotics

Clindamycin and Cefotaxime.

2.3. Reagents and Solutions

10 N Sodium hydroxide: NaOH (40 g) was dissolved in 100 mL of distilled water.

0.1 N HCl: A volume of 50 mL of distilled water was diluted to 0.41 mL of concentrated HCl.

1% Ferric ammonium citrate (FAC): FAC (0.1 g) was dissolved in 10 mL of deionized water and the solution was kept refrigerated in an amber bottle at 4°C until use.

2.4. Antibiotics

The antibiotics were prepared according to the manufacturer's instructions and be able to conduct research about bacteria diabetic foot.

2.5. Media

a) *L. acidophilus* culture: Composition of the medium used for *L. acidophilus* culture is described below: Dissolve in this order: 2.5 g Yeast extract, 0.50 g Sodium chloride, 0.5 g L-Cysteine Hydrochloride, 0.05 g Ascorbic Acid, 0.25 g Potassium Phosphate Dibasic, 0.15 g Potassium Phosphate Monobasic, 0.124 mg Ferric Ammonium Citrate, 10 g Casein Digest Peptone and 5.0 g Glucose, bring final volume to 250 mL in distilled water and pH to 7.00 using 1 N Sodium Hydroxide solution). Dispense in 5 mL amounts into 13 × 100 mm borosilicate glass tube and autoclave for 15 minutes at 121°C with 15 Lbs pressure. The sterile MPT-broth can be stored frozen at -20°C for several months.

b) Bacteria isolated from foot ulcer

Nutrient broth. For the bacteria culture nutrient broth was prepared by mixing 8 g of nutrient broth in 1 L of purified water and pH was adjusted to 7.0 with 10 N NaOH. Subsequently 5 mL aliquots were placed in borosilicate tubes for cultivation of 13 × 100 with screw-capped, immediately they were autoclaved at pressure of 15 pounds per square inch at 121°C for 20 min. Then they were allowed to temper and stored at 4°C until use.

Nutrient agar. 18 g of nutrient agar were mixed in 1 L of purified water and the pH was adjusted to 7.0 with 10 N NaOH. The medium was allowed to boil for a few seconds to homogenize the components, then it was sterilized by autoclaving at pressure of 15 pounds per square inch at 121°C for 20 min. Later when the agar was still hot (tempered to the limit of tolerating the flask with the cheek) it was deposited in disposable petri dishes, which was used immediately in the bioassay.

2.6. Methods

2.6.1. Isolation of Bacteria from Diabetic Foot Ulcer

Samples for microbiological processing were obtained by deeply gathering them from the ulcer performing a sweep of the affected area. Bacteriological sample obtained directly from the diabetic foot ulcer, was subsequently inoculated in MPT-agar containing 55 ppm per mL of Microdacyn 60. They were incubated at 37° for 24 hours and subsequently bacterial colonies resistant to treatment were selected and these bacteria were used for bioassay.

2.6.2. Maintenance

Bacteriological sample obtained directly from the diabetic foot ulcer, was subsequently inoculated in MPT-agar containing 55 ppm per mL of Microdacyn-60. They were incubated at 37° for 24 hours and subsequently bacterial colonies resistant to treatment were selected and these bacteria were used for bioassay. From a strain that were kept refrigerated at 4°C, successive inoculations were done in 13 × 100 mm borosilicate tubes with screw cap containing 5 mL of nutrient broth which were inoculated with a microstreaker and immediately incubated at 37°C for 18 to 24 hours.

2.6.3. Atomic Force Microscopy

For observations of Atomic Force Microscopy the samples were preparing according [19].

2.6.4. Bacterial Identification

The identification was made through the automated VITEK system (bioMerieux), using GNI (Gram Negative Identification) cards. The methodology was performed according to the manufacturer's specifications.

2.6.5. *L. Acidophilus*

- Preparation of culture media MPT-broth. The composition and Preparation procedure was previously described in the Section 2.5.
- Preparation of culture media MPT-agar. The same composition as the MPT-broth but supplemented with nutritive agar 23 g per Liter.
- Maintenance, growth kinetics of probiotics, obtaining probiotic conditioned medium, Obtaining lyophilized conditioned media of *L. acidophilus* and Preparation of stock solution of probiotics were made in accordance with [20].

2.7. Bioassay

Bioassays were performed in accordance with **Table 1**. Tubes of 13 × 100 mm were used, each one containing 5 mL of MPT medium and treated according MPT experimental strategy, then all tubes were incubated at 37°C/5hours. Absorbance readings for each treatment were taken immediately with the spectrophotometer at 635 nm (Spectronic-Genesys5®) and subsequently data analysis were performed.

2.8. Determination of the Percentage Inhibition (PI)

Value PI were determined according to the equation as below:

$$PI = 100 - \left[\frac{(\text{absorbance value of treatment})}{(\text{absorbance value of control})} \right] \times 100$$

Each bioassay was carried out in three independent events in triplicate.

2.9. Statistical Analysis

For the analysis of the bioassays three independent events are performed in

Table 1. Experimental strategy.

Treatments	MPT Medium	Inoculation of strains	LCMLa [mg/ml]			Antibiotics [µg/mL]	
			40	400	800	0.15	0.25
Control	Control						
Lyophilized Conditioned Media of <i>L. acidophilus</i>	LCMLa						
Antibiotics	Cefotaxime						
	Clindamycin						

triplicate, the results are analyzed by Analysis of variance with a $P < 0.05$ using SPSS program for Windows 2010.

3. Results

3.1. Biochemical and Morphological Features of Bacterium Isolated from Ulcer Foot Diabetic

Strains isolated from diabetic foot ulcer was identified as 1, 2 and 3, in **Table 2** shows the results of the microscopic analysis (morphology, measurement at AFM and determination of Gram reaction) and macroscopic description and requirement of oxygen.

Figure 1 shows the images of strains 1, 2 and 3 observed and analyzed under the atomic force microscope (AFM). Strain 1 which has a bacillus morphology and an average length of 3.74 μm and an average diameter of 0.9 μm . In strain 2 observed at AFM it is seen as an aggregate of yeast cells and have an average length of 3.26 μm . Strain 3 has a bacillary morphology and has an average length of 2.22 μm and a diameter of 1.12 μm .

3.2. Response of the Strains Isolated from Diabetic Foot Ulcer to Antibiotics and LMCLa

Table 3 and **Figure 2**, shows the concentrate analysis results of the biological response in strains 1, 2 and 3 to the treatment of cefotaxime and clindamycin and LCMLa. The results were taken and analyzed according to their treatment and dose.

Clindamycin only showed inhibition on strain 3, the doses of 0.25 and 50 $\mu\text{g/mL}$ did not differ between them, but they did with doses of 0.15 $\mu\text{g/mL}$, the percent inhibition was 88, 89 and 18 respectively.

Cefotaxime, in all concentrations, produced a major inhibition of the bacteria isolated from diabetic foot ulcers which indicates a high rate of toxicity because it does not allows growth, but strain 1 was the most sensitive, at the dose of 0.15, 0.25 and 0.5 $\mu\text{g/mL}$ evaluated there was a percentage inhibition of 85, 87 and 88

Table 2. Description of some characteristics of the strain 1, 2 and 3.

Characteristic	Strain 1	Strain 2	Strain 3
Morphology	Bacillus	Yeastlike cell	Bacillus
Gram	Negative	Negative	Negative
Measurement at AFM*	4.26 μm (length)	3.34 μm (diameter)	2.22 μm (length)
Macroscopic description	Small colonies, cream-colored, raised edges, creamy consistency	Colonies beige, glossy and rounded edges.	Colonies with smooth edges, high, white-creamy colored.
Oxygen requirements	Aerobic/facultative	Aerobic/facultative	Aerobic/facultative

*AFM = Atomic Force Microscopic.

Table 3. Percent inhibition (%) of antibiotics and LMCLa on strains isolated from diabetic foot ulcer.

Treatments	Doses	Strain 1	Strain 2	Strain 3
Clindamycin (µg/mL)	0.15	0	0	18
	0.25	0	0	88
	0.50	0	0	89
Cefotaxime (µg/mL)	0.15	85	70	55
	0.25	87	68	60
	0.50	88	65	76
LCMLa (mg/mL)	40	3	0	9
	400	34	0	18
	800	40	0	26

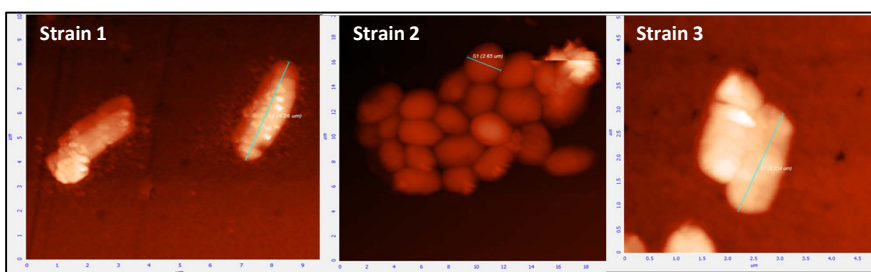


Figure 1. Microscopic appearance of the strains 1, 2, and 3 isolated from diabetic foot ulcer, which show at AFM the follow morphology: strains 1 and 3 present bacillary morphology, strain 2 present yeast like cell morphology.

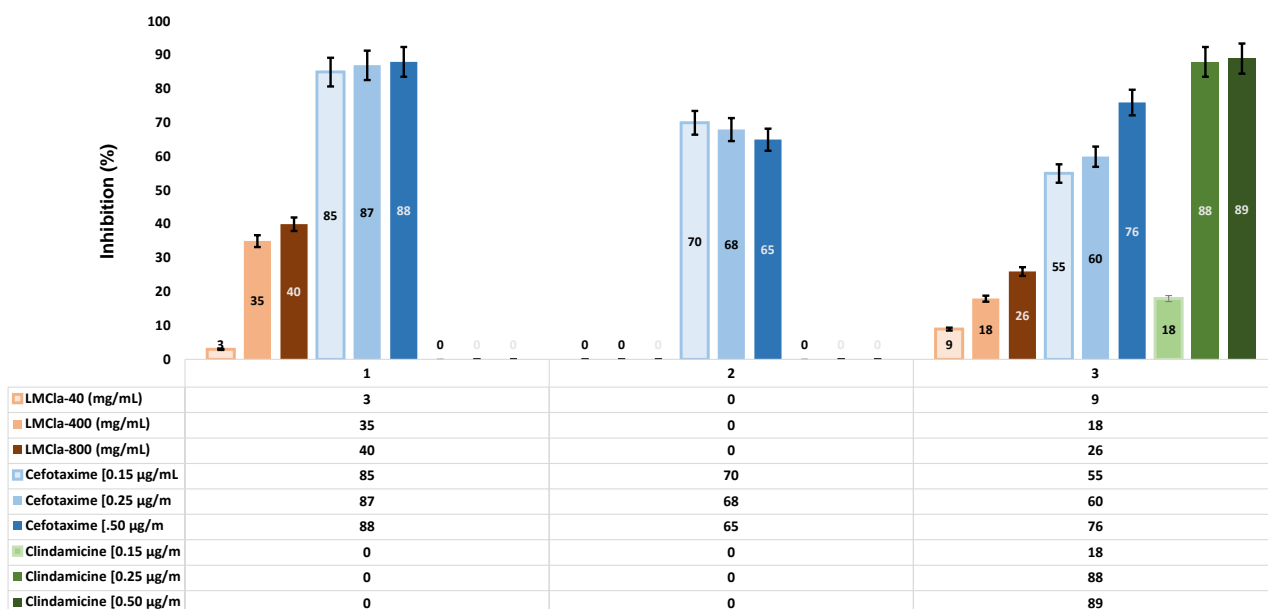


Figure 2. Comparison of the percentage of inhibition of strains 1, 2 and 3 by the antibiotics cefotaxime and clindamycin and the LMCLa at different doses evaluated.

respectively, between these values no significant difference was observed, and with respect to the inhibition observed in strains 2 and 3 significant difference is observed.

The LMCLa at the doses evaluated of 40, 400 and 800 mg/mL presented inhibition of strains 1 and 3, higher percentage of inhibition was observed on strain 1 at the dose of 800 mg/mL, the inhibition of LMCLa also inhibited the strain 3, but it has a lower percentage of inhibition, and it is important to emphasize that the 400 mg/mL dose of the LMCLa inhibited 18%, equal to the inhibition of clindamycin at a dose of 15 µg/mL.

Strain 1 was identified by the VITEK system, because it was the strain most susceptible to LMCLa and to cefotaxime, the result indicates that it is the *Pseudomonas sp.* and the strain 3 was identified as *Enterobacter sp.* This strain was the most susceptible to LMCLa and cefotaxime and clindamycin.

4. Discussion

Most probiotics from the genera Lactobacillus, Streptococcus and Bifidobacterium, are included in the category GRAS (Generally Regarded as Safe) by international regulatory bodies [21].

In this project was isolated from diabetic foot ulcers three microorganisms, identified in this work as strain 1, strain 2 and strain 3. According to the analysis under the optical microscope the morphology of the isolated microorganisms was identified, strains 1 and 3 were observed as a bacillus Gram-negative, strain 2 had yeast form or yeast like cell, both are Gram-negative.

The detailed morphology of these bacteria was obtained using an atomic force microscope, whose use reinforces the microscopic appearance of bacteria isolated from diabetic foot ulcers, although using this equipment we can identify that strain 1 and 2 are bacteria and strain 3 it is a yeast.

Furthermore, the antibiotics cefotaxime and clindamycin were evaluated, on microorganisms isolated from diabetic foot ulcer (strain 1, 2 and 3), the results obtained show that clindamycin did not inhibit the growth of both the strain 1 and 2, but it inhibits strain 3, observing that at a dose of 0.5 µg/mL has a greater inhibition of 89% [22].

Cefotaxime showed the highest inhibition (88%) of strain 1 to the concentration of 0.50 µL/mL. According to these results we believe that cefotaxime has greater antimicrobial activity as it presented inhibitory activity on the three isolates strains, however this drug showed a higher inhibitory activity on strain 1.

Cefotaxime concentration in the exudate of ischemic ulcers was evaluated in 9 patients with severe obstructive arterial disease. The drug administration was systemic (1 g in 250 mL of saline 30 min) or regional into a vein in the foot while applying a tourniquet at the thigh for 30 min. Hygroscopic discs were used to collect samples of ulcer exudates at intervals of one hour for 4 hours. The concentration of cefotaxime is determined by HPLC chromatography. Significantly greater antibiotic concentration was obtained from the regional sample compared

to systemic administration (46 ± 16 versus 25 ± 14 , p less than 0.01) and a higher percentage of patients achieved 90 MIC. A stable concentration of the drug is observed during the 4 hour period indicating a decreased rate of removal of the antibiotic from the tissue ulcer. Therefore, the regional administration of antibiotics allows a higher concentration than systemic administration, for the treatment of ischemic leg ulcers [23].

Moreover, according to the results obtained in this study, it was observed that the lyophilized of conditioned media of *L. acidophilus* (LCMLa) showed growth inhibition of strain 1 at [400 and 800 mg/mL].

According to the literature, there are papers that report wound healing in rats by using exopolysaccharides material (EPS) from *Lactobacillus plantarum* and *Lactobacillus brevis*, in which by using the EPS from *L. plantarum* total wound healing was demonstrated in 21 days, showing a marked improvement compared to the negative control (untreated/untreated wounds) and control (treated only with eucerin) in experiment 1 (eucerin + *L. brevis* [10 - 11 CFU/mL]) and experiment 2 (eucerin + *L. plantarum* [10 - 11 CFU/mL]). No infection was observed in the wounds of rats within 21 days of the trial, probably because probiotics prevent wound infection due to the antimicrobial mechanism that apparently works by secreting antimicrobial peptides produced by probiotics, preventing the infection caused by the bacteria which adheres to epithelial cells [24].

However it has not been demonstrated the effectiveness of topical antibiotics in infections on producing sensitization problems. Systemic antibiotics are used when there is suspicion of infection: increased pain in the area, perilesional erythema, lymphangitis, increased ulcer size or concentration $> 10^5$ microorganisms per gram of tissue. The approach of antibiotic therapy is performed according to the severity of the ulcer.

Mentioned that patients undergoing abdominal surgery (resection of the liver, stomach or pancreas) benefited from *L. plantarum* 299 in terms of a smaller number of infections, antibiotics and incidence of other complications as well as a shorter hospital stay [25].

According to the information displayed on this work, may think about the development of a topical alternative for the treatment of diabetic foot ulcer, owing to it is a major systemic disease. However, there are still gaps in our knowledge about efficacy, cost-effectiveness, possible benefit-risk, optimal dose, frequency and duration of treatment of probiotics in the prevention of infections of various diseases in critically ill patients.

Further studies are required for the contribution to these treatments with related bacteria to diabetic foot ulcers as they represent a health problem with high incidence, nevertheless here is presented a different alternative to antibiotics which in many cases are not effective in patients who are shown pathophysiological conditions or even resistance that could be developed by bacteria for this treatment.

In the results presented in this work, we report for the first time the inhibitory potential of the lyophilized medium conditioned with *L. acidophilus* on bacteria isolated from diabetic foot ulcers.

5. Conclusions

- Two bacteria were isolated from the diabetic foot ulcer: *Pseudomonas* sp (strain 1) and *Enterobacter* sp (strain 3) and a yeast like cell (strain 2).
- Lyophilized conditioned medium of *L. acidophilus* (LCMLa) inhibited the growth of *Pseudomonas* sp and *Enterobacter* sp isolated from diabetic foot ulcers.
- Cefotaxime inhibited the growth of *Pseudomonas* sp, *Enterobacter* sp and yeast isolated from diabetic foot ulcers.

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