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Development and Efficacy of a Shrimp Shell-Derived Chitosan Mouthwash: Antimicrobial and Antiplaque Properties against *Streptococcus mutans* and *Lactobacillus acidophilus*

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Abstract

Oral health is vital to wellness, with microbial biofilms driving dental diseases. While conventional mouthwashes use synthetic antimicrobials that may disrupt oral microbiota, chitosan - a natural crustacean shell polymer offers a sustainable alternative with antimicrobial and anti-biofilm properties. This study prepared chitosan from prawn shells through optimized deproteinization (83±10% yield), demineralization (68±8%), and deacetylation (53±9%). Thermal analysis revealed decomposition at 263.05°C (after deproteinization/deacetylation) and 303.86°C (full processing), with weight losses of 47.11 % and 84.88%, respectively. Thermogravimetric/differential thermal analysis (TG/DTA) confirmed standard chitosan properties. A 2% chitosan solution showed effective *in vitro* inhibition of cariogenic bacteria. In clinical testing with 20 volunteers (18-25 years), the chitosan mouthwash reduced *Streptococcus mutans* by 29.2% (vs 3.6% for commercial mouthwash) after 14 days. Both mouthwashes decreased *Lactobacillus acidophilus* populations (68.8% commercial vs 51.2% chitosan), though not significantly different. The chitosan formulation demonstrated comparable antiplaque effects to commercial products. These results confirm that shrimp shell-derived chitosan can be efficiently produced using modified industrial methods and exhibits superior targeted antimicrobial activity against *S. mutans*, while maintaining general antibacterial efficacy. The findings support potential of chitosan as an eco-friendly, effective oral care ingredient, particularly for caries prevention, while valorizing seafood processing waste through sustainable biomaterial production.

Keywords: Chitosan; Chitin; Modified gingival index; Mouthwash; O'leary plaque score; *Lactobacillus acidophilus*; *Streptococcus mutans.*
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1. Introductio

Chitosan, a natural polysaccharide derived from chitin, the primary component of crustacean exoskeletons, plays a vital role in advancing the circular economy by converting biowaste into high-value, eco-friendly products. One of the major environmental challenges today is the excessive generation of waste materials, a substantial portion of which comes from underutilized food waste.[1,2] Among these, seafood waste, particularly shrimp shells, could be a promising opportunity for sustainable utilization. The fishery sector has long served as a primary source of animal protein, yet its byproducts, often discarded as waste, can be repurposed in fields such as medicine and dentistry.[3-5]

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Its extraction and use support sustainable development goals by reducing environmental impact while adding economic value to what would otherwise be discarded.[6-9] These characteristics, combined with its antimicrobial, antifungal, anti-inflammatory, and bioadhesive properties, have generated significant interest in its application within the field of bio-dentistry.[10-12] Structurally similar to cellulose, chitosan is unique due to its nitrogen content, which enhances its functionality.[13,14]

Chitosan is increasingly used in sustainable, active, and smart packaging, often enriched with natural waste extracts to enhance food preservation, antioxidant activity, and spoilage detection.[1,15] In agriculture, chitosan biostimulants from seafood waste boost crop drought tolerance by regulating stress genes and nitrogen uptake.[16] Chitosan coatings with

orange peel extract extend frozen beluga sturgeon shelf life through strong antioxidant and antibacterial properties.[17] By integrating natural additives such as *Terminalia catappa* leaf extract, chitosan films gain mechanical strength and hydrophobicity, extending shrimp shelf life. These applications highlight chitosan's dual role in reducing synthetic preservatives while valorizing biowaste.[18] Additionally, chitosan-polylactic acid filaments with metal compatibility enabled 3D printing, showcasing a circular approach to bio-waste valorization.[19] Yang *et al.* developed chitosan-based smart films with fungal polysaccharides that offer UV resistance, antioxidant activity, and pH-responsive color changes for real-time spoilage detection in seafood. By stabilizing natural pigments and using agricultural waste, the films provide a sustainable alternative to synthetic freshness indicators.[20] Chitosan–fish gelatin films enhanced with mango peel-derived carbon dots improved strength, antioxidant, antimicrobial, and UV barrier properties.[21]

Advancements in green chemistry have enhanced its eco-friendly production, enabling chemical modifications for diverse applications. In dentistry, chitosan's biocompatibility, antimicrobial activity, bioadhesiveness, and low cytotoxicity support its use in periodontal therapy, wound healing, drug delivery, bone regeneration, caries prevention, and hemostasis.[5] Despite structural variability and limited clinical data, its integration into oral care products and surgical applications shows strong promise, warranting continued research in nanobiotechnology to unlock its full therapeutic potential.[22,23] In dentistry specifically, chitosan has been employed in multiple forms, such as gels, films, and nanoparticles—for applications ranging from periodontal therapy and wound healing to drug delivery, bone regeneration, and caries prevention.[23] Its anti-adherence capability arises from its ability to disrupt cell wall permeability, leading to intracellular leakage. Furthermore, its bioadhesive nature allows it to bind effectively to hydroxyapatite crystals on tooth surfaces, ensuring prolonged retention and sustained therapeutic action.[24]

Managing bleeding during dental surgery can be challenging, especially in patients with clotting disorders, medication-induced bleeding, or vascular issues, as uncontrolled hemorrhage may lead to serious complications like hematoma, septic shock, or even death. Chitosan-based hemostatic agents offer a superior solution, as studies confirm they reduce bleeding time more effectively than other materials while also being biocompatible, biodegradable, antimicrobial, and healing-promoting.[25]

The widespread acceptance of chitosan in medical and dental fields is further supported by its biocompatibility,[26,27] antibacterial and antifungal properties,[28-31] antioxidant activity, and hemostatic ability.[32] Recent innovations include chitosan-TiO_2 nanocomposites that enhance self-healing in dental materials without compromising cell viability, and polyphenol-chitosan mouthwashes that significantly reduce oral cancer cell viability, offering a dual therapeutic and preventive approach.[33] Chitosan-based hemostatic dressings have further advanced clinical practice by enabling rapid, localized bleeding control during extractions, periodontal surgeries, and implant procedures. Their value is particularly pronounced in patients with bleeding disorders or those on anticoagulants, offering safer and more efficient treatment outcomes.[25] Equally novel is the application of chitosan in denture care. Studies show that incorporating chitosan into abrasive toothpaste not only protects denture surfaces from wear but also enhances antifungal activity against *Candida albicans*. This dual-action approach, surface preservation and infection prevention—positions chitosan-modified toothpaste as a transformative solution for denture maintenance.[34]

Despite these breakthroughs, oral microbiome management remains a complex challenge. *S. mutans* and *Lactobacilli*, key contributors to dental caries, continue to complicate prevention efforts.[35] *S. mutans,* a primary cariogenic bacterium, plays a central role in initiating and advancing dental caries[36,37] and is a key component of dental plaque. While chitosan has shown promising antimicrobial effects against such pathogens, research on its application in oral healthcare products remains limited[38] highlighting the need for further investigation.

Given these advantages, researchers have increasingly explored the integration of chitosan into oral healthcare products. Extensive studies have investigated dentifrice formulations, such as toothpaste and mouthwash, incorporating natural ingredients such as propolis,[39] papain and guava,[40] green tea,[41] and neem.[42] A study on 5% chitosan toothpaste demonstrated a significant reduction in *S. mutans* (from 120.4 colony-forming unit to 11.4 colony-forming unit, p=0.000) in children with early childhood caries, confirming its potential as an effective antibacterial treatment.[43] Its antibacterial mechanism involves binding to bacterial DNA, inhibiting RNA synthesis, while its positively charged molecules interact with negatively charged bacterial

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cell walls, disrupting permeability and causing solute leakage.[30,44] Studies on commercially available chitosan-based toothpastes have demonstrated their efficacy in reducing total bacterial count, minimizing plaque formation, and inhibiting bacterial biofilm development. Despite these proven benefits, chitosan-based toothpastes are still largely in the research or niche market phase, with very few major commercial options available.[43,45-48] This presents a significant opportunity for a chitosan-based mouthwash, which offers distinct advantages, especially in pediatric oral care. A mouthwash formulation provides better penetration into hard-to-reach areas and offers easier administration for young children with developing brushing skills. Furthermore, it can deliver prolonged antimicrobial action throughout the day. Unlike fluoride products, which carry the risk of fluorosis, chitosan presents a natural and biocompatible alternative, making it particularly appealing for children. Currently, there are no major commercial chitosan mouthwashes available, creating a valuable opportunity to fill this critical gap in pediatric oral care. Future research should focus on optimizing chitosan concentration and rinsing protocols to maximize its caries-preventive effects while ensuring safety for children. Beyond its clinical efficacy as an antimicrobial agent, chitosan also aligns with circular economy principles. Optimizing its extraction and formulations can simultaneously improve treatment outcomes, reduce healthcare's ecological impact, and create economic value from waste streams, thereby addressing both clinical needs and environmental stewardship.

The study has three main objectives. First, the preparation of industrial-grade chitin and chitosan from shells will be undertaken, with the efficiency of their deproteinization and demineralization processes evaluated using thermogravimetric analysis/differential thermal analysis and Fourier transform infrared spectroscopy to thoroughly characterize the extracted materials. Second, the antimicrobial properties of the derived chitosan mouthwash will be assessed against *S. mutans* and *L. acidophilus*, to determine its potential in inhibiting oral pathogens. Finally, the anti-plaque effects of this chitosan-based formulation will be evaluated, providing valuable insights into its clinical applicability as a sustainable and effective oral healthcare solution.

2. Material and methods

2.1 Chemicals

Acetic acid (CAS: 64-19-7) and hydrochloric acid (CAS:7647-01-0) were obtained from R&M Chemicals, Malaysia. Sodium hydroxide, reagent grade ≥98 % pellets, anhydrous (CAS: 1310-73-2) was obtained from Merck, Sweden. Tryptic soy broth (CM0129) and tryptic soy agar (CM0131) were obtained from Thermo Scientific (USA). De Man, Rogosa and Sharpe (MRS) agar (Oxoid CM0361) and MRS broth (Oxoid CM0359) were obtained from Thermo Scientific (USA). Spectroscopic grade glycerol (CAS: 56-81-5) and potassium bromide (CAS: 7758-02-3) were obtained from Merck Sdn Bhd, Malaysia. Isopropanol (CAS: 67-63-0) was obtained from Systerm Chemical, Malaysia. Biuret reagent and Folin-Ciocalteu's phenol reagent (Sigma-Aldrich) was purchased from Merck Sdn Bhd, Malaysia. Food-grade peppermint (EA 129465-CL) was purchased from Bake with Yen Holdings Sdn Bhd, Malaysia. Food-grade sodium saccharine was obtained from SNJ Chemicals Mart, Malaysia. Natural, plant-based glycerine was purchased from DChemie Malaysia, and PEG 40 hydrogenated castor oil (CAS: 61788-85-0) was obtained from NineLife – Malaysia.

2.2 Microorganisms

Streptococcus mutans (ATCC 25175) and *Lactobacillus acidophilus* (ATCC 4356) were obtained from the American Type Culture Collection (ATCC, USA). *S. mutans* was cultured in tryptic soy broth (TSB) at 37 °C. For long-term storage, *S. mutans* was maintained in TSB supplemented with 30% glycerol at -80 °C. Similarly, *L. acidophilus* was cultured in MRS broth at 37 °C and stored in MRS broth with 30% glycerol at -80 °C.[49,50]

2.3 Cell lines

The HaCaT cell line (AddexBio, USA) was cultured in optimized DMEM (AddexBio, USA) supplemented with 10% fetal bovine serum (FBS; Sangon Biotechnology, China) and 1% penicillin-streptomycin (10,000 U/mL; Gibco, USA). The cells were cultured at 37 °C in a 5% CO_2 incubator and subcultured every 2 to 3 days.[51]

2.4 Chitosan preparation

Shrimp waste was collected and thoroughly washed with water at 25 °C. To ensure thorough preparation, samples were boiled twice at 100 °C for 30 minutes per boil. The boiled shrimp waste was then dried in an oven at 80 °C for 12 hours and subsequently ground into a fine powder for further processing.[52]

Deproteinization (DP) was performed using 5% NaOH at a liquid-to-solid ratio of 12 mL:1 g (w/v) at 90 °C for 24 hours, followed by washing with water, and overnight drying in oven. For demineralization (DM), sample was treated with 4% HCl at a liquid-to-solid ratio of 14 mL:1 g (w/v) at room temperature for 24 hours. The resulting mixture was washed with running tap water until neutral pH was achieved and then

dried in an oven. Finally, deacetylation (DA) was carried out using 70% NaOH at a liquid-to-solid ratio of 14 mL:1 g powder at room temperature for 75 hours. The resulting solid was collected and washed with distilled water. The obtained sample was dried in an oven overnight.[52,53]

2.5 Analysis of protein concentration during deproteinization

DP was performed using 5% NaOH at a liquid-to-solid ratio of 12 mL:1 g (w/v) at 90 °C for 24 hours. 500 µL samples were taken at 1, 2, 3, 4, 6, 20, and 24 hours, with an equal volume of 5% NaOH added back. A protein standard (60 mg/mL) was serially diluted in 0.85% sodium chloride solution. Protein samples were collected at 1, 2, 3, 4, 5, 20, and 24 hours following a reaction procedure. Biuret reagent (Sigma-Aldrich, product code B3934) was allowed to equilibrate at room temperature for 30 minutes before use. In a transparent 96-well plate, 110 µL of Biuret reagent was added to each well, followed by 10 µL of either protein sample or standard. The plate was incubated at room temperature for 10 minutes. Subsequently, 5 µL of Folin-Ciocalteu's phenol reagent (Sigma-Aldrich, product code F6678) was added to each well. After mixing, the plate was incubated at room temperature for 30 minutes. Absorbance at 700 nm was measured using a plate reader.[54,55]

2.6 Thermogravimetry/Differential Thermal Analysis (TG/DTA) of Deproteinization and Demineralization in Chitin and Chitosan Extraction

After removing loose tissue from shrimp shells, the shells were meticulously washed, dried, and grounded into a fine powder. Chitosan extraction was assessed using two distinct methods under varying conditions.

(1) Chitosan Extraction via Deproteinization, Demineralization, and Deacetylation[52]

DP was performed using 5% NaOH at a liquid-to-solid ratio of 12 mL:1 g (w/v) at 90 °C for 24 hours, followed by washing with water, and overnight drying in oven. For DM, dried sample was treated with 4% HCl at a liquid-to-solid ratio of 14 mL:1 g (w/v) at room temperature for 24 hours. The resulting mixture was washed with running tap water until neutral pH was achieved and then dried in an oven. Finally, DA was carried out using 70% NaOH at a liquid-to-solid ratio of 14 mL:1 g powder at room temperature for 75 hours. The resulting solid was collected and washed with distilled water. The obtained sample was dried in an oven overnight.

(2) Chitosan Extraction via Deproteinization and Deacetylation[52]

For DP, 3 g of shrimp shell powder was treated with 5% NaOH at a liquid-to-solid ratio of 12 mL:1 g (w/v) at 90 °C for 24 hours. The resulting deproteinized material was extracted and thoroughly washed with water. Subsequently, deacetylation (DA) was carried out using 70% NaOH at a liquid-to-solid ratio of 14 mL:1 g powder at room temperature for 75 hours. The final product was collected and washed with distilled water. The obtained sample was dried in an oven overnight. The efficiency of both procedures in chitin and chitosan production was evaluated through Thermogravimetry/Differential Thermal Analysis (TG/DTA) and Fourier Transform Infrared Spectroscopy (FTIR) to analyze thermal stability and chemical composition, respectively.

(3) Thermogravimetry/Differential Thermal Analysis (TG/DTA)

TG/DTA was conducted to evaluate the thermal stability, ash content, and residual weight of the products at each stage of the industrial chitosan extraction process. Thermogravimetric analysis was performed using a Perkin Elmer TGA 7 apparatus equipped with a platinum sample holder, and data were processed using the Pyris software. The measurements were carried out in a nitrogen atmosphere with a heating rate of 15 °C min^{-1}. Nitrogen was used as the purge gas at a flow rate of 20 mL min^{-1}. The samples were heated from 50 °C to a minimum of 750 °C. All measurements were repeated at least three times to ensure reproducibility.[56]

(4) Fourier Transform Infrared Spectroscopy (FTIR) analysis

FTIR analysis was conducted to confirm the synthesis of chitosan at the final stage of the industrial chitosan extraction process (post-deacetylation).[56] The FTIR spectra were obtained for chitin samples prepared as potassium bromide (KBr) pellets. The sample preparation involved grinding 2 mg of the chitin sample with 200 mg of KBr into a fine powder with particle sizes below 5 microns, which was then compressed to form a transparent disk. The FTIR spectra were recorded using a Perkin Elmer FTIR 2000 spectrometer, covering a wavenumber range of 4000–400 cm^{-1}.

2.7 Determination of minimum inhibitory concentration (MIC) of chitosan and mouthwash on *Streptococcus mutans* and *Lactobacillus acidophilus*

Extracted chitin/chitosan was dissolved in 1% acetic acid. Serial dilutions of the extracted chitosan (0, 0.25, 0.5, 1, 2.5, 5, 7.5, and 10 mg/mL) were prepared in deionized water and mouthwash base, respectively. In a 96-well plate, 100 µL of TSB was added to each well, followed by 25 µL of bacterial inoculum (previously calibrated to 10^5 cells/mL, equivalent to a 0.5 McFarland standard). The plate was incubated at 37 °C for 24 hours. After incubation, the wells were examined for

visible signs of growth/turbidity. The MIC was determined as the lowest concentration of chitosan that inhibited bacterial growth.[57]

2.8 MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H tetrazolium bromide) assay

HaCaT cells were seeded at a density of 5×10^4 cells/well and incubated for 24 hours. Following this, various concentrations of chitosan solution were added to the cells. After 20 hours of incubation, 0.01 mL of AB solution was added to each well, and the tray was gently tapped to ensure proper mixing. The plate was then incubated at 37 °C to facilitate the cleavage of MTT, with an optimal incubation period of approximately four hours. Post-incubation, MTT formazan crystals, appearing as black and fuzzy deposits, formed at the bottom of wells containing viable cells. To dissolve the formazan crystals and convert phenol red to yellow (preventing interference with MTT measurements), 0.1 mL of isopropanol containing 0.04 N HCl was added to each well and mixed thoroughly by repeated pipetting. Absorbance was measured within one hour using an ELISA plate reader, with a test wavelength of 570 nm and a reference wavelength of 630 nm.[58-60]

2.9 Preparation of mouthwash formulation

The chitosan mouthwash formulation in this study consisted of 2% chitosan, 10% glycerin, 0.008% sodium saccharin, 1% polyoxyethylene hydrogenated castor oil, and 0.3% peppermint in deionized water.[22]

2.10 Ethical approval and Study Population

This study employed a full-mouth, randomized, controlled parallel-group design, conducted in a blinded manner and approved by the Research Management Centre (RMC/EC07/2023). Healthy individuals aged 18 to 25 years who brushed their teeth regularly, had no known medical conditions, possessed a minimum of 24 teeth with at least five teeth in each quadrant, and presented a full mouth with a Modified Gingival Index (MGI) score of 1–2 were included.

Exclusion criteria were as follows: individuals with periodontal disease, regular use of oral antiseptics, presence of fixed or removable orthodontic appliances or dentures, use of medications affecting gingival inflammation, tobacco use or vaping, known allergies to any ingredients in the self-formulated chitosan mouthwash, and pregnancy or lactation.

Once the chitosan mouthwash was prepared, participants were provided with consent forms and underwent examinations after giving informed consent. A total of twenty participants were recruited and randomly assigned into two equal groups: Group 1 received commercial mouthwash, while Group 2 received 2% chitosan mouthwash. All participants were informed about the objectives, potential risks, and benefits of the study before signing the informed consent form.[23,24]

Participants were blinded to their group assignments. During the first clinical visit, gingival health was assessed using the MGI, and plaque levels were evaluated using the O'Leary Plaque Index. Saliva and plaque samples were collected on Day 1 and again on Day 14 for antibacterial testing. Sterile tubes were prepared to collect 1 ml each of saliva and plaque samples. Saliva was collected by having participants spit into sterile tubes, ensuring they had not consumed any food or flavored liquids after their first morning brushing. Plaque samples were obtained from the posterior teeth using sterile cotton swabs. Full-mouth ultrasonic scaling was performed after the initial examination. Each participant received 280 ml of mouthwash (either commercial mouthwash or 2% chitosan) and was instructed to rinse with 10 ml twice daily after brushing for 14 days. Daily reminders were provided throughout the study period to encourage compliance.[23,24]

After completing the 14-day mouthwash regimen, participants returned for a follow-up visit. Gingival and plaque scores were reassessed, and final saliva and plaque samples were collected. A concluding full-mouth scaling was performed, and the gingival and plaque scores were analyzed.

2.11 Antibacterial Susceptibility Testing of Saliva and Dental Plaque Samples

(1) Quantification of *S. mutans* colonies

Saliva samples were diluted 100-fold, and 50 µL of the diluted sample was spread onto an agar plate by using sterile swab. Following incubation at 37 °C for 24 hours, the number of colonies was counted. For plaque samples, 500 µL was added and incubated at 37 °C for 1 hour. Then, 50 µL of the incubated sample was spread onto a tryptic soy agar plate by using sterile swab. Following incubation at 37 °C for 24 hours, the number of colonies was counted.

(2) Quantification of *L. acidophilus* colonies

Saliva samples were diluted 100-fold, and 50 µL of the diluted sample was spread onto an agar plate by using sterile swab. Following incubation at 37 °C for 24 hours, the number of colonies was counted. For plaque samples, 500 µL of broth was added and incubated at 37 °C for 1 hour. Then, 50 µL of the incubated sample was spread onto a MRS agar plate by using sterile swab. Following incubation at 37 °C for 24 hours, the number of colonies was counted.

2.12 Statistical analysis

Data were analyzed using SPSS 15.0 for Windows (SPSS Inc., Chicago, IL, USA, 2001). The Mann-Whitney U test was employed to compare groups for nonparametric data. Statistical significance was set at $p \leq 0.05$.

3. Results and discussion

3.1 Preparation of chitosan from seafood waste

Table 1 illustrates a decreasing trend in chitin/chitosan retention during processing. The mean percentage retained was 83 ± 10% after deproteinization, 68 ± 8% after demineralization, and 53 ± 9% after deacetylation.

Table 1: Percentage of sample retained after deproteinization, demineralization and deacetylation (Number of replicate, n=3).

Sample	Procedure	Mean (%)	SD
1	Deproteinization	83	10
2	Demineralization	68	8
3	Deacetylation	53	9

In this study, the preparation of chitosan was carried out using an industrial-scale method that involved three sequential and critical steps: DP, DM and DA. Each step was meticulously designed and optimized to ensure the efficient extraction and modification of chitin, ultimately yielding high-quality chitosan with desirable properties. The DP step was the first critical procedure, aimed at removing the protective protein layer that coats the chitin. This step was essential to expose the underlying chitin structure, thereby enhancing its accessibility to hydrochloric acid in the subsequent DM process.[61] The removal of proteins not only facilitated more effective demineralization but also led to increased hydrolysis, which, while beneficial for protein removal, could result in some material loss in the solid chitin fraction. This trade-off between protein removal and material retention was carefully managed to optimize the overall yield and quality of the chitin.[2,62]

The DM process was employed to eliminate inorganic mineral content, primarily calcium carbonate and calcium phosphate, from the chitin.[63,64] These minerals must be eliminated to obtain purified chitin suitable for further processing. During the DM step, a notable observation was the binding of pigmented compounds to the solid matrix, resulting in residual coloration. This unintended effect highlighted the need for an additional treatment to address discoloration. Beyond mineral removal, the DM process plays a crucial preparatory role by producing a cleaner chitin substrate, thereby enhancing the efficiency of the subsequent DA stage.[65]

The final step in chitosan extraction, is critical for chitin into chitosan. This process involves the use of concentrated NaOH at elevated temperatures to cleave acetyl groups from the chitin polymer, thereby increasing the degree of DA and enhancing the solubility and functionality of the resulting chitosan. In addition to structural modification, the DA step also facilitates the removal of residual carotenoid pigments, which contribute to the coloration of the material. Consequently, this dual function of the DA process not only improves the purity and whiteness of the final product but also ensures the formation of high-quality chitosan with a high degree of DA, suitable for biomedical and industrial applications.[66,67]

As a result of these three sequential procedures, the final residual weight of the material was significantly reduced. This reduction was attributed to the removal of proteins, minerals, and color compounds, which collectively accounted for a substantial portion of the initial raw material. Despite the weight loss, the process was optimized to ensure that the final chitosan product was of high quality, with minimal impurities and excellent functional properties. The industrial method employed in this study demonstrated a well-balanced approach to chitin extraction and chitosan production. Each step—DP, DM, and DA—was carefully designed to address specific challenges, such as protein removal, mineral elimination, and pigment extraction, while maintaining the integrity and quality of the final chitosan product. This systematic approach not only enhanced the efficiency of the process but also ensured the scalability and reproducibility of chitosan production for industrial applications.

3.2 Analysis of protein concentration during deproteinization

The protein concentration during the DP process is depicted in Fig. 1. Initially, the concentration steadily declined from 1.36 ± 0.06 mg/mL at the first hour to 1.32 ± 0.03 mg/mL by the third hour. However, a notable spike was observed at the 4th hour, where the concentration increased to 1.65 ± 0.10 mg/mL. This unexpected rise could be attributed to protein re-solubilization or the partial release of bound proteins from the substrate under the prevailing processing conditions. Following this peak, the protein concentration consistently declined, reaching 1.10 ± 0.50 mg/mL by the 24th hour.

The rate of protein reduction was non-uniform throughout the process. The most significant decrease occurred between the 4th and 5th hour, indicating a critical phase for protein removal. This was followed by a more gradual decline from the 5th to the 20th hour, after which the reduction rate slightly accelerated between the 20th and 24th hour. These findings suggest that the majority of DP occurs within the first few

hours, particularly between the 4th and 5th hour. Given this trend, it may be feasible to optimize the DP process by reducing its duration to approximately 5 hours without significantly compromising protein removal efficiency. Such an adjustment could enhance process efficiency, reduce energy consumption, and lower operational costs, making the process more economically viable.

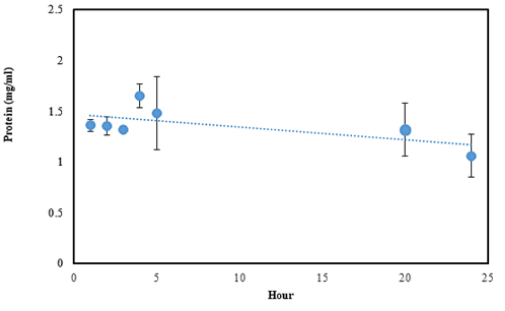

Fig. 1: Protein concentration (mg/ml) during deproteinization at different time points.

The industrial extraction of chitin and chitosan, as applied in this study, is widely utilized due to its cost-effectiveness and scalability, enabling large-scale production. However, the process is not without its challenges. In this study, the DP process involved harsh treatment conditions—5% NaOH for 3 hours at 85–95 °C, followed by 17 hours at room temperature. While these conditions were effective in protein removal, achieving complete DP and DM remains challenging. The use of highly concentrated acids and bases at elevated temperatures may degrade chitin/chitosan, potentially compromising its functional properties. Therefore, future research should explore alternative DP strategies, such as enzymatic or mild alkaline treatments, to enhance extraction efficiency while minimizing degradation.[68] For regions with medium technological capacity, traditional chemical processing offers superior scalability as it utilizes widely available reagents and standard industrial equipment, unlike advanced methods requiring precision instruments or controlled bioprocessing facilities. The current extraction method, is chemically less intensive, offers a reasonable compromise—delivering functional chitosan suitable for industrial use, while remaining within the reach of local technological capabilities.

The traditional chitin/chitosan extraction process faces several drawbacks that can hinder its industrial adoption. These include high energy consumption, as the requirement for sustained heating significantly increases operational costs.

Furthermore, excessive chemical usage, requiring large quantities of acids and bases, leads to elevated raw material costs and complicates operations. Environmental concerns also arise from the discharge of chemical-laden effluents, which contribute to pollution, especially under alkaline conditions. Lastly, prolonged processing times extend chemical treatment durations, reducing production efficiency and throughput. These challenges are particularly problematic in regions with stringent environmental regulations or where sustainable manufacturing practices are prioritized.[2]

The balance between chemical concentration, treatment temperature, and processing duration is critical to yield functional chitosan, as excessive conditions degrade polymer chains (reducing molecular weight and viscosity), while insufficient treatment compromises purity and degree of deacetylation. The current findings highlight potential optimizations to improve the DP process while addressing its limitations. One key strategy involves using lower concentrations of alkaline solutions and reducing treatment durations, which can significantly mitigate the risk of chitin/chitosan degradation. Shorter exposure to strong alkaline conditions helps preserve the structural integrity and functional properties of chitin and chitosan, including biocompatibility, antimicrobial activity, and film-forming capabilities.[67,69]

Beyond improving product quality, these adjustments also offer economic and environmental benefits. Lower chemical usage and reduced processing time translate into cost savings by minimizing reagent consumption and reducing energy demand. Additionally, shorter treatment durations contribute to a lower environmental footprint by decreasing effluent discharge and energy requirements, making the process more sustainable. These improvements align with the principles of green chemistry and sustainable manufacturing, enhancing both eco-friendliness and economic viability.

3.3 Thermogravimetry/Differential Thermal Analysis (TG/DTA)

The TGA curve indicated that significant deterioration occurred at a single step for the chitosan shell. The decomposition temperature of the product obtained after DP and DA was recorded at 263.05 °C. In contrast, the decomposition temperature of the product resulting from the combined treatment of DP, DM, and DA was significantly higher at 303.86 °C. The increase in decomposition temperature indicates enhanced thermal stability in the product treated with DP, DM, and DA, compared to the one treated with only DP and DA (Fig. 2). The thermal degradation peak observed at 300 °C corresponds to the primary breakdown of the chitosan macromolecular structure. This stage is characterized by the release of gases such as CO_2,

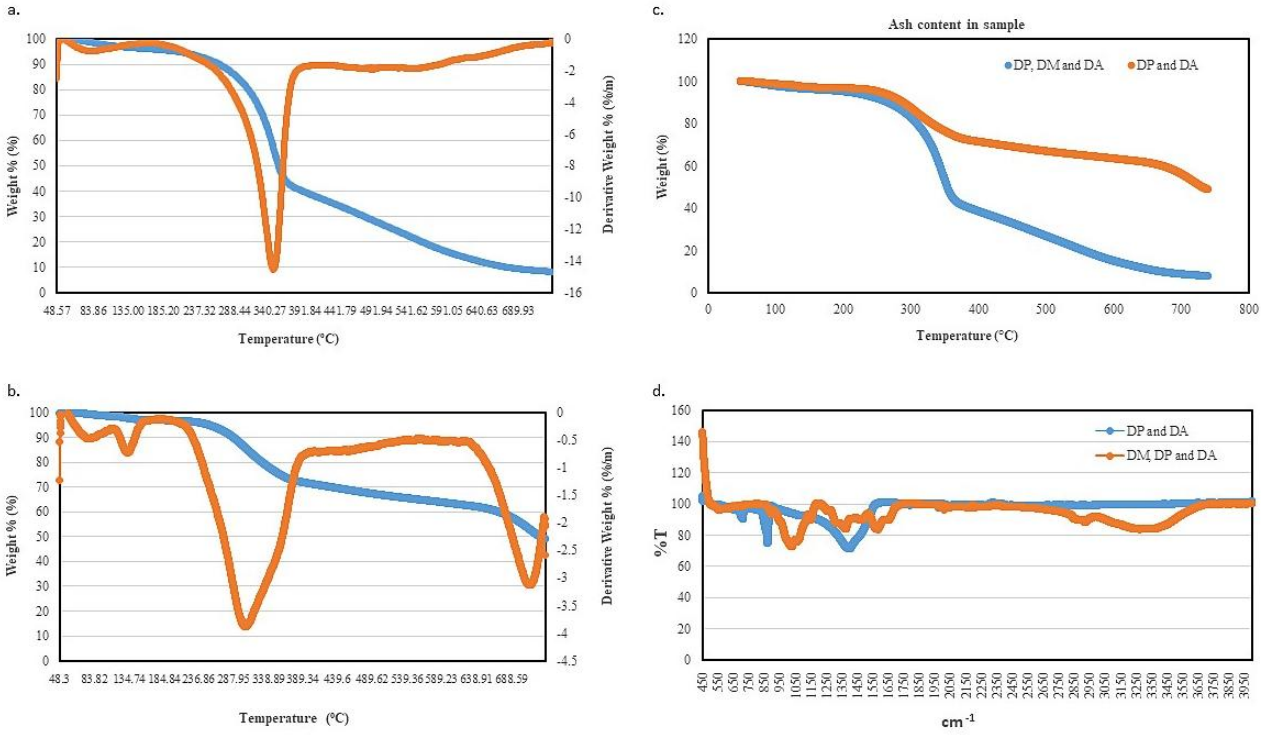

Fig. 2a-d: Characterization of chitosan extraction products. Thermogravimetric analysis (TGA) of samples after (**Fig. 2a**) combined deproteinization (DP), demineralization (DM), and deacetylation (DA), and (**Fig. 2b**) combined DP and DA. **Fig. 2c** presents differential thermal analysis (DTA) of these products. **Fig. 2d** shows FTIR spectra of chitosan extracted from shrimp waste using (i) DP and DA, and (ii) combined DM, DP, and DA methods.

NH_3, and H_2O, resulting from the rupture of chemical bonds. In certain conditions, intermediate or volatile compounds may temporarily condense on the sample surface before decomposing at higher temperatures, giving the appearance of a temporary mass increase.[70] A similar pattern was reported by Rahman *et al.*, who observed that the initial degradation at this temperature involved depolymerization, dehydration, and cleavage of hydroxyl groups, ultimately leading to char formation.[71]

Likewise, Liyanage *et al.* noted that chitin exhibited a distinct exothermic peak at 360.41 °C (54.48 µV), associated with glycosidic bond cleavage and degradation of the polymer backbone. In contrast, chitosan showed a lower-temperature exotherm at 312.79 °C (28.11 µV), indicating reduced thermal stability due to its deacetylated structure[72].

Furthermore, Fig. 2 illustrates that the weight loss percentage for DP was 50.96%, leaving a residual weight of 49.04%. In comparison, the product obtained after DP, DM, and DA treatment exhibited a substantial increase in weight loss percentage to 91.74%, with a residual weight of only 8.16%. These findings suggest that the product resulting from DP and DA treatment retains a higher percentage of residual weight compared to the DP, DM, and DA-treated product. This indicates that the DP and DA-treated product contains a greater proportion of carbonaceous materials and inorganic metal oxides (ash content), whereas the inclusion of DM in the treatment process contributes to increased degradation and reduced residual weight.

3.4 Fourier Transform Infrared Spectroscopy Analysis (FTIR)

The FTIR spectra of the chitosan synthesized in this study are presented in Fig. 2. Table 2 lists the identified functional groups and their respective wavelengths. A comparative analysis was conducted, comparing the wavelengths of these functional groups observed in current study with standard chitosan functional group wavelengths, also shown in Table 2. A peak of 3275.57 cm^{-1} was observed as the main functional group of chitosan, which was due to O-H group and stretching of N-H group. The presence of 2919.27 cm^{-1} was due to stretching of C-H and C=O of the amide group, CONH-R. The peak at 1581.10 cm^{-1} was responsible for the vibrational mode of amide C═O stretching and amide II group (N-H bending). The peak at 1374.14 cm^{-1} was recognized as C-N stretching of amide III group. Besides that, 1151.70 cm^{-1} was responsible as C-H group stretching of the glycosidic linkage. The presence of peak at 1023.78 cm^{-1} was due to the free amino group at C_2 of glucosamine while 564 cm^{-1} was due to out-of-plane bending N-H, C–O groups.[73,74]

Table 2: Standard wavelength of functional groups present in chitosan compared to wavelength of chitosan obtained from current study via FTIR analysis.[73,74]

Standard Wavelength (cm^{-1})	Functional group	Wavelength (cm^{-1}) of respective functional group of chitosan obtained after combination DP, DM and DA
3300-3250	NH Stretching, OH	3275.57
2919-2868	Stretching band C-H and C=O of the amide group, CONH-R	2919.27
1650-1550	Vibrational mode of amide C═O stretching, amide II (N-H bending) aromatic	1581.10
1390-1370	Amide III, (C-N stretching)	1374.14
1153-1156	Glycosidic linkage, C-H Stretch	1151.70
1029	free amino group at C_2 of glucosamine	1023.78
500	out-of-plane bending NH, out-of-plane bending C–O	564 (Sharp peak)

For sample only went through DP and DA, the FTIR analysis of the sample revealed high absorption peaks at 1650 cm^{-1} (Amide I) and 1550 cm^{-1} (Amide II), indicating a significant presence of acetyl groups. This suggests that the DA process was incomplete, leaving the sample with a composition closer to chitin rather than fully converted chitosan. The presence of a broad peak between 3200–3600 cm^{-1}, corresponding to hydroxyl (-OH) and amine ($-NH_2$) groups, confirms some degree of DA, but the strong amide signals suggest that a large fraction of the chitin structure remains intact. These findings indicate that the applied DA conditions were not sufficient to fully remove the acetyl groups from chitin.

The absence of a DM step could significantly contribute to the incomplete deacetylation observed in the FTIR results. Demineralization is essential for removing calcium carbonate ($CaCO_3$) and other minerals that can interfere with the deacetylation process by limiting NaOH penetration into the chitin structure. Without this step, residual minerals may consume part of the alkali, reducing its effectiveness in breaking acetyl groups and leading to uneven deacetylation. Additionally, mineral deposits can create structural heterogeneity, preventing uniform conversion to chitosan and resulting in the persistence of strong amide peaks at 1650 cm^{-1} (Amide I) and 1550 cm^{-1} (Amide II).[75] The functional groups of chitosan obtained in this study were similar to reported FTIR data on chitosan.[68,73,74] Therefore, it can be inferred that the chitosan obtained from the study was valid and ready to be applied into oral healthcare products.

3.5 Minimum inhibitory concentration (MIC) of chitosan solution on *Streptococcus mutans* and *Lactobacillus acidophilus*

As depicted in Fig. 3, the higher concentration of chitin/chitosan extract in sample, a general decreasing trend in optical density was observed, indicating a reduction in bacterial growth. At a concentration of 2 mg/ml, the optical density reached its lowest point compared to the control group. This led to conclude that 2 mg/ml is the MIC for *S. mutans* in current study. The MIC is a critical parameter for evaluating the antimicrobial efficacy of a compound, as a lower MIC signifies greater potency. Chitosan has demonstrated significant antimicrobial properties, including antibacterial, antifungal, and antioxidant effects, as supported by previous studies.[28-31] The proposed mechanisms of action include enzyme inactivation, chelation of essential metal ions, and the formation of polyelectrolyte complexes with bacterial surface compounds.[76] Furthermore, the cationic amino groups of chitosan interact with the anionic components of the bacterial cell wall (phospholipids and carboxylic acids), causing structural damage, altered permeability, and leakage of cellular contents.[77,78]

It is important to note that variations in MIC values for chitosan against *S. mutans* have been reported in the literature. Costa *et al.* cited higher effective concentrations of 3.0–5.0 mg/ml.[79] Bae *et al.* reported an MIC of 1.25 mg/ml for chitosan with a 70% degree of deacetylation,[38] while Aliasghari *et al*. corroborated this finding. In contrast, Abedian *et al.* reported a lower MIC of 0.62 mg/ml, chitosan effectively inhibits the growth of *S. mutans* and *Streptococcus sobrinus*, with high molecular weight chitosan being more effective than low molecular weight chitosan. The MIC and minimum bactericidal concentration varied slightly between the bacterial species and chitosan types, with *S. sobrinus* showing more resistance to low molecular weight chitosan. Additionally, chitosan significantly reduced bacterial

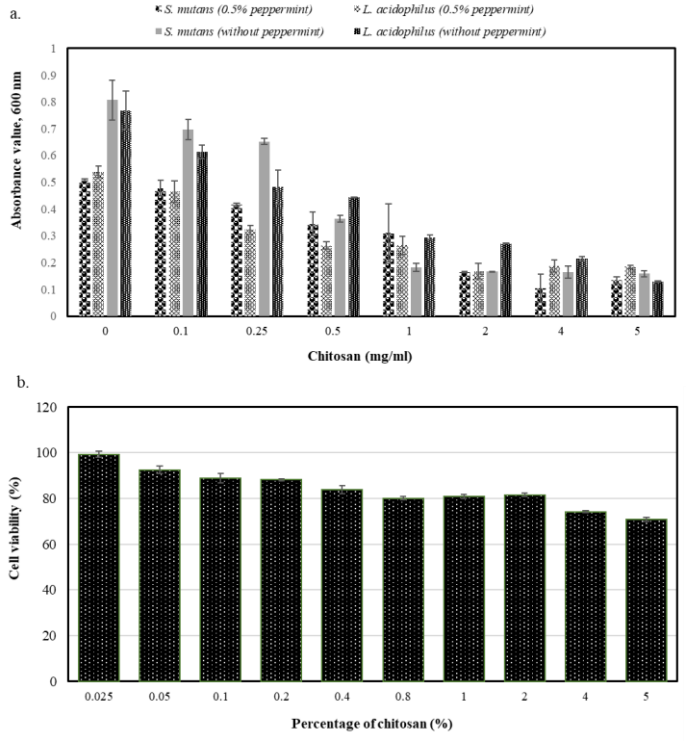

Fig. 3 a-b: Antibacterial and cytotoxicity assessment of chitosan. (a) Determination of the minimum inhibitory concentration (MIC) of chitosan *against Streptococcus mutans* and *Lactobacillus acidophilus*. This figure illustrates the antibacterial efficacy of chitosan to identify the optimal concentration for effective inhibition of these oral bacteria. (b) MTT cytotoxicity assay with HaCaT cells. HaCaT cells were seeded at a density of 5×10^4 cells/well in 96-well plates and incubated for 24 hours (37 °C, 5% CO_2) to facilitate cell attachment. Subsequently, the cells were subjected to an MTT assay to assess the cytotoxicity of varying chitosan concentrations (0.025%–5%).

adhesion and biofilm formation, with higher concentrations (1.25–2.50 mg/mL) completely inhibiting biofilm development.[80]

These discrepancies highlight the influence of chitosan's structural properties, such as the degree of DA and molecular weight, on its antimicrobial activity. For instance, a higher degree of DA increases the number of free amino groups, enhancing chitosan's positive charge and its interaction with bacterial cell walls.[81] Additionally, the particle size of chitosan plays a significant role in its efficacy, with nano-chitosan demonstrating a larger inhibition zone against *S. mutans* compared to conventional chitosan.[82] The current MIC finding of 2 mg/mL falls within the reported range of effective concentrations for chitosan against *S. mutans* and *S. sobrinus*, which varied from 0.62 to 2.50 mg/mL depending on molecular weight and bacterial strain. This suggests that the chitosan obtained through the present extraction method retains its antimicrobial functionality, including its ability to inhibit bacterial growth and biofilm formation.

Current findings indicate that the MIC of chitosan against *L. acidophilus* approximate 5 mg/mL, while chitosan possesses antimicrobial properties, its efficiency against *L. acidophilus* is lower than against *S. mutans*. The reduced antimicrobial effect of chitosan on *L. acidophilus* compared to *S. mutans* may be attributed to differences in cell wall composition and bacterial physiology. *L. acidophilus*, being a Gram-positive bacterium, has a thick peptidoglycan layer that may provide resistance to chitosan's cationic interactions, requiring higher concentrations for effective inhibition.[83] Additionally, some studies suggest that probiotic strains like *L. acidophilus* may have adaptive mechanisms to tolerate chitosan exposure, further reducing its susceptibility.[84] Current findings contribute to the evidence supporting chitosan as a promising antimicrobial agent. However, further research is needed to address the variability in chitosan's efficacy and to optimize its application in oral healthcare.

For mouthwash solution preparation, the incorporation of 0.5% peppermint flavoring in the chitosan mouthwash serves a dual purpose: enhancing sensory attributes and potentially contributing to antimicrobial activity. Peppermint (*Mentha piperita*) is widely recognized for its refreshing sensation and its ability to improve the overall texture and palatability of oral care products. The cooling effect of peppermint, primarily attributed to menthol, not only enhances user experience but

also provides a mild anesthetic property, which can improve comfort during rinsing. This makes the mouthwash more appealing and user-friendly, encouraging consistent use.[85]

From an antimicrobial perspective, peppermint oil contains bioactive compounds such as menthol, menthone, and pulegone, which exhibit antibacterial activity against a broad spectrum of oral pathogens.[53] These compounds disrupt bacterial cell membranes, increasing permeability and ultimately compromising cell viability. Given these properties, the MIC of chitosan in combination with 0.5% peppermint was evaluated.

The results revealed a synergistic effect between chitosan and peppermint, significantly reducing the overall bacterial population.[86] However, this combination did not substantially alter the MIC values of chitosan against *S. mutans* and *L. acidophilus*. This finding aligns with prior studies indicating that at lower concentrations (*e.g.*, 0.5%), peppermint oil primarily serves as a flavoring agent to enhance taste and freshness rather than as a primary antimicrobial. Thus, while peppermint contributes marginally to antibacterial activity, its key role lies in improving sensory appeal and user compliance.[87]

Therefore, the incorporation of 0.5% peppermint in the chitosan mouthwash strikes an optimal balance between sensory enhancement and minor antimicrobial synergy. Although it does not significantly influence chitosan's MIC against major oral pathogens, its refreshing properties and potential to boost patient adherence make it a valuable addition to the formulation.

3.6 MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H tetrazolium bromide) assay

In order to assess the safety profile of chitosan mouthwash, an MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) cytotoxicity assay was conducted following the manufacturer's guidelines. Various concentrations of chitosan were tested by incubating them with keratinocytes for 20 hours (Fig. 3). The results indicated that a chitosan concentration of 2 mg/mL maintained approximately 80% cell viability. This level of viability is generally considered acceptable for biomedical applications, suggesting that chitosan is a safe candidate for use in oral rinse formulations. It was highlighted chitosan's excellent biocompatibility, noting that its cationic nature allows for favorable interactions with mucosal surfaces without significant cytotoxicity at low to moderate concentrations. Their findings align with current results, reinforcing that chitosan concentrations around 2 mg/mL are well-tolerated by keratinocytes.[88] The degree of deacetylation of chitosan is frequently recognized as a key factor influencing various physicochemical and biological characteristics of chitosan, including its crystallinity, hydrophilicity, degradation behavior, and cellular interactions.[89]

Previous studies have also reported the biocompatibility of chitosan in oral and biomedical applications, highlighting its low cytotoxicity and favorable interactions with epithelial cells.[90,91] A 0.2% chitosan solution may be used with many final cleaning techniques to improve bond strength between fiber posts and root canal dentin, especially in the cervical third.[92] Additionally, the ability of chitosan to preserve cell viability at this concentration suggests that it may provide antimicrobial and anti-inflammatory benefits while maintaining biocompatibility. This aligns with existing literature that supports chitosan's role in promoting wound healing, inhibiting microbial growth, and enhancing oral health without significant toxicity concerns.[93,94] Given these findings, chitosan-based mouthwash presents a promising alternative to conventional oral rinses, particularly for individuals seeking biocompatible and naturally derived options.

3.7 Antibacterial susceptibility testing

Statistical analysis was performed using the Mann-Whitney U test, a non-parametric test selected due to the small sample size and the non-normal distribution of the data. A 95% confidence level was established to assess statistical significance. The results demonstrated a reduction in both gingival index and plaque score following the use of chitosan mouthwash (Table 3). However, despite these observed reductions, the statistical analysis revealed no significant difference between chitosan mouthwash and commercial mouthwash, indicating that the efficacy of chitosan mouthwash is comparable to that of the commercially available mouthwash. These findings are consistent with prior research that has highlighted the antimicrobial and anti-inflammatory properties of chitosan in oral health applications, further supporting its potential as a viable alternative to conventional mouthwashes.[95,96]

In addition to clinical parameters, microbial analysis was conducted to evaluate the impact of chitosan mouthwash on oral microbiota. The results showed a notable reduction in the colony count of *S. mutans*, a primary cariogenic pathogen, after the use of chitosan mouthwash. This reduction was more pronounced compared to that observed with the commercial mouthwash. This finding aligns with previous studies that have demonstrated chitosan's bactericidal activity against *S. mutans*, underscoring its potential role in preventing dental caries. However, chitosan mouthwash was less effective in reducing the colony counts of *L. acidophilus* compared to

Table 3: O'Leary Plaques index, MGI score and (CFU)/ml: Mann-Whitney test 2-tailed, 95% confidence level (Before and After using mouthwash).

Description	Group 1 (Commercial mouthwash)	Group 2 (2 % Chitosan added mouthwash)	U value	P value
MGI score	0.7	1.0	39.5	>0.05
O'Leary Plaques index	16.7 %	29%	33	>0.05
Saliva sample (CFU)/ml				
S. mutans	- 8	-37	66	>0.05
L. acidophilus	-159	-80	37	>0.05
Plaque sample (CFU)/ml				
S. mutans	- 27	- 79	62.5	>0.05
L. acidophilus	- 59	- 23	37.5	>0.05

commercial mouthwash, suggesting potential differences in its antimicrobial spectrum. Despite these variations, the differences between the two mouthwashes were not statistically significant, reinforcing the conclusion that chitosan mouthwash exhibits antimicrobial properties similar to those of commercial mouthwash.[93] Current finding aligns with report from Uraz *et al.*, the comparison indicates that the 2% chitosan mouthwash had antiplaque properties equivalent to the established 0.2% chlorhexidine mouthwash in a short-term, non-brushing research, suggesting it as a viable option, especially for people sensitive to chlorhexidine side effects.[97] A 0.5% (5 mg/mL) chitosan mouthwash has demonstrated superior efficacy compared to chlorhexidine (CHX) mouthwash in reducing plaque, gingivitis, and staining. This enhanced effectiveness against gingivitis is likely attributed to chitosan's favorable bioadhesive properties, allowing it to effectively adhere to oral mucosal surfaces. Furthermore, both chlorhexidine and chitosan mouthwashes were equally effective in reducing halitosis, possibly due to their potent antimicrobial characteristics that target oral microorganisms responsible for producing odor-causing volatile sulfur compounds.[98] However, it is worth noting that using a higher percentage of chitosan may present dissolving issues during preparation, a factor important for further discussion and research.

While these results are promising, the study has certain limitations, most notably the small sample size, which may limit the generalizability of the findings. To draw more definitive conclusions, future studies with a larger and more diverse cohort are recommended. Additionally, further research should investigate the effects of varying chitosan formulations, concentrations, and long-term usage to better understand its potential as an alternative to conventional mouthwashes. Exploring these factors could provide deeper insights into optimizing chitosan-based oral care products and their efficacy in maintaining oral health.

Overall, the findings of this study suggest that chitosan mouthwash is a promising alternative to commercial mouthwash, with comparable efficacy in reducing gingival inflammation, plaque accumulation, and *S. mutans* colonization. However, further research is needed to address the limitations of the current study and to fully elucidate the potential of chitosan in oral health applications.

4. Conclusion

The present study successfully demonstrated that chitin can be efficiently extracted and converted to chitosan using industrial methods. The efficiency of chitosan production was validated through comprehensive laboratory analyses, including protein concentration determination after the deproteinization process, TG/DTA, FTIR. Furthermore, chitosan derived from shrimp waste exhibited significant antimicrobial activity against *S. mutans*, with a MIC of 2 mg/mL, while for *L. acidophilus*, the MIC was 5 mg/mL. These findings highlight the potential of CS as a key ingredient in oral healthcare formulations, particularly a 2% chitosan-based mouthwash, which demonstrated promising effects in combating *S. mutans*, reducing plaque scores, and contributing to overall oral health improvement.

Beyond its therapeutic potential, this study underscores the broader implications of utilizing seafood waste as a source of high-value, natural bioactive products. The successful transformation of shrimp waste into an effective antimicrobial agent presents a sustainable approach to reducing environmental waste while simultaneously advancing oral healthcare innovation. This aligns with global efforts toward eco-friendly and circular economy practices, reinforcing the viability of chitosan as a multifunctional biomaterial.

Further research should focus on optimizing chitosan formulations to enhance their efficacy and biocompatibility. Modifications such as altering the molecular weight, degree of deacetylation, and solubility parameters could significantly influence antimicrobial activity and adherence to oral surfaces. Additionally, long-term clinical trials are necessary to evaluate the sustained effects of chitosan-based mouthwashes in real-world settings, particularly in

comparison to established antimicrobial agents such as chlorhexidine and essential oil-based rinses. Investigating the potential synergistic effects of chitosan with other bioactive compounds, such as fluoride, probiotics, or herbal extracts, could also enhance its therapeutic potential while preserving the balance of the oral microbiome.

Moreover, advancements in formulation technologies could further improve chitosan's application in oral healthcare. The development of smart and targeted delivery systems, such as nanoparticle-based chitosan formulations, could prolong antimicrobial action and improve bacterial specificity. Additionally, large-scale production feasibility, cost-effectiveness, and regulatory considerations must be explored to facilitate the commercialization of chitosan-based oral care products. Given the increasing demand for sustainable and eco-friendly healthcare solutions, chitosan derived from seafood waste represents a viable and innovative alternative that aligns with both environmental conservation and public health improvement efforts.

Ethics approval and consent to participate

Research Management Centre (RMC/EC07/2023).

Human and animal rights

Not applicable.

Declaration of AI and AI-assisted technologies in the writing process

The English language of the article was improved with ChatGPT and Gemini AI. Upon generating draft language, the author reviewed, edited, and revised the language to their own liking and takes ultimate responsibility for the content of this publication.

Availability of data and materials

The datasets supporting the conclusions of this article are available from the corresponding author on reasonable request.

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Conflict of Interest

There is no conflict of interest.

Supporting Information

Not applicable.

CRediT Statement

The authors confirm their contribution to the papers as follow: **Fong Fong Liew, Hsiao Wei Tan and Santhosh Kotian:** Study concept and Design; **Fong Fong Liew; Yeong Kit Foong, Kin Man Shu, Ee Hua Tan, Jing Shi Tan**: Carrying out experiments; **Fong Fong Liew, Hsiao Wei Tan and Santhosh Kotian:** Data analysis and Interpretation; **Fong Fong Liew and Hsiao Wei Tan:** Statistical analysis and Figures preparation; **Fong Fong Liew:** Manuscript preparation; **Fong Fong Liew and Santhosh Kotian:** Supervision; **Fong Fong Liew:** Cell lines, Chemical and Reagent purchasing. All authors contributed, critically reviewed the manuscript and approved the final version.

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