Journal of Dentistry and Oral Biology

Research Article
Published: 20 Sep, 2018

Antibacterial Efficacy of Lauroyl Arginine Ethyl (LAE) Contained In the Liquid Mouthwash In Relation to the Exposure Time - *In Vitro* Study

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Abstract

The aim of this study was to evaluate the bactericidal and fungicidal activity of the commercial mouthwash containing 0.147% v/v of LAE against potentially pathogenic microorganisms of the oral cavity, in relation to the exposure time. Studies were conducted with the use of standard strains of microorganisms (*Streptococcus oralis* ATCC 6249, *Staphylococcus aureus* ATCC 25923, ATCC 33384 *Aggregatibacter actinomycetemcomitans*, *Candida albicans* ATCC 10231), the suspension was contacted with 4 ml of mouthwash containing 0,147% v/v LAE or 18% ethanol or 0, 2% chlorhexidine solution. After 5, 10, 20, 30 and 60 min of incubation periods of each test sample and the control samples at the room temperature, a volume of 20 µl was placed on a Columbia agar base with 5% sheep blood (bacteria) or Sabouraud agar (fungi). After 24 hrs or 48 hrs of incubation at 37°C (bacteria) or 35°C (fungi), the grown colonies of microorganisms were counted using an "aCOLyte" colony counter (Symbios, Cambridge, UK). The total reduction in the growth of all tested strains of reference microorganisms was already visible after 5 min of incubation with mouthwash containing 0,147% v/v LAE and 0.2% chlorhexidine solution, while the incubation with 18% ethanol did not show such an effect even after 60 min.

Keywords: Lauroyl arginate ethyl; Chlorhexidine; Microbes; Biofilm

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Received Date: 30 *Aug 2018*
Accepted Date: *17 Sep 2018*
Published Date: *20 Sep 2018*

Citation:
Mocny-Pachońska K, Kuśka-Kielbratowska A, Trzcionka A, Mertas A, Tanasiewicz M. Antibacterial Efficacy of Lauroyl Arginine Ethyl (LAE) Contained In the Liquid Mouthwash In Relation to the Exposure Time - In Vitro Study. J Dent Oral Biol. 2018; 3(7): 1149.

ISSN: 2475-5680

Introduction

Oral health is one of the most important factors in the overall health of the body [1]. The environment of the oral cavity is colonized by a large number of microorganisms, with bacteria as the most common group. The microbes create a biofilm called plaque, which is a well-organized structure. Growing in stages, it adheres closely to the hard tissues of the tooth. Acquired film (pellicle), an amorphous and bacteria-less structure appears directly on the surface of exposed enamel and cementum. Due to its properties, it is colonized by bacteria of the *Streptococcus genus*, followed by *Actinomyces* and *Veillonela*. At the beginning, the colonizers modify the living conditions in the oral cavity, creating opportunities for relative anaerobes and anaerobes. The permanent bacterial flora is replaced by new a new species, leading to bacterial succession [2-4]. The lack of plaque elimination is a major cause of dental caries, as well as gingivitis and periodontitis [5]. The most common, as well as most effective way to eliminate it is mechanical removal procedures, such as brushing and flossing [1]. They do not, however, completely remove bacteria, so in order to promote the elimination of pathogenic micro-organisms, liquid mouthwash is recommended to control dental plaque and to prevent gum and periodontal diseases. A lot of research on oral hygiene focuses on commonly known substances, i.e. chlorhexidine, essential oils and cetylpyridinium hydrochloride [6]. A few years ago a new substance called L-Arginine ethyl ester was introduced to the dental market to be used in mouthwash preparations. Lauroyl arginine ethyl ester, referred to as LAE, is a novel substance derived from lauric acid and arginine. It belongs to the cationic surfactants with strong antimicrobial properties. About a decade ago, LAE was introduced to the food industry for food preservation. The huge success of LAE is related to its lack of smell, very fast and long-lasting antimicrobial effect, and lack of toxicity to the human body. It is a synthetic compound, but the human body decomposes it into natural endogenous compounds (lauric acid, arginine), which means it is safe for use [7-9].

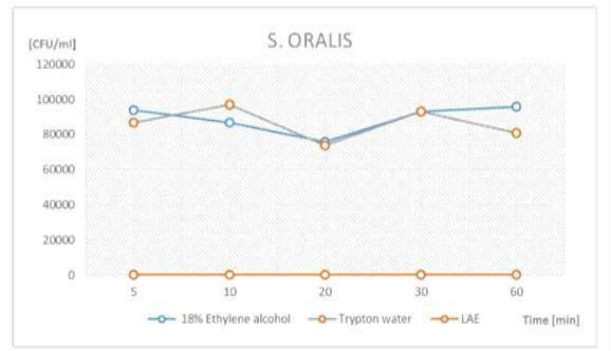

Figure 1: The efficacy of mouthrinse containing LAE against *Streptococcus oralis* depending on time of exposure.

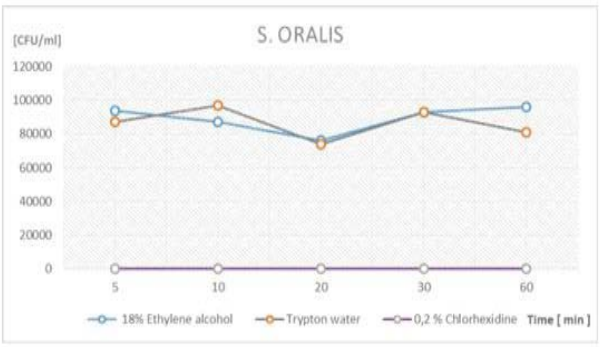

Figure 2: The efficacy of chlorhexidine against *Streptococcus oralis* depending on time of exposure.

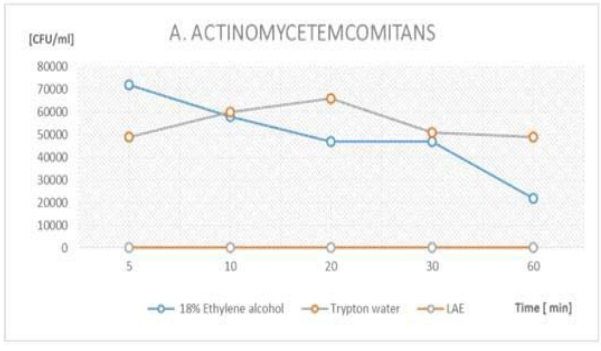

Figure 3: The efficacy of mouthrinse containing LAE against *Aggretatibacter actinomycetemcomitans* depending on time of exposure.

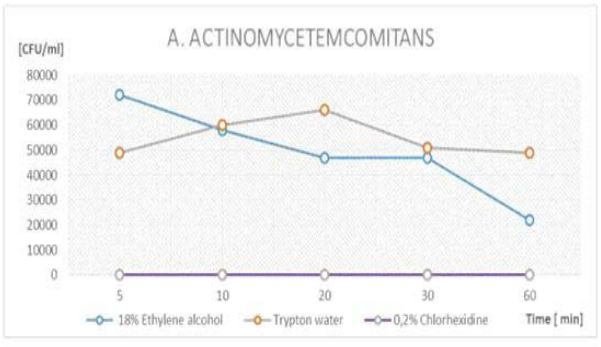

Figure 4: The efficacy of chlorhexidine against *Aggregatibacter actinomycetemcomitans* depending on time of exposure.

The aim of the study was to evaluate the bactericidal and fungicidal activity of LAE contained in the liquid mouthwash against the potentially pathogenic microorganisms of the oral cavity, in relation to the exposure time.

Evaluation of the bactericidal and fungicidal activity of Lauroyl Arginine Ethyl ester contained in the liquid mouthwash in relation to the exposure time.

Materials and Methods

The material used in the study comprised of standard strains of microorganisms present in the oral environment: *Aggregatibacter actinomycetemcomitans* ATCC 33384, *Candida albicans* ATCC 10231, *Streptococcus oralis* ATCC 6249 and *Staphylococcus aureus* ATCC 25923. The *Streptococci* strains were chosen due to their well known role in the etiology of dental caries [10]. Candida albicans and *A. actinomycetemcomitans* are mentioned as the main pathogens in the discussion on the etiology of periodontal disease. Oral infections caused by yeast of the *Candida albicans* (oral candidiasis) genus is a common problem [11]. In contrast, *Aggregatibacter actinomycetemcomitans* is a bacterium responsible for localized periodontitis [12]. A suspension was prepared with each of those strains and, subsequently, it was contacted with the mouthwash preparation containing LAE in its composition. The mouthrinse preparation contains (in accordance with the manufacturer's information): 0.147% Lauramide Arginine Ethyl Ester Hydrochloride (LAE), purified water, sorbitol, 18% denatured alcohol, glycerol, poloxamer 407, flavor, benzoic acid, sucralose and sodium benzoate [13]. Due to the 18% ethyl alcohol content in the product, a control test of the suspension containing each of the microorganisms with the same alcohol concentration was conducted. The effectiveness of the antimicrobial activity of mouthrinse containing LAE was compared with the effect of chlorhexidine, which is defined as the "gold standard" of antimicrobial activity. A concentration of 0.2% chlorhexidine was used for the purpose of the control test performed with use of the same diluent, which was tryptone water. Each of the materials used for the study was applied in a volume of 4 ml [14]. All research and control tests conducted as parts of the experiment were performed in triplicate. Then, both tested and control samples underwent 5, 10, 20, 30 and 60 min incubation at room temperature and 20 µl cultures of microorganisms were seeded on a suitable substrate medium. The suspensions of bacterial strains were plated onto Columbia agar with 5% sheep blood, and incubated at 37°C for 24 hrs. In contrast, fungal suspension was placed on Sabouraud agar and incubated at a lower temperature of 35°C for 48 hrs. After this time, the grown colonies were counted with an automatic "aCOLyte" colony counter (Symbios, Cambridge, UK).

Results and Discussion

In the group tested with mouthrinse containing LAE, no strains of the reference microorganism Streptococcus oralis grew on the media, regardless of the incubation time. Similar efficacy was observed for the 0.2% solution of chlorhexidine, while the use of an 18% ethanol component of mouthrinse containing LAE did not result in the complete inhibition of the strain growth. Even in the case of the microorganisms that had a contact time of 60 minutes with alcohol, 9.6 × 104 [CFU/mL] *Streptococcus oralis* count was noted (Figure 1, 2). In the case of *Staphylococcus aureus*, there was no growth of microorganisms, both in the test group that used LAE preparation

Table 1: The number of *Staphylococcus aureus* bacteria [CFU/ml] depending on various solutions and time of exposure.

Exposure time	***Staphylococcus aureus* ATCC 25923 [CFU/mL]**			
	Mouthrinse with	18% ethanol	0,2% chlorhexidine	Control +
	0,147% LAE			
5 minutes	0	$3,7\times10^4$	0	$4,3\times10^4$
10 minutes	0	$3,7\times10^4$	0	$3,5\times10^4$
20 minutes	0	$3,1\times10^4$	0	$3,9\times10^4$
30 minutes	0	$3,6\times10^4$	0	$3,4\times10^4$
60 minutes	0	$3,4\times10^4$	0	$3,5\times10^4$

Table 2: The number of *Candida albicans* [CFU/ml] depending on various solutions and time of exposure.

Exposure time	***Candida albicans* ATCC 10231 [CFU/mL]**			
	Mouthrinse with 0,147% LAE	18% ethanol	0,2% chlorhexidine	Control +
5 minutes	0	$0,4\times10^3$	0	$4,6\times10^3$
10 minutes	0	$0,3\times10^3$	0	$5,9\times10^3$
20 minutes	0	$0,4\times10^3$	0	$5,3\times10^3$
30 minutes	0	$0,2\times10^3$	0	$5,3\times10^3$
60 minutes	0	$0,3\times10^3$	0	$5,3\times10^3$

and after application of the 0.2% solution of chlorhexidine. The contact of microorganisms with the 18% ethanol component did lead to complete inhibition. After 60 mins of the contact with the 18% ethyl alcohol 3.4 × [104 CFU/ml] *Staphylococcus aureus* were grown. Similar values were obtained in the positive sample, where the strain was incubated in tryptone water. After 60 min of incubation 3.5 × [104 CFU/ml] bacteria were grown (Table 1). The antibacterial effectiveness of mouthrinse containing LAE and 0.2% chlorhexidine was also confirmed in contact with *Aggregatibacter actinomycetemcomitans*. The conducted study showed no microbial growth in both cases. During the 60 minute test, both alcohol and tryptone water gave the following values for *Aggregatibacter actinomycetemcomitans*: 2.2 × [104 CFU/ml] and 4.9 × 104 [CFU/mL], respectively (Figure 3,4). The tested preparations also have a high antifungal efficacy. The use of mouthrinse containing LAE and the 0.2% solution of chlorhexidine completely inhibited the growth of *Candida albicans* either after 5, 10, 30 and 60 mins into the test. However, the 18% ethyl alcohol and tryptone water do not have the same efficiency. After 60 mins of testing with alcohol 0.3 × 104 [CFU/mL] the *Candida albicans* count was recorded, while in the case of tryptone water a value of 5.3 × 104 [CFU/ml] was obtained (Table 2). In a test with the mouthrinse containing LAE preparation, 0.2% chlorhexidine, 18% ethyl alcohol and tryptone water microbes did not grow on any of the media after 60 mins of incubation.

Caries and periodontal diseases are still a major problem for both dentists and patients. It is well known that one of the major causes of these diseases is dental plaque. Plaque is a well-organized and systematic structure with high microbial diversity, emerging from and adjacent to hard structures of the oral cavity. There are many ways of removing it; the simplest mechanical means is surface cleaning of teeth with a toothbrush and toothpaste. However, it does not completely eliminate the bacteria, hence the need to use additional measures in everyday oral hygiene in order to maintain proper hygiene, especially in places difficult to clean with a brush. Another way is to use antiseptic rinses to wash the oral cavity [15,16].

The market currently offers a wide range of antiseptics added to liquid mouthwashes. Their task is either to inhibit the formation of the plaque biofilm or to eliminate it. Their effectiveness in elimination of plaque and gingivitis is relatively well known, as is their mechanism of action. These compounds include chlorhexidine, essential oils, metal ions, phenol derivatives (triclosan), quaternary ammonium compounds (cetylpyridimum) [4,17]. LAE is a relatively recently new material introduced into the composition of the liquid mouthwashes. LAE is a synthetic substance, which is derived from lauric acid and arginine. LAE is a substance with a distinctive mechanism of action, as it forms a coating on the amorphous acquired film (pellicle); thereby preventing further adhesion of bacteria to biofilm and the maturation of plaque and, simultaneously, it do not cause staining [13]. Research in the field of food control has shown that LAE significantly reduces surface tension thus causing the destruction of microbial cell membranes, consequently acting as a perfect preservative [7]. Its efficacy does not depend on the size of the inoculum and the time required for full activity is very short. Moreover, other studies have demonstrated that LAE maintains its antimicrobial properties in the pH range of 3-7, suggesting that it can be used in the variable environment of the oral cavity. LAE is safe to use as it decomposes into compounds found naturally in the body: lauric, fatty and arginine amino acids, necessary for proper growth during the maturation period. In 2013, the European Commission issued an opinion in which it recognized the importance of safety aspects as related to the use of hygienic compounds with LAE in their composition. One of them was to establish the limit values for oral rinses (0.1% to 0.15 %), where they do not cause tissue irritation and intolerance. In addition, a survey was conducted were the participants did not report any discomfort while using preparations with these concentrations. According to SCCS, it was revealed that a dose of 0.0575 mg/kg/day, delivered to the body during normal use of hygienic product: 2 times daily, 15 ml for 30 sec is neither fetotoxic nor negatively affects fertility. Toxic doses amount to 691 mg/kg/day and 207 mg/kg/day, respectively [9]. At the moment, there are only a few published studies on the application and the effectiveness of LAE as a substance used in dentistry. In 2015, results of randomized *in vivo* studies were published, in which half of the subjects underwent a

positive test consisting of rinsing the mouth twice a day with 15 ml of 0.15% solution of LAE after teeth brushing. The negative group used a 5% solution of aqueous alcohol. A 29.1% reduction of dental plaque was observed in the positive group after 2 weeks and after 4 weeks it amounted to 42.6%. The bleeding rate was also reduced by 36% after 2 weeks and by 50.9% after 4 weeks of using the preparation. The results of these studies show a significant reduction in plaque build-up, the severity of gingivitis and the rate of bleeding. In our study, we used chlorhexidine for positive control. It is a substance with a well-known and proven effectiveness. Some authors consider CHX as the gold standard among antiseptics in various fields of dentistry [14]. This is due to the strong and effective influence it has against both G+ and G bacteria, as well as fungi. Chlorhexidine (CHX) is one of the most commonly used antiseptics in dentistry. Due to its chemical structure it is classified to the biguanide group. A strong positive charge allows it to combine with negatively charged structures, e.g. the bacterial cell membrane. The result of such a combination is an increase of the permeability of the microbial cell membrane for small inorganic particles such as potassium ions and cytosolic components: amino acids or nucleotides. With the use of high concentrations of chlorhexidine, the cell membrane is disrupted, which in turn results in cell death. This occurs as a result of a non-specific reaction of CHX with the acidic phospholipids of the cell membrane. Chlorhexidine shows a superior therapeutic effect against gram-positive bacteria, since the cell membranes of these bacteria are more negatively charged to a greater degree. In addition, binding to bacteria hampers their ability to absorb on the surface of teeth. Chlorhexidine reversibly binds to salivary mucins, minimizing the formation of the basal layer and inhibits colonization of dental plaque [18]. It is slowly released there from so that its antibacterial activity is maintained for 6 hr to 8 hrs. However, this feature is also the cause of adverse reactions: the formation of colored deposits on the teeth, taste perversion, burning sensation of the tongue. These symptoms do not occur during a reasonably short (2-3 weeks) treatment and disappear after termination of the treatment. However, the resulting discoloration requires professional cleaning. Despite its capacity to damage the bacterial cell membrane, chlorhexidine does not have such an effect on the human mucous membrane. This is because it has a structure different from the bacterial cells. Furthermore, the outer layer of the oral mucosa is composed of dead cells which are quickly exfoliated. They provide a layer of insulation and protect against environmental and chemical damages [19]. Despite the many advantages of rinses with CHX content, the use of most of the available preparations is time-limited to approximately 2-3 weeks because, when used for prolonged periods, it causes staining of tooth structures and fillings [4]. Therefore, is there an alternative to chlorhexidine? *In vitro* studies conducted by the authors show the high efficacy of LAE against oral pathogens, which may indicate the existence of an equivalent alternative to chlorhexidine. Further clinical studies on a large scale and increasing recognition among dentists gives hope to find other effective antiseptic substances with a broad spectrum of effects, which could support the course of periodontal treatment.

Conclusion

The *in vitro* studies show high antimicrobial efficacy of LAE. The use of a formulation containing LAE may be an alternative to the gold standard in dentistry, which is chlorhexidine.

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