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MICROBIOLOGICAL AND PHYSICOCHEMICAL EVALUATION OF CHITOSAN NANOPARTICLES OF CHEMICAL AND FOOD GRADE, LOADED WITH LIQUID SMOKE AND THEIR APPLICATION IN TROUT FILLETS (*Oncorhynchus mykiss*)

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ABSTRACT

The growing demand for natural and safe preservatives has increased interest in biopolymer-based delivery systems capable of enhancing the stability and activity of natural antimicrobials. This study evaluated food-grade chitosan (QGA) and chemical-grade chitosan (QGQ) nanoparticles (NPs) as carriers for liquid smoke (LS), with a focus on their potential use in extending the shelf life of rainbow trout fillets (Oncorhynchus mykiss). The objective was to determine whether QGA nanoparticles offer a safer and more effective option for food applications. Nanoparticles were prepared using ionic gelation and characterized by dynamic light scattering (DLS), polydispersity index (PDI), zeta potential, and scanning electron microscopy (SEM). The LS encapsulation efficiency averaged 68.3% with particle sizes ranging from 178 to 423 nm, PDI values between 0.23 - 0.34, and both formulations showed zeta potentials above +40 mV, confirming good colloidal stability. Both nanoparticle types exhibited high antioxidant activity (~95%) and strong antimicrobial effects, achieving roughly 90% inhibition against Gram-positive and Gram-negative bacteria. Minimum inhibitory concentrations were 12.5% for Gram-positive and 50% for Gram-negative strains. Microbiological tests on trout fillets treated with the nanoparticles showed marked preservative effects, suppressing the growth of mesophilic aerobic bacteria, lactic acid bacteria, *Escherichia coli*, *Staphylococcus aureus*, and *Salmonella spp*. Fillets treated with QGA-LS and QGA nanoparticles consistently showed better stability than those treated with QGQ, indicating that food-grade chitosan provides a more suitable matrix for LS encapsulation. Overall, the results demonstrate that food-grade chitosan nanoparticles are effective carriers for liquid smoke, combining high encapsulation efficiency, strong bioactivity, and significant improvements in the microbial stability of perishable foods. This work supports the development of natural, chitosan-based preservative systems for the food industry.

Key words: Encapsulation, trout, liquid smoke, nanoparticles, chitosan, food grade chitosan, antioxidant activity, microbiological stability, chitosan

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INTRODUCTION

Currently, the world population has surpassed 8 billion people, leading to a substantial increase in the demand for food products. Within this demand, the fishing industry has experienced significant growth due to the nutritional and protein contribution it provides [1]. However, its production has reached critical points that, if increased, could cause irreversible effects on marine biodiversity. One potential solution to this is the aquaculture industry, of which trout is one of the most appreciated fishes due to its high nutritional content and quality of animal protein. Additionally, it is relatively easier to cultivate and renew [2]. The shelf life of food products is an essential factor of great importance to consumers. However, in fish, the high content of proteins and unsaturated fatty acids, compared to other food products, makes them degrade more rapidly through oxidative deterioration or sometimes due to pathogenic microorganisms. These are the main issues for both the fishing industry and consumers [3,4].

Different processes and methods have been used for decades for fish processing and long-term preservation [5]. Recently, among the applied methods, antimicrobial substances, natural and synthetic additives have been utilized, such as coatings that inhibit bacterial growth [6], as well as packaging materials (films) based on biopolymers like chitosan, starch, alginate among others [7]. Furthermore, many researchers have shown great interest in new nanotechnological materials such as nanoemulsions, nanoparticles, among others based on biomaterials (organic and inorganic), loaded with various bioactive compounds, aiming to delay bacterial growth and even the chemical and physical degradation of fish fillets like trout [3,4]. Additionally, these nanotechnological materials have the capability to maintain the flavor and sensory properties of the fillet.

Natural preservatives have become one of the best alternatives for preserving and extending food shelf life. One of these preservatives is liquid smoke (LS), a flavoring derived from burning hardwood components like hemicellulose, cellulose and lignin [8]. Liquid smoke (LS) enhances food products like fish fillets by providing flavor, antioxidant, and antimicrobial effects, due to its rich composition of phenolics, carbonyls, flavonoids, and aldehydes [9,10]. Indeed, LS has a positive and significant inhibitory effect on gram-positive bacteria (*S. aureus* and *B. subtilis*) and gram-negative bacteria (*E. coli* and *Salmonella spp.*), albeit with slight inhibitory differences between them, determined by the Kirby–Bauer method [11]. Likewise, LS combined with carrageenan was able to reduce bacterial growth in meatballs from 13.54 % to 99.93 % after 36 hours [8]. In addition, Moringa oil-loaded chitosan nanoparticles embedded in gelatin nanofibers effectively combat *Listeria monocytogenes* and *Staphylococcus aureus* on cheese without altering its sensory

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quality. Similarly, oregano essential oil-loaded zein-pectin-chitosan nanoparticles coated onto Harbin red sausage inhibit lipid oxidation and spoilage bacteria growth, extending shelf life and improving flavor retention. These findings highlight the potential of nanoparticle coatings for enhancing food safety and quality [7,12].

Chitosan is a polysaccharide-type biopolymer derived from chitin and has numerous properties, including being non-toxic, biocompatible, biodegradable, among many other biological functions. However, one of the most prominent is its strong antimicrobial and preservative activity, making it unique for the food industry as it can extend the shelf life of various types of meat such as fish fillets, chicken and others [1,13]. Recent advances in nanotechnology have enabled the development of diverse matrices, such as nanoemulsions, nanoparticles and nanofibers that improve the encapsulation and application of preservatives such as liquid smoke (LS). For instance, Ceylan *et al.* [3] reported that LS combined with thymol encapsulated in chitosan nanofibers delayed the microbiological deterioration of sea bass fillets. Similarly, studies have shown low counts of mesophilic and psychrophilic bacteria during the first three days of storage of fish fillets at 6 °C, confirming its strong bactericidal and preservative effects. Moreover, the combination of chitosan with LS has emerged as a promising alternative for meat preservation, as their synergy significantly enhances antibacterial activity [6].

In this context, the objective of the present study was to evaluate the effect produced by chitosan nanoparticles of chemical grade (QGQ) and food grade (QGA) loaded with LS on the biological and antimicrobial activity in trout fillets, by determining the minimum inhibitory concentration (MIC), antioxidant capacity and evaluating microbiological stability through the enumeration of microbial indicators over a period of 20 days.

MATERIALS AND METHODS

Reagents

The reagents used in the present research work were low molecular weight food-grade chitosan (QGA), chemical-grade chitosan (QGQ), glacial acetic acid, benzo(a)pyrene, sodium tripolyphosphate (TPP) (99 %, Sigma-Aldrich®), liquid smoke LS (Special Smoke 08696, Frutarom®), acetonitrile (ACN, HPLC grade, J.T. Baker), diphenylpicrylhydrazyl (DPPH, Sigma-Aldrich®), and Mueller-Hilton agar (Biolife).

Preparation of chitosan nanoparticles

The preparation of liquid smoke-loaded nanoparticles (QGA-LS, QGQ-LS) was conducted following the method of ionic gelation described by Tuesta-Chavez *et al.* [14] with certain modifications. First, a solution of 1.5 mg mL^{-1} of chitosan (chemical

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or food-grade) was prepared with a 2.4 mg mL^{-1} acetic acid solution under magnetic stirring for 24 hours. After the specified time, the solution was centrifuged for 30 minutes at 6000 rpm. From the supernatant, 10 mL was taken and mixed with 8.77 µL of LS. This chitosan and LS solution were placed under constant magnetic stirring at 300 rpm. Subsequently, 6 mL of TPP 0.25 mg mL^{-1}, previously adjusted to pH 5 with acetic acid, was added dropwise, and the mixture was left for an additional hour under magnetic stirring at 300 rpm. The preparation of smoke-free nanoparticles (QGA, QGQ) was carried out following the same previous procedure without adding the 8.77 µL of LS.

Characterization of nanoparticles

The particle size, polydispersity index (PDI), and zeta potential of the QGA, QGA-LS, QGQ, QGQ-LS nanoparticles were measured by dynamic light scattering (DLS) using a Zetasizer instrument (Malvern® Instruments), Nano-ZS90 model, with a 633 nm laser at a scattering angle of 90°. The morphological analysis of the nanoparticles was performed using scanning electron microscopy (SEM - JEOL).

Loading capacity and efficiency

The loading capacity and efficiency of QGA-LS, QGQ-LS were evaluated in a single experiment based on the method described by Deng *et al.* [15] with some modifications, To conduct this experiment, the nanoparticle suspensions of each formulation (10 mL) were dried at 50°C for 48 hours and then weighed (nanoparticle mass). The nanoparticles were redissolved by adding 10 mL of deionized water, and the mixture was subjected to magnetic stirring at 200 rpm for 48 hours. The amount of loaded LS was estimated using a calibration curve from 12.5 to 500 µg mL^{-1} of LS at λ = 271 nm by UV-Vis spectrophotometry (Shimadzu®, PharmaSpec UV-1700), and applied in equations (1) and (2) to determine the loading efficiency (% LE) and loading capacity (% LC).

$$\% \text{ LE} = \frac{(\text{Total liquid smoke mass} - \text{released liquid smoke mass})}{\text{Total liquid smoke mass}} \times 100\% \quad .. (1)$$

$$\% \text{ LC} = \frac{(\text{Total liquid smoke mass} - \text{released liquid smoke mass})}{\text{nanoparticles mass}} \times 100\% \quad ..... (2)$$

The total mass of LS corresponds to the mass added during the preparation of the nanoparticles; the mass of released LS corresponds to the mass of LS released over a period of 48 hours and measured from the absorbance in the UV-Vis spectrophotometer; and the mass of nanoparticles corresponds to the mass of chitosan nanoparticles loaded with LS after drying.

Study of controlled release of LS

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The release of LS was carried out following the method described in a previous work [14] .In summary, 10 mL of QGA-LS and QGQ-LS were placed into a dialysis membrane with a molecular weight cutoff of 12 kDa in 50 mL of deionized water at 4°C. Samples were taken at 0, 10 and 30 minutes, and then every 60 minutes up to 9 hours, then at 21, 22, 23 and 24 hours. Analysis of the released smoke was conducted using UV-Vis spectrophotometry at 271 nm.

Antioxidant capacity

The antioxidant activity was determined using the diphenylpicrylhydrazyl (DPPH) assay for QGA-LS and QGQ-LS, following the method described by Jiin-Tzong Guo *et al.* [16]. Initially, 1 mL of 0.1 mM DPPH solution was mixed with 1 mL of QGA-LS or QGQ-LS solution, then was kept for 1 hour in a dark place. Absorbance was measured at 517 nm. The antioxidant capacity was determined as a percentage of the DPPH radical scavenging capacity using the following equation (3):

$$\text{Radical scavenging activity } \% = \left(\frac{A_{DPPH} - A_{sample}}{A_{DPPH}}\right) \text{ x } 100\% \text{ ..... (3)}$$

Where, A_{DPPH} and A_{sample} are the absorbances of DPPH and QGA-LS, QGQ-LS, respectively.

Antimicrobial capacity

The antimicrobial activity of the different samples was carried out through the broth microdilution assay described by the Clinical and Laboratory Standards Institute [17]. First, an inoculum was prepared at a concentration of 0.5 Mc Farland Unit of *St. aureus*, E. coli, *B. cereus*, and *Salmonella spp*. strains, whose equivalence is 1-2 x 10^8 CFU mL^{-1}. Then, 90 µL of Mueller Hinton broth was filled into the 96 wells of the plate except for the wells in the 2nd column, to which 180 µL of extract was added. Serial dilutions were made from this; that is, 90 µL was transferred from one column to another until the 11th column. Subsequently, the initial inoculum was diluted to a concentration of 10^6 CFU mL^{-1} of each strain and 10 µL was added to all wells except those in the 1st column, to obtain a final volume of 100 µL in each well. The prepared plate was incubated at 37°C for 24 hours, and the minimum inhibitory concentration (MIC) was determined by reading the turbidity spectrophotometrically at 600 nm in the microplate reader (Biotek Power Wave XS2 Model). The analyses were performed in triplicate.

It should be noted that the absorbance values obtained from the 1st column represent the control and those from the 12th column represent the Positive Control. The values obtained from the other columns represent the antimicrobial effect from a concentration of 100 % to 0.20 % against a microorganism. The results were expressed as a percentage of inhibition, calculated using the following equation (4):

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$$\% \text{ Inhibition} = \left(1 - \frac{A_S - A_{CN}}{A_{PC} - A_{NC}}\right) x\ 100\% \ldots\ldots\ldots (4)$$

A_{PC}: Average Absorbance of the Positive Control, A_S: Average Absorbance of the Sample, and A_{NC}: Average Absorbance of the Negative Control.

Application of QGA-LS and QGQ-LS NPs in trout fillets

Collection and Treatment of Trout

Trout at their commercial stage (approximately 1 kg each one) were collected from the SHAGA Aquaculture, Oxapampa province, Pasco region, Peru following good aquaculture practices. Subsequently, they were descaled, manually eviscerated, and filleted using a dorsal cut (approximately 200 g each one); thereafter, they were immersed in a disinfectant solution of 0.125 mol L^{-1} chlorine dioxide, except for one fillet which was directly vacuum-packed. This fillet was referred to as the "no cleaning solution" sample and was frozen until analysis.

The total disinfected fillets were separated into 6 groups of 20. The first group was untreated and labeled as the control group. For the second group, 5 mL of QGA nanoparticle was added to each vacuum-packed bag along with the fresh trout fillet, then vacuum-packed and sealed. Lastly, the packaged fillets were stored in a polystyrene foam cooler box with ice distributed in layers. The same procedure was also carried out for the remaining groups labeled as: QGQ (Chemical-Grade Chitosan Nanoparticles), QGA-LS (Food-Grade Chitosan Nanoparticles loaded with Liquid Smoke), QGQ-LS (Chemical-Grade Chitosan Nanoparticles loaded with Liquid Smoke), and LS (Liquid Smoke). The 6 polystyrene foam cooler boxes were sealed with transparent film and kept at 4°C until microbiological analysis.

Determination of microbiological stability

The microbiological stability of trout fillets was assessed by enumerating microbial indicators over a 20-day period following direct treatment with the control, QGA, QGA-LS, QGQ, and QGA-LS matrices. The analysis was conducted against viable mesophilic aerobic bacteria using the 3M Petrifilm method (AOAC 990.12, 2021), similarly for *E. coli* (AOAC 991.14, 2021) and *S. aureus* (AOAC 2003.11, 2007), while lactic acid bacteria were assessed using the method established in ISO/FDIS 15214 (1998), and for *Salmonella spp*. through the method described in the Bacteriological Analytical Manual [18], with some modifications.

RESULTS AND DISCUSSION

Characterization of nanoparticles

Table 1 presents the increase in particle size between loaded and unloaded nanoparticles, demonstrating clear size changes between them and indicating the

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existence of interactions between LS and chitosans [19]. Additionally, the Zeta potential of all four samples exceeded + 30mV, indicating high stability [20].

On the other hand, the PDI of the samples was less than 0.5, indicating relatively homogeneous dispersion [21]. These results were confirmed by SEM, as shown in Figure 1, where the surface morphology of QGA, QGA-LS, QGQ, and QGQ-LS nanoparticles can be observed to be spherical with some degree of agglomeration, like the findings reported by Morales-Olan *et al.* [22].

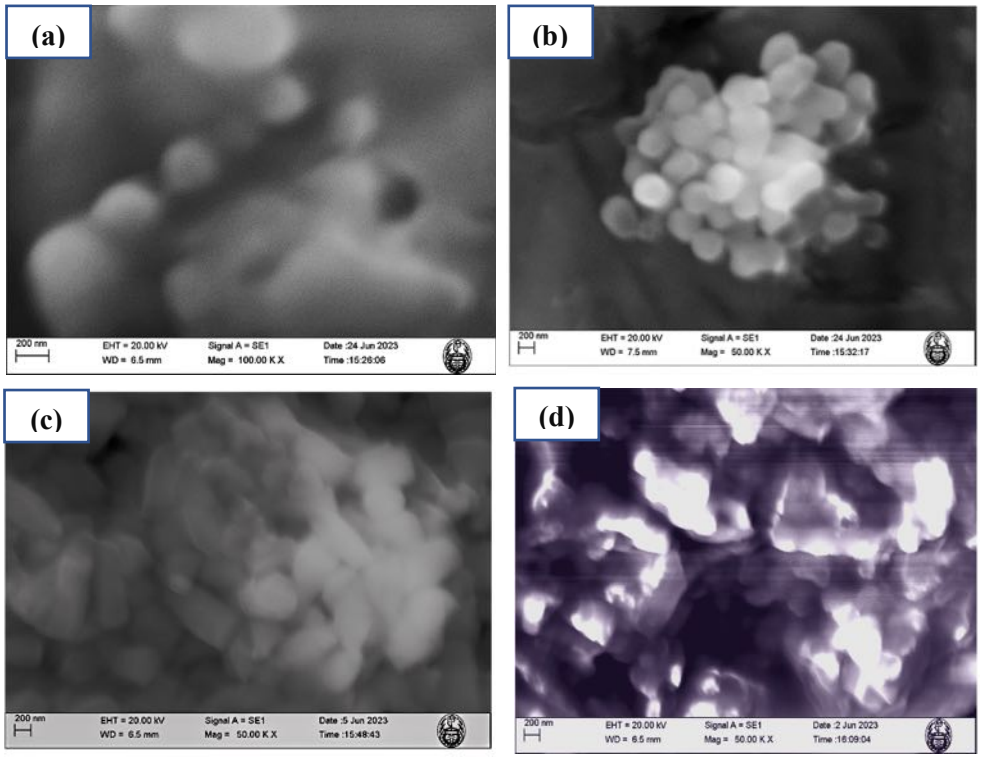

Figure 1: Micrograms SEM of (a) QGA, (b) QGA-LS, (c) QGQ y (d) QGQ-LS

Loading Efficiency and Capacity

The loading efficiency of LS was 68.20% and 68.39% in QGA-LS and QGQ-LS, respectively, while the loading capacity was 83.46% for QGA-LS and 50.08% for QGQ-LS. The results indicate that although both chitosan exhibit similar loading efficiency, the loading capacity of food-grade chitosan is much higher. This difference may be associated with the molecular weight, as the molecular weight for QGA was 152 kDa, while for QGQ was 496 kDa. Therefore, less QGA-LS will be required to release a specific amount of LS compared to QGQ-LS [23].

Study of Controlled Release of LS

The study of controlled release indicates how much of the LS loaded into chitosan nanoparticles can be released within a specified time. For the current study, all

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measurements were conducted in triplicate within the first 24 hours. The release profile of both chitosan, as shown in Figure 2, revealed a kinetics comprising two stages: a rapid one during the first 240 minutes due to the release of LS compounds adsorbed more superficially in the nanoparticles, followed by a slower stage starting from 360 minutes. At this point, the percentage of release in both samples began to stabilize until reaching 31.80 ± 2.89 % for QGA-LS and 31.62 ± 1.36 % for QGQ-LS. Nevertheless, the quantity of LS released surpasses the minimum amount specified by Saloko *et al.* [24] for LS to function as an antioxidant and antimicrobial agent in tuna meat.

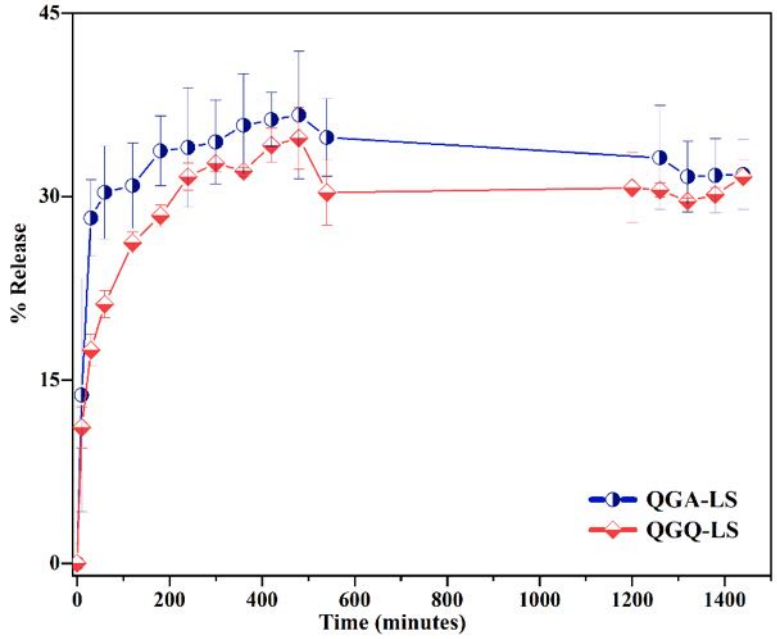

Figure 2: Controlled release of liquid smoke from QGA-LS and QGQ-LS

Antioxidant Capacity

Liquid smoke, besides its preserving and sensory properties, is also known for its antioxidant action. However, to confirm if this activity persists when encapsulated in chitosan, the DPPH test was conducted, resulting in antioxidant capacity values of 95.36 % in QGA-LS and 95.56 % for QGQ-LS. These results are attributed to the fact that the activity mainly comes from the LS loaded in the chitosan nanoparticles, as it has been reported that unloaded chitosan nanoparticles exhibit very low antioxidant activity [25].

Antimicrobial Capacity

According to Table 2, it has been observed that all four matrices exhibit remarkable antimicrobial activity, above 80 % inhibition, against *St. aureus*, up to a dilution of 12.50 %, reaching average inhibition rates of 91.91 % (QGQ-LS) to 96 % (QGQ).

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Meanwhile, against *B. cereus*, inhibition rates range from 85.55 % (QGQ-LS) to 92.07 % (QGA-LS), except for the QGA nanoparticle treatment, which showed an inhibition value below 80 %; therefore, the minimum inhibitory concentration would be 25 %. On the other hand, there is a directly proportional relationship between concentration and inhibition rate; that is, decreasing the antimicrobial reduces its effect, consequently allowing microbial growth at concentrations below 6.25 %. Against both gram-positive bacteria, loading LS into QGA nanoparticles increases antimicrobial activity compared to unloaded nanoparticles, while in the case of QGQ, this effect was not clearly manifested. Finally, it can be affirmed that the minimum inhibitory concentration is at 12.50 %, except for the QGA treatment against *B. cereus*, which was observed at 25 % dilution compared to the original concentration.

According to Table 3, it has been found that all four matrices show remarkable antimicrobial activity against *E. coli*, up to a dilution of 12.50 %, except for QGA, which resulted in an inhibition of 40.38 %. However, the minimum inhibitory concentration was observed at a 25 % initial dilution. On the other hand, in the case of *Salmonella spp*., a concentration of 50 % was required to achieve an inhibition rate higher than 80 %. Although gram-negative bacteria showed more resistance to the antimicrobial action of the agents, as in the previous case, a directly proportional relationship between concentration and percentage of inhibition was noted. Likewise, the loading of LS in QGA nanoparticles increased antimicrobial activity compared to unloaded nanoparticles, while in the case of QGQ, its activity decreased. Finally, it can be stated that the minimum inhibitory concentration is 25 % and 50 % for *E. coli* and *Salmonella spp*., respectively.

Table 4 shows that the antimicrobial effect of LS was moderate at a direct exposure of 100 % concentration. Additionally, the inhibition rate against gram-negative bacteria (*E. coli* and *Salmonella spp*.) is higher compared to gram-positive ones (*St. aureus* and *B. cereus*). However, it has been observed that its initial dose is more and less effective against *E. coli* and *B. cereus*, respectively.

In summary, the results obtained demonstrated that the antimicrobial agents QGQ and QGA-LS were more effective as they showed higher inhibition percentages against all four microorganisms. Likewise, it was shown that a diluted concentration of 50 % is effective for the elimination of gram-negative bacteria, while a dilution of 12.5 % is effective for gram-positive ones. The difference in effectiveness is related to the natural resistance from the cell wall of gram-negative bacteria [26]. Finally, it was observed that the effectiveness in inhibiting microorganisms follows the following decreasing order: *St. aureus* > *B. cereus* > *E. coli* > *Salmonella spp*.

The study confirmed that loading LS into QGA nanoparticles slightly increased the inhibition percentage across all four microorganisms. This finding aligns with

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literature reports suggesting that LS, as an antimicrobial agent, enhances the antimicrobial capacity of chitosan nanoparticles [27,28]. Conversely, loading into QGQ nanoparticles had a negative impact on activity, suggesting that the interaction between the phenolic compounds of LS and chitosan counteracts the antimicrobial effect of QGQ nanoparticles [24]. In summary, the results suggest that nanoparticles have the potential to inhibit the growth of *E. coli* and *Salmonella spp*., which are commonly associated with food safety issues. Additionally, the high inhibition percentages observed for the antimicrobials indicate a more effective means for their control [6,24,28].

Microbiological Stability

Table 5 illustrates the results of the Analysis of Variance (ANOVA) statistical analysis, depicting the probability values (P-value) for microbial count indicators in trout fillets treated with control, QGA, QGQ, LS, QGA-LS, and QGQ-LS over a 20-day period, along with the minimum and maximum averages. Initially, on day zero, the matrices show no significant differences regarding viable aerobic mesophilic bacteria count ($P > 0.05$). This finding was corroborated by confidence interval (CI) analyses and Fischer's mean differences (Figure 3, a1- a2), indicating a uniform antimicrobial behavior among the treatments.

However, over time, from day 1 to 20, a notable probability ($P < 0.05$) emerged, suggesting significant differences in microbial indicator counts, with a minimum and maximum mean load of 2.92 and 8.47 log (CFU g^{-1}), respectively. In essence, this reflects varying effectiveness in controlling the growth of viable aerobic mesophilic bacteria among the treatments. Notably, these variations were further supported by analyzing confidence intervals and Fischer's mean differences on day 20 (Figure 3, b1-b2), revealing that treatments with QGA-LS and QGQ-LS present the most effectiveness against bacterial growth, potentially due to the synergistic effect of chitosan and LS. However, it is worth noting that microbial loads exceeded the maximum established limits (log (UFC g^{-1}) < 5) for aerobic mesophilic bacteria in meats [29].

In terms of lactic acid bacteria (LABs) in trout fillets, the analysis showed a range of 1 to 5.84 log (CFU g^{-1}) over the 20-day period. Initially, between day 0 and 7, all matrices exhibited similar growth behavior ($P > 0.05$), indicating no significant differences in their effect against LABs. This consistency was affirmed by confidence intervals and Fischer's mean comparison (Figure 3, c1 - c2). However, slight variations were attributed to inherent microbiological analysis variability.

Subsequently, between day 13 and 20, significant differences ($P < 0.05$) in the samples' effect for each treatment were observed, highlighting substantial variances in antibacterial activity. This trend was consistent with Fischer mean comparison

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analysis (Figure 3, d2), where each treatment differed, except for QGQ and QGA. Consequently, confidence intervals (Figure 3 d1) for the 20-day showed a microbial load > 5 log (CFU g^{-1}), indicating considerable presence of LABs. However, despite the high bacterial load, LABs are known for their inhibitory effect against various pathogens and have long been considered natural food preservatives [30,31].

The enumeration of *E. coli* in trout fillets revealed a range from 1 to 2.06 log (CFU g^{-1}), respectively. Initially, no appreciable bacterial growth was detected during the first 7 days, attributed to conservation conditions and microbial competition from LABs [31]. Moreover, the reported antibacterial capacity of chitosan and LS contributed to this inhibition [10,32]. However, from day 13 onwards, *E. coli* counts increased rapidly, with day 20 showing the highest population. Nonetheless, statistical analysis indicated no significant differences in *E. coli* counts among treatments ($P > 0.05$). These results were further supported by confidence intervals and Fischer comparative means (Figure 3, e1-e2), respectively, indicating levels within acceptable limits for fresh food consumption [33]. Additionally, similar results have been reported in Africa for different meat products or smoked fillets [34].

Moreover, the study revealed mean *S. aureus* counts ranging between 1 and 1.43 log (CFU g^{-1}), indicating good handling of trout fillets and adherence to established consumption limits [33]. Additionally, counts were statistically similar across all treatments ($P > 0.05$), emphasizing the importance of evaluating *S. aureus* presence due to potential health hazards associated with high concentrations [35].

Foods such as poultry, fish and other fresh meat products are common sources of salmonellosis since they are rich in nutrients and do not contain inhibitory agents to prevent their growth [36]. Additionally, an inoculum between 10^6 to 10^8 of *Salmonella spp*. can lead to the development of symptomatic diseases such as fever, diarrhea, vomiting, among others, which can be chronic if contamination exceeds acceptable limits [33]. In the present study, no *Salmonella spp*. was found in each of the 25 g samples analyzed, indicating that trout is not a carrier species of this group of pathogenic bacteria. This underscores the effectiveness of the treatments in inhibiting *Salmonella spp*. growth in trout fillets [29,33].

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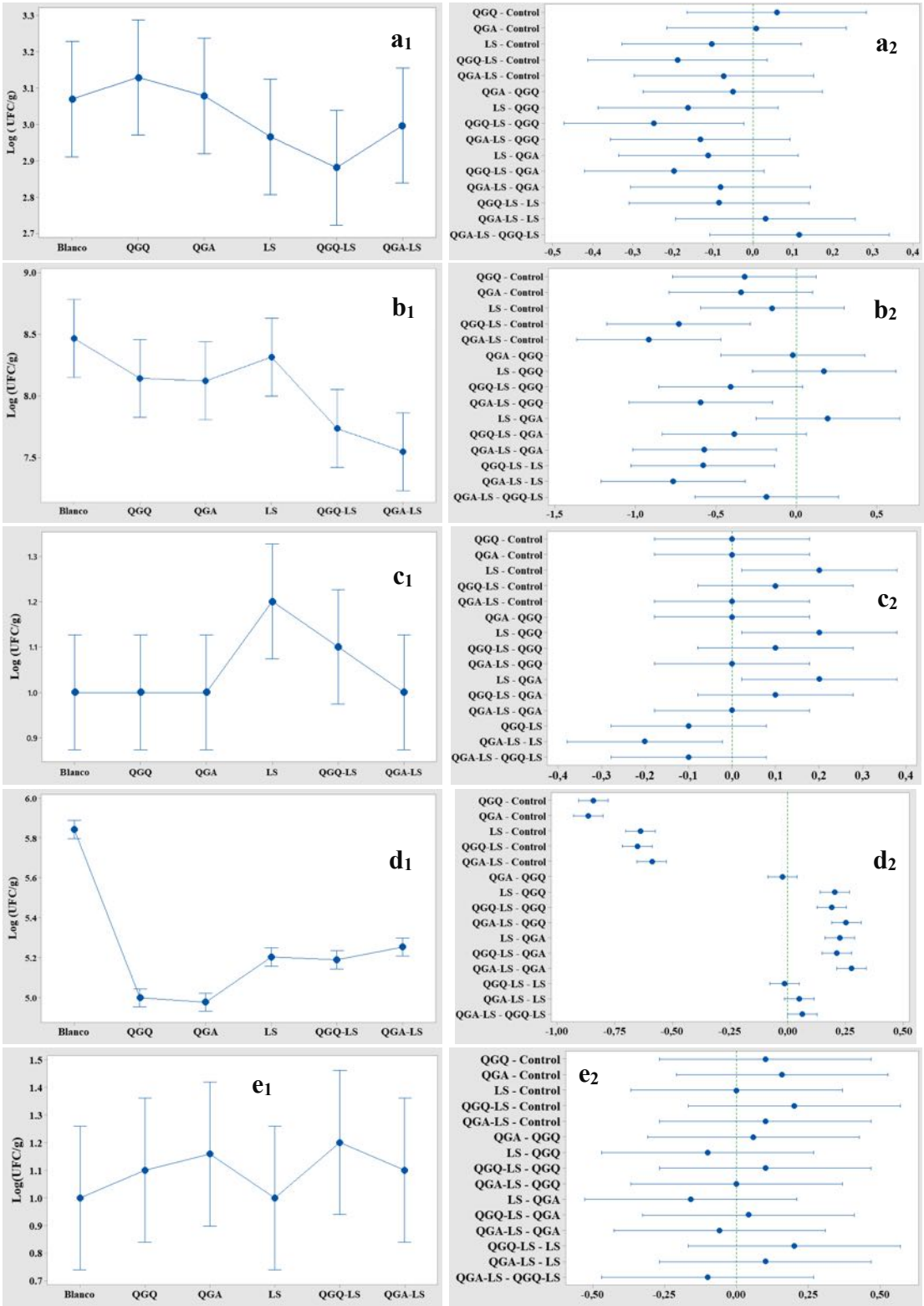

Figure 3: Confidence intervals and Fischer's mean comparison for the bacterial count of mesophilic aerobic bacteria (a1 and a2, day 0; b1 and b2, day 20), lactic acid bacteria (c1 and c2, day 0; d1 and d2, day 20), and *E. coli* (e1 and e2, day 20), respectively

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CONCLUSION AND RECOMMENDATIONS FOR DEVELOPMENT

The present study successfully demonstrated that loading LS into chitosan nanoparticles significantly contributes to their biological and physicochemical properties, positioning them as a potential matrix for preserving trout fillets. From a physicochemical perspective, the prepared nanoparticles exhibited high stability in suspension with a Z potential greater than + 40 mV and a PDI between 0.2 – 0.34. They exhibited a mostly spherical morphology with some aggregation observed by SEM. Both QGA and QGQ nanoparticles showed a loading efficiency of ~68.3% and similar biphasic release profiles, with an initial rapid release up to 240 minutes followed by a slower phase, reaching 31.8% total release after 24 hours. On the other hand, from a microbiological standpoint, there was high antimicrobial activity with an average of 90 % inhibition against gram-positive bacteria (*St. Aureus* and *B. Cereus*) at concentrations of 12.5 % and gram-negative bacteria (*E. Coli* and *Salmonella spp*.) at concentrations of 50 %. Finally, the evaluation of microbiological stability in trout fillets shows that QGQ-LS, QGQ, QGA-LS, QGA, and LS nanoparticles have a positive inhibitory effect over time against mesophilic aerobic bacteria, *E. coli*, lactic acid bacteria, Staphylococcus aureus, and *Salmonella spp*.; with QGA-LS and QGQ-LS matrices generally exhibiting the best preservation effect for trout fillets.

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Table 1: Particle size, PDI and Zeta potential of the nanoparticles

	Particle diameter (nm)	PDI	Zeta potential (mV)
QGA	178.20 ± 4.50	0.235 ± 0.019	48.07 ± 2.56
QGA-LS	217.53 ± 13.10	0.279 ± 0.018	43.80 ± 6.22
QGQ	350.37 ± 10.29	0.345 ± 0.012	47.66 ± 2.87
QGQ-LS	423.87 ± 7.52	0.329 ± 0.033	44.99 ± 2.27

Table 2: Inhibition of 4 matrices (QGQ, QGQ-LS, QGA, QGA-LS) against gram-positive bacteria

St. aureus					
Concentration	100%	50%	25%	12.50%	6.25%
QGQ	89.48%	93.75%	95.66%	96.00%	58.45%
QGQ-LS	90.34%	89.53%	89.53%	91.91%	0.00%
QGA	88.79%	91.57%	90.88%	93.15%	10.99%
QGA-LS	91.19%	96.47%	93.96%	94.38%	12.34%
B. cereus					
Concentration	100%	50%	25%	12.50%	6.25%
QGQ	91.04%	91.20%	90.73%	90.66%	38.73%
QGQ-LS	88.57%	87.97%	86.46%	85.55%	23.77%
QGA	88.80%	84.35%	85.49%	62.80%	4.35%
QGA-LS	89.96%	88.26%	88.84%	92.07%	5.70%

* The values in red are those below 80 %, therefore do not show significant antimicrobial activity for their use

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Table 3: Inhibition of 4 matrices (QGQ, QGQ-LS, QGA, QGA-LS) against gram-negative bacteria

			E. coli		
Concentration	100%	50%	25%	12.50%	6.25%
QGQ	98.43%	99.05%	94.81%	85.74%	37.21%
QGQ-LS	90.23%	93.24%	94.75%	81.95%	19.33%
QGA	93.24%	91.78%	94.92%	40.38%	18.24%
QGA-LS	98.74%	95.96%	97.14%	95.82%	16.63%
			Salmonella spp		
Concentration	100%	50%	25%	12.50%	6.25%
QGQ	98.83%	99.06%	71.62%	46.42%	18.49%
QGQ-LS	97.34%	87.26%	36.59%	22.93%	7.24%
QGA	97.24%	83.17%	50.00%	4.71%	0.00%
QGA-LS	96.97%	95.53%	53.79%	12.78%	0.00%

* The values in red are those below 80 % and therefore do not present a significant antimicrobial activity for use

Table 4: Inhibition of liquid smoke at direct exposure (100 %) against four microorganisms

Microorganism	Inhibition
E. coli	56.65%
Salmonella. Spp.	54.74%
St. aureus	46.61%
B. cereus	44.46%

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Table 5: ANOVA and overall mean log (CFU g^{-1}) load of bacterial indicator counts in trout fillets treated with control, QGA NPs, QGQ NPs, LS, QGA-LS, and QGQ-LS

Bacteria	Time (Days)							Media (Log (CFU g^{-1}))	
	0	1	2	5	7	13	20		
	P-valor							Min	Max
Mesophilic Aerobic	0.253	0.005	0.027	0.012	0.049	0.000	0.006	2.92	8.47
Lactic acid	0.136	0.573	0.834	0.505	0.393	0.000	0.000	1	5.84
Escherichia Coli	-	-	-	-	-	0.008	0.794	1	2.06
Staphylococcus aureus	-	0.345	-	-	-	-	-	1	1.43
Salmonella spp.	Absence/25 g								

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