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Improvement of Antimicrobial and Anti-biofilm Potentials of Mouthwashes by Chitosan Produced by Lactic Acid Bacteria: An *in vitro* Study

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Abstract: *Introduction:* Modern dentistry emphasizes the importance of dental plaque control to improve oral health. To that end the development of oral care formulations has been geared toward the incorporation of antiplaque agents that may play a crucial role in oral health maintenance. *Aims:* The aims of this work were to incorporate chitosan produced by *Lactobacillus plantarum* into a mouthwash matrix and assess its effect upon microbial adherence and biofilm formation of oral microorganisms. Additionally, the action of the chitosan mouthwash was compared with two commercially mouthwashes. *Methods:* A total of 38 lactic acid bacteria, belonging to *Lactobacillus* species, isolated from 24 samples of traditional Egyptian dairy products, were screened for chitin degradation. *Lactobacillus plantarum* is the best producer of the enzyme chitin deacetylase so as to release chitosan. *Results:* The chitosan containing mouthwashwes capable of interfering with all microorganisms' growth, adherence and biofilm formation and showing vastly superior activity than both chitosan and commercial mouthwashes assayed. *Conclusions:* Chitosan mouthwashes show great potential as a natural and efficient alternative to traditional mouthwashes.

Keywords: Shrimp Waste, Chitosan, Lactic Acid Bacteria, Anti-adherence, Biofilm Formation

1. Introduction

Dental plaque is a structurally and functionally organized multi-species microbial biofilm and plays a major role in the etiology of oral diseases [1]. Therefore, its elimination is the decisive component in the prevention and treatment of these diseases. Additions of chemical antimicrobial agents such as essential oils, triclosan and chlorhexidine to toothpaste or mouthwash formulations have been used for the management of periodontal diseases. However, the widespread use of these methods was lead to several side effects as well as enhancement of microbial resistances [2]. As such the search for new, natural alternatives to the existing mouthwashes formulation is of great importance.

Natural polymers have received much attention because they can be an alternative to synthetic polymers in many technological processes [3]. Chitosan (CH) refers to the group of natural polycationic polysaccharides with high molecular weight, different viscosities and degrees of acetylation [4]. It is produced by the deacetylation of chitin found in the exoskeleton of arthropods, crustacean shell, insects, algae and fungi [5]. Chitosan has pronounced bactericidal effect against bacteria and fungi [6]. This behavior, along with its biocompatibility, biodegradability and lack of toxicity, has led to the usage of chitosan in diverse fields, such as technology, food, cosmetics, medicine, biotechnology, agriculture and the paper industry [7].

Chitosan's antimicrobial activity is well established against a variety of microorganisms. However, most of the published works concerning the effects of chitosan report its bactericidal action against planktonic microorganisms, but limited information is known about its activity upon oral bacterial adhesion and biofilm formation [8]. With that in mind, the purpose of this work was to fully assess chitosan's potential as a means to prevent several known oral potential pathogens through the control of their growth, adhesion and biofilm formation and evaluate the impact of adding chitosan to commercially mouthwashes.

2. Materials and Methods

2.1. Raw Material

Shrimp waste from processing of *Penaeus semisulcatus* was collected from a fish restaurant. Following cooking in boiling salt water for 10 minutes, the shell and meat portions were separated. The shell material was collected and dried at 50°C in an oven for 24h and homogenized in a blender until small sized pieces (10−20 mm) were obtained. These were then kept frozen until used.

2.2. Bacterial Cultivation and Chitosan Production

The potentiality of 38 lactic acid bacteria, belonging to *Lactobacillus* to produce chitosan was investigated. The lactobacilli species were isolated from 24 samples of traditional Egyptian dairy products collected from Cairo markets as described by Rushdy and Gomaa [9]. They were sub- cultured on de Man, Rogosa and Sharpe (MRS) broth medium and incubated at 35-37°C for 48-72 h in the presence of 5% CO_2. For chitosan production, 5 ml of MRS broth containing each of *Lactobacillus spp*. (OD_{600}=0.8-1) were added to the fermentative medium culture (50 ml distilled water + 5g of shrimp waste powder) and incubated for 5 days at 37°C.

2.3. Extraction of Chitosan

The fermented broth from each flask was centrifuged at 10,000 rpm for 15 minutes. The supernatant was discarded and the pellets contained mixture of bacteria, chitin, and chitosan were collected. To each of these pellets, 10 ml of 0.1N NaOH was added. The contents were mixed thoroughly and autoclaved for 15 minutes. Most of the cells were solubilized during the alkaline treatment. The tubes were again centrifuged at 10,000 rpm for 15 minutes. The supernatants were carefully removed and pellets containing chitin, chitosan, and small amount of cell debris were mixed with 10 ml of 2% acetic acid and the mixtures were left on a shaker overnight at room temperature to solubilize chitosan in acetic acid. The extracted slurry was centrifuged at 10,000 rpm for 15 minutes and the acid insoluble material was discarded. The pH of the supernatant fluids was adjusted to 7, centrifuged at 10,000 rpm for 15 minutes, washed twice with distilled water and dried at 65°C until constant weight achieved [10]. To confirm the presence of chitosan, 2-3 drops of iodine/potassium iodide solution were added to the dried precipitate, mixed and the mixture was acidified with 2-3 drops of 1% H_2SO_4. After addition of iodine/potassium iodide solution, the precipitate change color to dark brown and the solution becomes colorless and on addition of sulfuric acid the dark brown color turns to dark purple. This indicates the presence of chitosan [11].

2.4. Microorganisms and Mouthwash Formulations

Four oral clinical microorganisms *Streptococcus mutans*, *Streptococcus salivarius*, *Lactobacillus acidophilus* and *Candida albicans* were used as model microorganisms. All these strains were obtained from Fermentation Biotechnology and Applied Microbiology (FERM-BAM) Centre, Al-Azhar University, Cairo, Egypt. *S. mutans* and *S. salivarius* were grown in nutrient broth, *L. acidophilus* was grown in MRS broth and *C. albicans* was grown in Yeast Malt broth. Chitosan solutions (CH) 1% (w/v) were prepared in 1% (v/v) solution of glacial acetic acid 99%. Afterwards, the solution was stirred overnight at 50°C to promote complete dissolution of chitosan. The pH was adjusted with NaOH to a final value of 5.6–5.8, and stored at refrigerated temperature. The commercial mouthwashes tested had either essential oils as active principle (MW1) or chlorhexidine (MW2). The chitosan containing mouthwash (CHMW) (1%), prepared for a final pH of 5, contained 0.5% (w/v) salt (NaCl), 1% (w/v) stabilizer (arabic gum), 5% (w/v) sweetener (mannitol).

2.5. Antimicrobial Activity

Antimicrobial activity assays were performed by the agar well diffusion assay (AWDA) as described by Ennahar et al. [12]. Culture suspension (200 μl) of the tested microorganisms (10^6 CFU/ml) of cells was spread on Muller–Hinton agar medium. Then, bores were made using a sterile borer and loaded with chitosan, chitosan containing mouthwash or one of the two commercial mouthwashes (50 μl). The Petri dishes were kept, first for 1 h at 4°C, and then incubated for 24 h at 37°C for bacteria and 48 h at 30°C for yeast strain. Antimicrobial activity was evaluated by measuring the diameter of growth inhibition zones in millimeters.

2.6. Determination of MIC

Minimal Inhibitory Concentration (MIC) was performed using the broth microdilution assay as described by Costa et al. [6]. Briefly, an inoculum of 0.5 MacFarland (ca.1.5×10^8 CFU/ml) of each microorganism was prepared from overnight cultures and inoculated in the proper medium with either chitosan, chitosan containing mouthwash or one of the two commercial mouthwashes at concentrations ranging from 0.1 to 10 (mg/ml). Two controls were simultaneously assessed: one with 0.1 mg/ml chitosan, but without inoculum, and another where chitosan was replaced by sterile water and with added inoculum. The MIC was determined by observing the lowest concentration which inhibited visible growth.

2.7. Anti-adherence Activity

The effect of mouthwashes and /or chitosan upon bacterial adhesion was tested in accordance with protocol described by Costa et al. [8]. Briefly, 1 cm aluminum disks were dipped for 60s in wells containing either chitosan, chitosan containing mouthwash or one of the two commercial mouthwashes at sub-MIC concentration (1/2 MIC). Following that disks were rinsed with sterile water and submerged in a well containing inoculum for 60s, after which disks were placed in wells containing the appropriated medium and incubated for 24 h at 37°C. Two controls were simultaneously assessed. In the first disks were dipped in

sterile water, inoculated and incubated and in the second disks were dipped in the test solutions, rinsed and then incubated without inoculum. After 24 h disks were retrieved and viable counts were assessed. Results were given as inhibition percentages using the following formula:

Adhesion inhibition percentage = 100 – (log CFU sample / log CFU control) × 100.

2.8. Biofilm Inhibition Assay

Quantification of anti-biofilm activity was carried out by adapting the microtiter biofilm formation protocol described by Stepanovic et al. [13]. Briefly, in a flatbottom 96 microplate, wells were filled with 200 µl of tested solutions at sub-MIC concentration (1/2 MIC) with inoculum being added at 2% (v/v). Following this the microplate was incubated at 37°C for 48h. To visualize biofilms, the contents of each well were discarded and the well washed 3 times with sterile deionized water in order to remove non-adherent cells. The remaining attached bacteria were fixed with 200 µl of ethanol for 15 min. Ethanol was then discarded and the wells air dried. After that, 200 µl of crystal violet solution were added to the wells for 5 min. Excess stain was removed by rinsing the plate under tap water and the air dried. Adherence was quantified by measuring the optical density (OD) at 660 nm using a microplate reader. Control was performed by the same protocol without adding tested solution. Results for this test were given as percentage of biofilm formation inhibition applying the following formula:

Biofilm formation inhibition percentage = 100 – (OD assay / OD control) × 100

2.9. Statistical Treatment

All experiments were performed in triplicate and data were analysed using one-way analysis of variance (ANOVA) at 5% significance level. ANOVA data with a $p < 0.05$ were classified as statistically significant.

3. Results and Discussion

The production of chitosan-based biomaterials has been the focus of numerous studies, since this biopolymer and its derivatives have proved to be promising agents in the treatment and prevention of various diseases because they have numerous cellular actions [14]. Chitosan is produced from chitin via a harsh thermo chemical procedure. This process shares most of the disadvantages of a severe chemical procedure; it is environmentally unsafe and not easily controlled, leading to a broad and heterogeneous range of products. Also, the chitosan manufactured by chemical methods gives the product of inferior quality with respect to its properties like viscosity, molecular weight, and degree of deacetylation. The chemical method also produces alkaline wastes that could be minimized with biological degradation of sugar chain [15].

Biotransformation of chitin to chitosan by bacteria can be used in an economical and environmentally friendly process. Bacteria are easier and faster than fungi to grow in a large-scale fermentation system. Additionally, bacteria can be utilized without the necessity of purifying the enzyme. In the present study, out of 38 *Lactobacillus* species isolated from traditional Egyptian dairy products collected from Cairo, only three *Lactobacillus* species named *L. plantarum*, *L. brevis* and *L. rhamnosus* were chitin degrader. The yield of chitosan by *L. plantarum*, *L. brevis* and *L. rhamnosus* were 460, 290 and 350 mg/g, respectively, when grown on shrimp wastes so it was presumed that they would produce the enzyme chitin deacetylase so as to release chitosan (Table 1). *L. plantarum* was chosen for further study as it produced the highest chitosan yield (460 mg/g). This yield is considered as superior to that previously reported. Moreover, chitosan production from shrimp waste certainly saves the environment from serious water pollution problem [16], thus these bacteria can be exploited for biotransformation of chitin to chitosan at industrial scale.

Table 1. Chitosan yields from microbial extraction method as compared to chemical one.

Methods		Biomass of shrimp waste (mg)	Chitosan yield (mg/g)
Microbial method	*Lactobacillus plantarum*	5000	460
	Lactobacillus acidophilus	5000	290
	Lactobacillus rhamnosus	5000	350
Chemical method		5000	400

Chitosan has received much more attention as an antimicrobial agent that provide clinical benefits for dental plaque control. Chitosan contains many amino groups, which interact with the negatively charged residues of macromolecules at the surface of bacteria such as proteins, anionic polysaccharides fatty acids, bile acids and phospholipids causing disturbances in cellular permeability and subsequently inhibit bacterial growth [17]. Moreover, it is also important to emphasize that chitosan may cause blocking of transcription of RNA from DNA by adsorption of penetrated chitosan to DNA molecules [5].

Despite these potent antibacterial and antiplaque properties, the antibacterial activity of chitosan is influenced by a number of factors including the species of bacteria, concentration, pH, solvent and molecular weight. Chitosan can only be dissolved when the pH is less than 6.5 in which its antibacterial activity is limited [18]. However, antibacterial properties and biocompatibility of chitosan are highly desirable in dental materials as it is safe and nontoxic in the body and it is capable of promoting regeneration of oral soft tissue and alveolar bone. Moreover, chitosan has an extended retention time on the oral mucosa because of its insolubility in water. Meanwhile, most of oral antibiotics have a limited action spectrum, cannot be taken/used over a

prolonged period of time or have side effects [19].

In the present study, the antimicrobial potency of chitosan produced by *Lactobacillus plantarum*, commercial mouthwashes and chitosan containing mouthwashes against four tested oral pathogenic microorganisms *S. mutans*, *S.*

salivarius, L. acidophilus and *C. albicans* was quantitatively assessed. The results presented in Table 2 indicated that chitosan exhibited higher activity than commercial mouthwashes against all tested microorganisms. *S. mutans* was the most sensitive strain with 10.2 mm inhibition zone.

Table 2. *Antimicrobial activity of chitosan (CH), commercial mouthwashes (MW1 & MW2) and chitosan based mouthwashes (CHMW1 & CHMW2) against some oral potential pathogens.*

Microorganisms	Inhibition zone diameter (mm)				
	CH	MW1	MW2	CHMW1	CHMW2
Streptococcus mutans	10.2±1.10	7.5±0.00	9.1± 0.2	11.6±0.03	13.1±0.02
Streptococcus salivarius	7.8± 0.8	4.6± 1.66	6.8± 1.98	8.2± 1.00	10.7±0.9
Lactobacillus acidophilus	8.0±0.01	5.9± 1.56	7.3± 1.77	8.9±1.27	11.6±0.7
Candida albicans	9.2±0.6	6.5± 0.56	8.4± 0.01	9.6±1.82	12.8±0.1

Values are means± SD of 3 separate experiments

Streptococcus mutans, one of the many etiological factors of dental caries, is a microorganism which is able to acquire new properties allowing for the expression of pathogenicity determinants determining its virulence in specific environmental conditions. Through the mechanism of adhesion to a solid surface, *S. mutans* is capable of colonizing the oral cavity and also of forming bacterial biofilm. Additional properties enabling *S. mutans* to colonize the oral cavity include the ability to survive in an acidic environment and specific interaction with other microorganisms colonizing this ecosystem [20].

The MIC values, obtained by broth micro-dilution method for chitosan activity were in the range of 0.312 mg/ml for *S. mutans* to 1.25 mg/ml for *S. salivarius* that is superior to

those previously reported; Ji et al. [21] reported MIC values of 2.5 mg/ml for *S. mutans* and *P. intermedia*, Tayel et al. [5] reported a MIC of 1.50 mg/ml for *C. albicans*.

It is note worthy to state that when comparing the MIC's obtained for the commercial mouthwashes, one can see that the chlorhexidine containing mouthwash (MW2) presented significantly lower MICs than the essential oils containing mouthwash (MW1) (Table 3). This result was in agreement with conducted by Verkaik et al [22]. As a mechanism of action for chlorhexidine, it has been seen that its cationic molecule is quickly attracted by the negative charge of the bacterial surface, being adsorbed to the cell membrane through electrostatic interactions, probably by hydrophobic bonds or hydrogen bridges and thus causing bacterial death [23].

Table 3. *Minimum inhibitory concentration (MIC) of chitosan (CH), commercial mouthwashes (MW1 & MW2) and chitosan based mouthwashes (CHMW1 & CHMW2) against some oral potential pathogens tested in the microdilution assay.*

Microorganisms	MIC (mg/ml)				
	CH	MW1	MW2	CHMW1	CHMW2
Streptococcus mutans	0.312	0.625	0.312	0.312	0.156
Streptococcus salivarius	1.25	5.0	2.50	0.625	0.625
Lactobacillus acidophilus	0.625	2.50	1.25	0.625	0.625
Candida albicans	0.625	1.25	0.625	0.312	0.312

The chitosan containing mouthwash presented a higher range of antimicrobial activity for all studied microorganisms when compared with either chitosan or mouthwash (Table 2). The chitosan containing mouthwash presented consistently lower MIC values than either chitosan or commercial mouthwashes (from 0.156 to 0.625 mg/ml) (Table 3). These results seem to indicate that chitosan was successfully incorporated and stabilized into the mouthwash matrix as it did not register any loss in antimicrobial activity.

From here, the ½ the MIC were calculated to be used in the adherence and biofilm assays, as previously described by Cerca *et al.* [24]. At the present time there are little previous reports regarding the effect of the incorporation of chitosan in a mouthwash matrix upon its *in vitro* antimicrobial activity. Nevertheless, some comparisons can be made about the chitosan concentration present in each MIC and the chitosan MICs found in previous works. Costa *et al.* [6] reported that MICs values obtained for the chitosan mouthwash were 0.5 mg/ml for *S. mutans* and 1 mg/ml for *P. intermedia*.

3.1. Adherence to Surfaces

Several authors have reported on the problem raised by bacterial adhesion to surfaces, with the oral cavity being given as one of the most problematic sites for adherence and consequential biofilm management [25, 26]. It is known that hydrophobic property and ionic bond act in these processes. Considering the importance of these microorganisms in dental caries, oral candidiasis and periodontitis the capability to inhibit its establishment in the oral cavity is of upmost importance. So the efficacy of the addition of chitosan to mouthwash on these properties was assessed. As can be seen from Figure 1 that chitosan containing mouthwashes were capable of inhibiting adherence of all studied microorganisms. The highest anti-adhesive activity was recorded for *S. mutans* (98%) with CHMW1. Additionally, comparing these results with those obtained for the commercial mouthwashes, one can see that chitosan containing mouthwashes presented a significantly higher

anti-adherence capability than MW1 and MW2 presenting at

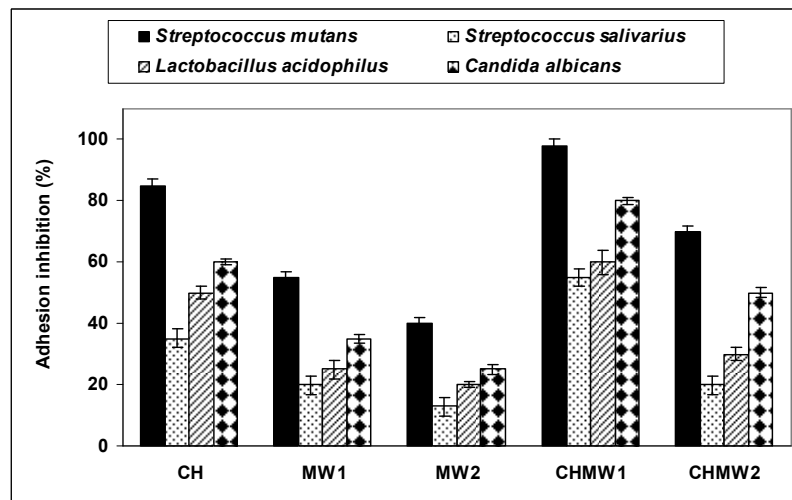best an inhibition percentage of 55 and 40% respectively.

Figure 1. Inhibitory effect of chitosan upon oral microorganisms' adhesion. Chitosan (CH), commercial mouthwashes (MW1 & MW2) and chitosan based mouthwashes (CHMW1 & CHMW2). Results are averages of triplicate experiments and error bars represent the standard deviation values.

3.2. Biofilm Formation

Biofilms in the oral cavity are characterized as being a protective environment, where microorganisms are protected from host and antimicrobials, which allows for the colonization of more fastidious bacteria [27, 28]. As such is of the upmost importance the development of valid biofilm control strategies.

Chitosan containing mouthwashes presented the highest range of action on the four tested microorganisms (Figure 2). The highest inhibition percentage (80%) was obtained for *S. mutans* followed by inhibition percentage of 70% for *C.*

albicans. Commercially mouthwashes had both a significant lower antibiofilm activity when compared with chitosan. Actually, chlorhexidine containing mouthwash (MW2) showed significantly lower antibiofilm activity than the essential oils containing mouthwash (MW1). In the same line, Dong et al. [29] stated that chlorhexidine may be responsible for the formation of an extensive extrapolymeric matrix in *S. mutans* biofilms and anup regulation of the genes related to *S. mutans* biofilm formation. This may help explain why the lack of antibiofilm activity found for the chlorhexidine containing mouthwash used in this assay.
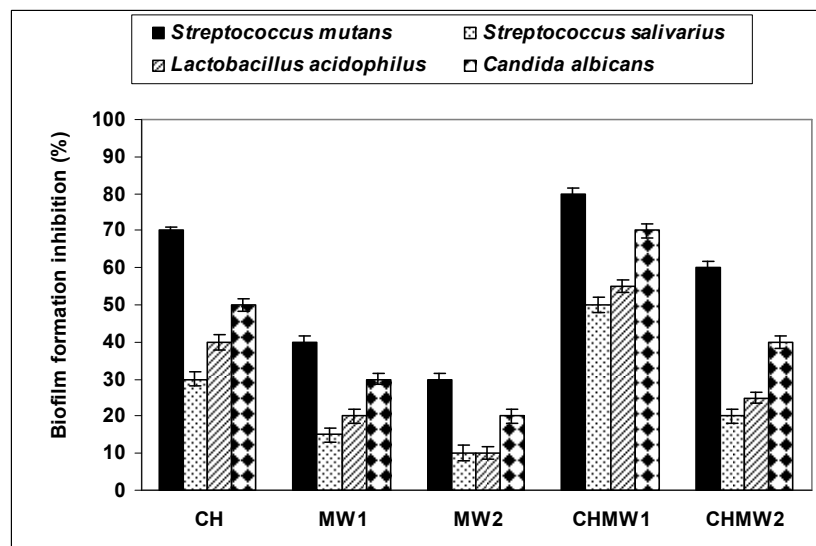

Figure 2. Inhibitory effect of chitosan upon oral microorganisms' biofilm formation. Chitosan (CH), commercial mouthwashes (MW1 & MW2) and chitosan based mouthwashes (CHMW1 & CHMW2). Results are averages of triplicate experiments and error bars represent the standard deviation values.

4. Conclusions

The present study demonstrated the potential of chitosan containing mouthwashes to act as better antimicrobial, anti-adherence and antibiofilm agent than commercial

mouthwashes products currently available. The chitosan produced by lactobacillus strains can be used as an alternative natural product to be used in mouthwashes formulations. However, further investigations are needed to evaluate the potential value of chitosan as an effective

antiplaque mouth rinse in industrial applications.

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