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Preservation Effect of Nisin and Chitosan on the Quality of *Patin* (*Pangasius hypophthalmus*) Fillets During Cold Storage

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Abstract

Patin is a type of fish commonly processed into fillets for export purposes. *Patin* fillets are prone to deterioration during cold storage. Nisin is a natural preservative that inhibits microbial growth, particularly Gram-positive bacteria; however, it remains ineffective against Gram-negative bacteria. A combination of nisin and chitosan has been reported to inhibit Gram-negative bacteria. This study aimed to analyze the effect of combining nisin from *Lactococcus lactis* subsp. *lactis* and chitosan, applied by bottle-spraying, on the quality parameters (total plate count/TPC, pH, total volatile base nitrogen/TVB-N, and thiobarbituric acid/TBA) of *patin* fillets during cold storage (4±1 °C) for 16 days. The treatments consisted of 2% chitosan, combinations of nisin–chitosan (125 IU ml^{-1} nisin and 1.5% chitosan, 250 IU ml^{-1} nisin and 1% chitosan, 500 IU ml^{-1} nisin and 0.5% chitosan), and 1,000 IU ml^{-1} nisin. The results showed that both the combined treatments of nisin and chitosan, as well as the individual applications of 1,000 IU ml^{-1} nisin and 2% chitosan, slowed the increase in TVB-N and TBA values. However, these treatments did not significantly affect pH or microbiological quality (TPC). The combination treatment effectively delayed chemical spoilage (TVB-N, TBA) during cold storage; however, microbial limits (TPC) were reached by day 12, indicating that the overall shelf life of *patin* fillets could not be extended beyond this period.

Keywords: chitosan; fish; natural preservative; nisin; Pangasius

INTRODUCTION

Patin (*Pangasius hypophthalmus*) is one of the freshwater fish species widely cultivated in Indonesia because the demand is relatively high (Aryani et al., 2023), particularly in the regions of Jambi, South Sumatra, South Kalimantan, West Kalimantan, East Kalimantan, West Java (Suryaningrum, 2008), and East Java (Fattah et al., 2021). *Patin* fillets from Indonesia are in considerable demand overseas, especially in the Middle East, where they reached 150 tons (Mansur, 2019). The advantage of *patin* fillets lies in their versatility as raw materials for semi-processed products such as meatballs, tempura, and others (Martha, 2006). The filleting process produces boneless, uniform muscle blocks that minimize waste and simplify further processing, making it more time- and labor-efficient (Rathod et al., 2018; Jenita et al., 2022; Vu et al., 2023).

The physical, chemical, and microbiological quality of *patin* fillets is highly susceptible to deterioration and spoilage (Tahiluddin et al., 2022). Chemical and enzymatic spoilage cause loss of freshness, whereas microbiological spoilage leads to the decomposition of fillets (Ghomi et al., 2011). Efforts to preserve the quality of fish fillets generally involve frozen or

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chilled storage, which can inhibit enzymatic and microbial activities (Handayani et al., 2010; Dawson et al., 2018; Nurjanah et al., 2020; Oveland et al., 2024). However, frozen storage may cause quality degradation due to structural changes in the fish muscle (Cheng et al., 2014; Dawson et al., 2018; Liu et al., 2020; Xie et al., 2023) and the loss of fat-soluble vitamins (Dobreva et al., 2013; Duarte et al., 2020). Therefore, chilled storage is preferred as an alternative method for preserving *patin* fillets and can also be combined with other preservation methods to enhance its effect.

Preservation methods can also employ natural preservatives that are safe for human consumption, as demand for them has increased in the long term (Meira et al., 2017). Bacteriocins produced by lactic acid bacteria are considered safe for food applications because they are non-toxic and do not produce harmful toxins. When used as food preservatives, bacteriocins offer several advantages: they are heat-stable, active under acidic conditions, and do not alter the flavor or texture of food products (Garsa et al., 2014; Bhattacharya et al., 2022; Reuben and Torres, 2024).

Among the bacteriocins currently used in food preservation, nisin (produced by *Lactococcus lactis* subsp. *Lactis*) is one of the most widely used due to its proven activity against Gram-positive spoilage and pathogenic bacteria, yeast and fungi, *Mycobacterium* (Todorov et al., 2019), even some viruses (Todorov et al., 2022), regulatory approvals, and demonstrated applications in foods and active packaging (Suganthi et al., 2012; Ibarra-Sánchez et al., 2020; Negash and Tsehai, 2020; Popa et al., 2022; Field et al., 2023). The mechanism of nisin against Gram-positive bacteria involves its interaction with lipid II (a peptidoglycan precursor) in the target bacteria, leading to pore formation in the cell membrane. This increases membrane permeability, causing the leakage of essential cytoplasmic components, such as amino acids, nucleotides, and ions, from damaged cells. As a result, these cells lose their ability to generate energy effectively, ultimately leading to cell death (Punyauppa-path et al., 2015; Li et al., 2018). However, the application of nisin alone remains less effective against Gram-negative bacteria because their outer cell wall acts as a permeability barrier, making it more difficult for bacteriocins to penetrate (Fawzya, 2010; Li et al., 2018; Charest et al., 2024). Therefore, nisin is often combined with other antimicrobial compounds to extend its inhibitory activity against both Gram-negative and Gram-positive bacteria (Pinilla and Brandelli, 2016; Yap et al., 2022), such as chitosan (Hui et al., 2016).

Chitosan, a cationic polysaccharide derived from the deacetylation of chitin, consists of N-*acetylglucosamine* and D-*glucosamine* monomers (Trisnawati et al., 2013; Yarnpakdee et al., 2022; Wu et al., 2024). The advantage of chitosan as a natural preservative, aside from its safety, is that it can inhibit Gram-negative bacteria (Li and Zhuang, 2020; Yan et al., 2021) through its amine functional group ($-NH_2$), which binds to amino acid residues that produce negatively charged proteins in bacteria. The amine functional group can also bind to the free electrons of Mg^+ contained in the Ca^{2+} ribosomes of the bacterial cell wall and form coordinated covalent bonds, leading to bacterial death due to leakage of essential cellular components (Sarwono, 2010; El-Araby et al., 2024). Chitosan can inhibit Gram-negative bacteria more than Gram-positive bacteria because the outer membrane components of Gram-negative bacteria contain lipoproteins and lipopolysaccharides that are more sensitive to chitosan, thereby causing structural changes in the outer membrane (Killay, 2013; Ardean et al., 2021).

To enhance their effectiveness, antimicrobial agents such as chitosan are often applied by dipping, spraying, or coating. Spraying antimicrobial compounds can inhibit the growth of spoilage and pathogenic microorganisms, particularly in dairy products, cereal-based products, soup, sauces, juices, and fresh-cut fruits and vegetables (Lucera et al., 2012; Salas et al., 2017; Aslam et al., 2021; Osimani et al., 2022). More recently, studies confirm that spraying techniques offer better control over contact uniformity and reduced cross-contamination compared to dipping, making them highly suited for industrial-scale application (Weng et al., 2025).

Previous studies have investigated the application of antimicrobial compounds using the spray method. Silvia et al. (2014) applied chitosan at a concentration of 2.5% by spraying on catfish (*Clarias batrachus*) and mackerel (*Rastrelliger* sp.). Similarly, the application of nisin and rosemary essential oil through spraying on carp fillets (*Cyprinus carpio*) stored at 4 °C was reported to maintain fillet quality for up to 9 days

while inhibiting the growth of mesophilic and *Staphylococcus* bacteria (Rezaei and Shamloofar, 2016). However, no studies have yet examined the combined use of nisin and chitosan through spraying on microbiological and chemical quality of *patin* fillets. Therefore, this study was conducted to evaluate the effect of combining nisin derived from *Lactococcus lactis* subsp. *lactis* and chitosan on the microbiological and chemical quality (total plate count/TPC, pH, total volatile base nitrogen/TVB-N, and thiobarbituric acid/TBA) of *patin* fillets during cold storage (4±1 °C) for 16 days.

MATERIALS AND METHOD

Materials

This study used nisin produced by *Lactococcus lactis* subsp. *lactis* (product number N5764) obtained from Sigma-Aldrich Pte Ltd. Fresh *patin* fish weighing approximately 1 to 2 kg per fish and aged less than one year were sourced from Mina Sejahtera, Sragen, Central Java. Chitosan was obtained from CV. Chemix Pratama, Yogyakarta. Acetic acid (1%) was used as a solvent for chitosan, while 0.02 N HCl served as a solvent for nisin. Other materials used included Plate Count Agar (Merck), 0.85% NaCl solution, distilled water, 7% trichloroacetic acid (TCA) solution, boric acid solution, saturated potassium carbonate (K_2CO_3) solution, N/70 HCl solution (0.01428 N HCl, where 1 ml of 1/70 N HCl solution is equivalent to 0.2 mg nitrogen), TBA reagent (0.02 M thiobarbituric acid in 90% glacial acetic acid), and 4 M HCl.

Patin fish preparation

The filleting process began with gutting the fish using a knife, cutting off the head and tail, and removing the entrails. The filleting process then involved removing the skin and bones, then washing the fish with running water to produce Pangasius fish fillets (Suryaningrum, 2008). Fish fillets weighing between 300 and 500 g were cut into 50 g pieces per sample. The fillets were then packaged in polypropylene ziplock bags and placed in a cool box containing ice gel.

Nisin suspension preparation

The preparation of nisin combination solutions was carried out following the method of Adilla (2016) with slight modifications. One gram of nisin powder (Sigma) with an activity of 10^6 IU g^{-1} was dissolved in 100 ml of 0.02 N HCl solution to obtain a nisin solution with an activity of 10^4 IU ml^{-1}. Subsequently, aliquots of 0.5, 1, 2, and 4 ml of the nisin solution were diluted with 0.02 N HCl to a final volume of 40 ml, resulting in nisin solutions with activities of 125, 250, 500, and 1,000 IU ml^{-1}, respectively. The Pangasius fillets were then treated with each nisin solution by spraying until the entire surface was evenly covered (40±1 ml of nisin solution for 25 skinless fillets).

Chitosan solution preparation

The preparation of the chitosan solution was conducted according to the modified procedure of Bonilla et al. (2018). One gram of chitosan powder was dissolved in 100 ml of 1% acetic acid solution and homogenized to obtain a 1% chitosan solution. The preparation of chitosan solutions at 0.5%, 1.5%, and 2% followed the same procedure, using 0.5, 1.5, and 2 g of chitosan powder, respectively. Each chitosan solution was then applied to the *patin* fillets by spraying until the entire surface was evenly coated.

Application of nisin and chitosan on *patin* fillets

The application procedure for nisin and chitosan suspensions on *patin* fillets was carried out according to the modified method of Rezaei and Shamloofar (2016). Nisin suspensions at the desired concentrations were sprayed onto the fillets (40±1 ml of nisin solution for 25 skinless fillets), followed by spraying 40±1 ml of chitosan solution, alternating between the two, until the entire surface of the fillets was evenly coated. The treatments consisted of 2% chitosan, combinations of nisin–chitosan (125 IU ml^{-1} nisin and 1.5% chitosan, 250 IU ml^{-1} nisin and 1% chitosan, 500 IU ml^{-1} nisin and 0.5% chitosan), and 1,000 IU ml^{-1} nisin. The sprayed fillets were then placed on plastic trays, wrapped in plastic film, and stored at 4±1 °C. Triplicate samples were prepared for each treatment and storage condition. Microbiological and chemical quality analyses were conducted on days 0, 4, 8, 12, and 16, including TPC following Fardiaz (1993), pH measurement using a Hanna pH meter, TBA analysis following Tarladgis et al. (1960) and Sudarmadji et al. (2010), and TVB-N determination using the Conway dish method SNI 7388:2009 (Indonesian National Standard, 2009).

Data analysis

The data obtained were analyzed using separate one-way analysis of variance (ANOVA) for treatments within each storage day and for storage day within treatments. When significant differences were observed among treatments or storage days, Duncan's multiple range test (DMRT) was performed at a 5% significance level ($p \leq 0.05$).

RESULTS AND DISCUSSION

TPC value

Fresh fish naturally contain microflora that inhabit the skin, gills, and digestive tract, or originate from the water in which they live. The types of microflora found on fresh Pangasius are mainly Gram-positive bacteria, including *Micrococcus*, *Staphylococcus*, *Streptococcus*, *Clostridium*, and *Bacillus alvei* (Noseda et al., 2012; Dambrosio et al., 2016). Meanwhile, Gram-negative bacteria commonly found in Pangasius (*Pangasius pangasius*) include the genera *Psudomonas*, *Acinobacter*, *Flavobacterium*, *Vibrio*, *Enterobacter*, *Serratia*, and *Aeromonas* (Dambrosio et al., 2016; Jalal et al., 2017).

Table 1 shows an increase in TPC in all samples treated with nisin, chitosan, or their combination. The ANOVA results at the 5% significance level indicated that storage duration had a significant effect on the TPC of *patin* fillets. The increase in TPC during the 16-day storage period was presumably due to the post-mortem condition of the fish, which provided nutrients for microbial or bacterial growth that could not obtain sufficient nutrients from the surrounding environment. Consequently, bacteria could grow more rapidly by utilizing the fish tissue as a new nutrient source (Hadiwiyoto, 1993) and during the intermediate stages of spoilage (Kontominas et al., 2021). Zhong et al. (2023) also reported that the total number of spoilage bacteria colonies increased with increasing storage time. Moreover, the pH, water activity (Aw), and moisture content of the fillets also influenced the increase in the TPC (Wally et al., 2015).

Based on the ANOVA results at the 5% significance level, all treatments had no significant effect on the TPC of *patin* fillets during 16 days of storage. On day 0, the TPC ranged from 3.16 to 3.74 log CFU g^{-1}. According to the Indonesian National Standard (SNI 2729:2013) (Indonesian National Standard, 2013), the maximum allowable limit for fresh fish is 5.7 log CFU g^{-1} (equivalent to 5×10^5 CFU g^{-1}), indicating that *patin* fillets treated with 1,000 IU ml^{-1} nisin, 2% chitosan, and the combination of nisin and chitosan were still considered fresh and safe for consumption. On days 4 and 8, the TPC of the treated samples also remained within the "satisfactory" category, corresponding to fresh fish.

On day 12 of storage, the TPC ranged from 6.05 to 7.05 log CFU g^{-1}. This indicates that samples treated with 2% chitosan and the combination of nisin and chitosan were still within the borderline or consumer acceptance limit category, as defined by the Centre for Food Safety (2014), which ranges between 6 and 7 log CFU g^{-1}, except for the sample treated with 1,000 IU ml^{-1} nisin, which reached 7.05 log CFU g^{-1}. By day 16 of storage, the samples treated with 1,000 IU ml^{-1} nisin (7.09 log CFU g^{-1}) and 2% chitosan (7.26 log CFU g^{-1}) had exceeded the acceptable limit and were classified as unsatisfactory or unfit for consumption (Centre for Food Safety, 2014). In contrast, samples treated with combinations of nisin and chitosan at 125 IU ml^{-1} and 1.5%, 250 IU ml^{-1} and 1%, as well as 500 IU ml^{-1} and 0.5%, recording TPC of 6.87, 6.63, and 6.61 log CFU g^{-1}, respectively, remained within the borderline category.

A study by Rezaei and Shamloofar (2016), which applied control, 1.5% rosemary essential

Table 1. TPC value of *patin* fillet during refrigeration storage (4±1 °C)

Nisin (IU ml^{-1}); Chitosan (%)	TPC (log CFU g^{-1})				
	Day-0	Day-4	Day-8	Day-12	Day-16
0; 2	$3.74\pm0.32^{a}{}_{A}$	$4.46\pm0.17^{b}{}_{A}$	$4.86\pm0.10^{b}{}_{A}$	$6.05\pm0.30^{c}{}_{A}$	$7.26\pm0.71^{d}{}_{A}$
125; 1.5	$3.60\pm0.34^{a}{}_{A}$	$4.40\pm0.42^{b}{}_{A}$	$5.39\pm0.54^{c}{}_{A}$	$6.57\pm0.44^{d}{}_{A}$	$6.87\pm0.17^{d}{}_{A}$
250; 1	$3.16\pm0.07^{a}{}_{A}$	$4.00\pm0.25^{b}{}_{A}$	$6.01\pm0.13^{c}{}_{A}$	$6.56\pm0.44^{cd}{}_{A}$	$6.63\pm0.23^{d}{}_{A}$
500; 0.5	$3.17\pm0.15^{a}{}_{A}$	$3.84\pm0.16^{b}{}_{A}$	$5.04\pm0.46^{c}{}_{A}$	$6.28\pm0.62^{d}{}_{A}$	$6.61\pm0.20^{d}{}_{A}$
1,000; 0	$3.69\pm0.33^{a}{}_{A}$	$4.58\pm0.44^{b}{}_{A}$	$5.53\pm0.52^{c}{}_{A}$	$7.05\pm0.03^{d}{}_{A}$	$7.09\pm0.61^{d}{}_{A}$

Note: The same lowercase letters in the same row and the same uppercase letters in the same column indicate no significant difference ($\alpha = 0.05$)

oil, 1.5% nisin, and a combination of 1.5% nisin with 1.5% rosemary essential oil on common carp fillets using a spraying technique, reported TPC ranging from 7.4 to 8.7 log CFU g^{-1} on day 9 of storage. In comparison, the present study showed lower TPC, ranging from 6.61 to 7.26 log CFU g^{-1} in samples treated with the combination of nisin and chitosan, 1,000 IU ml^{-1} nisin, or 2% chitosan after 16 days of storage. The effect of nisin on the quality of *patin* fillets is associated with its antimicrobial properties. Nisin is active against Gram-positive bacteria and their spores. Its function has been reported to disrupt cell wall biosynthesis by binding to the peptidoglycan precursor (lipid II). At the same time, its inhibitory effect on spores occurs through interactions with sulfhydryl-containing membranes that damage spore germination (Adilla et al., 2017).

The antibacterial mechanism of chitosan involves its amine groups (-NH_2), which can bind negatively charged amino acids, thereby inhibiting microbial protein synthesis. These amine functional groups also contain free electron pairs that can coordinate with Mg^{2+} or Ca^{2+} ions present in microbial cell walls and ribosomes, forming coordination covalent bonds that lead to cell death due to leakage of vital intracellular components (Sarwono, 2010; Ardean et al., 2021; Wu et al., 2024). The combination of nisin and chitosan provides enhanced inhibition of microbial contamination compared to either compound alone or the control. This effect is likely due to the complementary antimicrobial spectra of nisin, which is effective against Gram-positive bacteria, and chitosan, which inhibits Gram-negative bacteria. The result is supported by the research of He et al. (2016) on chilled mutton, which found that the combination of nisin, tea polyphenols, and chitosan can be used as preservatives to efficiently inhibit the growth of spoilage microorganisms and pathogens in meat, thereby improving its safety and shelf life.

pH value

The pH of *patin* fillets on day 0 ranged from 6.40 to 6.55. These results are not significantly different from those of Adilla et al. (2017) on nisin-treated *patin* fillets, which showed a pH of 6.35 to 6.44 on day 0. According to Liviawaty and Afrianto (2010), the pH value of Pangasius in the pre-rigor phase ranges from 6.4 to 6.8. It also depends on the fish species, harvesting method, and biological condition, which can influence initial pH variations. The pre-rigor phase is characterized by the fish flesh remaining firm, elastic, and flexible (Liviawaty and Afrianto, 2014). According to ElShehawy et al. (2016), fresh fish are characterized by a pH below 7. This shows that in this study, the *patin* fillets on day 0 can be classified as fresh, as their pH values ranged from 6.40 to 6.55.

Shown in Table 2, the pH values of *patin* fillets treated with nisin, chitosan, and their combination were observed over 16 days of storage. According to the ANOVA results at a 5% significance level, no significant increase in pH was found in samples treated with 2% chitosan, the combination of 125 IU ml^{-1} nisin and 1.5% chitosan, or 1,000 IU ml^{-1} nisin during the 16-day storage period. However, a significant increase in pH was observed in the combination of 250 IU ml^{-1} nisin and 1% chitosan on day 12, and in the combination of 500 IU ml^{-1} nisin and 0.5% chitosan on day 4 of storage.

This increase in pH is presumed to indicate that the samples had entered the post-rigor phase, during which bacterial and enzymatic activity contribute to pH elevation. Proteolytic enzymes break down proteins into smaller molecules, weakening the structure of muscle fibers and cell walls. Similar findings were reported by Zhuang et al. (2023), who demonstrated that endogenous

Table 2. pH value of *patin* fillet during refrigeration storage (4±1 °C)

Nisin (IU ml^{-1}); Chitosan (%)	pH				
	Day-0	Day-4	Day-8	Day-12	Day-16
0; 2	$6.64\pm0.06^{a}{}_{A}$	$6.78\pm0.18^{a}{}_{A}$	$6.80\pm0.01^{a}{}_{A}$	$6.83\pm0.20^{a}{}_{A}$	$7.01\pm0.12^{a}{}_{B}$
125; 1.5	$6.60\pm0.20^{a}{}_{A}$	$6.73\pm0.02^{a}{}_{A}$	$6.75\pm0.07^{a}{}_{A}$	$6.75\pm0.03^{a}{}_{A}$	$6.83\pm0.03^{a}{}_{A}$
250; 1	$6.52\pm0.06^{a}{}_{A}$	$6.53\pm0.03^{a}{}_{A}$	$6.55\pm0.04^{a}{}_{A}$	$6.73\pm0.05^{b}{}_{A}$	$6.76\pm0.02^{b}{}_{A}$
500; 0.5	$6.40\pm0.10^{a}{}_{A}$	$6.59\pm0.04^{b}{}_{A}$	$6.66\pm0.05^{b}{}_{A}$	$6.68\pm0.05^{b}{}_{A}$	$6.69\pm0.02^{b}{}_{A}$
1,000; 0	$6.65\pm0.07^{a}{}_{A}$	$6.69\pm0.03^{a}{}_{A}$	$6.71\pm0.10^{a}{}_{A}$	$6.71\pm0.04^{a}{}_{A}$	$6.79\pm0.07^{a}{}_{A}$

Note: The same lowercase letters in the same row and the same uppercase letters in the same column indicate no significant difference ($\alpha = 0.05$)

and microbial proteases hydrolyze muscle proteins, producing volatile basic compounds that increase pH and accelerate spoilage. Furthermore, during the post-rigor process, phosphatases hydrolyze creatine phosphate and ATP, resulting in the conversion of creatine phosphate to creatine and phosphate, and of ATP to ADP and organic phosphate. ADP is subsequently degraded into ribose, phosphate, ammonia, and hypoxanthine. An elevated hypoxanthine level is associated with a decline in fish quality (Muchtadi et al., 2011). Based on the research of Rasul et al. (2022) on grass carp (*Ctenopharyngodon idella*) during ice storage, the pH will continue to increase during the preservation period. Muscle pH changes from an acidic to an alkaline range in the post-rigor stage due to the production of volatile basic nitrogenous chemicals by bacterial metabolic activity.

Based on the ANOVA analysis at the 5% significance level, all treatments had no significant effect on the pH of Pangasius fillets from day 0 to day 12 of storage. However, on day 16, a significant difference among treatments was observed, with the sample treated with 0 IU ml^{-1} nisin and 2% chitosan showing the highest pH, which was significantly higher than the other treatments. This result is likely due to chitosan's relatively higher antibacterial activity against Gram-negative bacteria than against Gram-positive bacteria, resulting in a less effective overall inhibitory effect on bacteria in Pangasius (Damayanti et al., 2016).

The antibacterial mechanism of chitosan involves increasing cell membrane permeability by binding to bacterial membrane proteins and phosphatidylcholine components, leading to the leakage of essential cytoplasmic constituents. Over time, this process causes cell lysis, inhibits cell regeneration, and ultimately results in cell death (Suptijah, 2006). This finding aligns with Hui et al. (2016), who reported that treatment of yellow croaker (*Pseudosciaena crocea*) with 1% chitosan combined with 0.6% nisin did not significantly alter pH compared with the control. The pH values of *patin* fillets on day 16 ranged from 6.69 to 7.01. According to ElShehawy et al. (2016), fish are considered fresh when the pH is below 7, whereas values above 7 indicate spoilage. Therefore, the pH values of all treatments remained within the acceptable freshness range after 16 days of cold storage.

TVB-N value

On day 0, the TVB-N values of *patin* fillets ranged from 7.36 to 9.76 mg N 100 g^{-1} fillet. According to Farber (1965), fish classified as very fresh have TVB-N values below 10 mg N 100 g^{-1}, indicating that the *patin* fillets in this study were still in a very fresh condition. These results are consistent with those reported by Utami et al. (2018), who investigated the effect of an edible coating containing nisin applied by spraying on the quality of *patin* fillets and found initial TVB-N values ranging from 9.97 to 12.16 mg N 100 g^{-1} on day 0.

Table 3 shows increased TVB-N values in all samples treated with nisin, chitosan, or their combination. ANOVA with a 5% significance level revealed that storage time significantly affected TVB-N values. The ANOVA results were then followed up with a DMRT test. According to Susanti et al. (2013), the increase in TVB-N values in fish fillets is caused by the formation of volatile bases, including histamine sulfides, trimethylamine, ammonia, and histamine, which originate from protein degradation in the fillets or fish meat, resulting in an unpleasant odor. Recent studies also reported that volatile nitrogenous compounds, such as trimethylamine, ammonia, and dimethylamine, accumulate due to microbial and enzymatic activity during storage, thereby increasing TVB-

Table 3. TVB-N value of *patin* fillet during refrigeration storage (4±1 °C)

Nisin (IU ml^{-1}); Chitosan (%)	TVB-N (mg N 100 g^{-1})				
	Day-0	Day-4	Day-8	Day-12	Day-16
0; 2	$7.36\pm0.72^{a}{}_{A}$	$9.57\pm0.19^{a}{}_{B}$	$16.55\pm0.00^{b}{}_{C}$	$32.62\pm3.08^{c}{}_{D}$	$82.57\pm1.36^{d}{}_{E}$
125; 1.5	$8.78\pm0.26^{a}{}_{BC}$	$9.84\pm0.10^{a}{}_{BC}$	$10.02\pm0.67^{a}{}_{A}$	$27.28\pm1.06^{b}{}_{C}$	$51.02\pm1.24^{c}{}_{D}$
250; 1	$9.23\pm0.02^{a}{}_{C}$	$8.59\pm0.03^{a}{}_{A}$	$9.29\pm0.05^{a}{}_{A}$	$24.28\pm0.68^{b}{}_{BC}$	$30.57\pm2.45^{c}{}_{B}$
500; 0.5	$7.63\pm0.29^{a}{}_{AB}$	$10.55\pm0.31^{b}{}_{CD}$	$12.88\pm0.45^{c}{}_{B}$	$21.71\pm0.63^{d}{}_{AB}$	$25.04\pm0.57^{e}{}_{A}$
1,000; 0	$9.76\pm0.57^{a}{}_{C}$	$11.09\pm0.68^{a}{}_{D}$	$13.35\pm0.02^{b}{}_{B}$	$20.19\pm0.09^{c}{}_{A}$	$30.58\pm1.64^{d}{}_{C}$

Note: The same lowercase letters in the same row and the same uppercase letters in the same column indicate no significant difference ($\alpha = 0.05$)

N and decreasing fish freshness (Walayat et al., 2023; Kim et al., 2024).

The ANOVA results showed that all treatments had a significant effect on the TVB-N values of *patin* fillets during 16 days of storage. The DMRT results revealed that on day 0, the sample treated with 0 IU ml^{-1} nisin and 2% chitosan had the lowest significant TVB-N value among the treatments. On day 4 of storage, the sample with a nisin activity of 250 IU ml^{-1} and 1% chitosan showed the lowest significant value compared to other combinations. The samples with a combination of 250 IU ml^{-1} nisin and 1% chitosan, and 125 IU ml^{-1} nisin and 1.5% chitosan, showed the lowest significant difference compared to other treatments on day 8 of storage. On day 12, the combination of 1,000 IU ml^{-1} nisin and 0% chitosan showed the lowest significant TVB-N value, while on day 16, the combination of 500 IU ml^{-1} nisin and 0.5% chitosan showed the lowest significant TVB-N value. This trend is likely due to the synergistic effect between nisin, which inhibits Gram-positive bacteria, and chitosan, which suppresses the growth of Gram-negative microorganisms, thereby slowing protein degradation and maintaining lower TVB-N levels. Cold storage is also believed to help inhibit damage caused by enzymatic reactions, physiological damage, and microbiological damage (Buntu et al., 2017).

In this study, the TVB-N values of *patin* fillet samples in all treatments on day 0 ranged from 7.36 to 9.76 mg N 100 g^{-1}. According to the Chinese standard (Ministry of Primary Industries, 2015), fresh freshwater fish typically exhibit TVB-N values below 20 mg N 100 g^{-1} fillet, indicating that the samples at day 0 were still in a very fresh state. The measurements on days 4 and 8 also showed that the fish remained fresh. By day 12, the TVB-N values ranged from 20.19 to 32 mg N 100 g^{-1}. As reported by Kim et al. (2024), TVB-N values in yellow croaker increased significantly from 4.41 to 24.76 mg 100 g^{-1} during cold storage, indicating protein degradation and the progressive formation of volatile bases such as trimethylamine and ammonia, indicators of spoilage. As reported by Liviawaty and Afrianto (2010), the acceptable limit for fish freshness is 30 to 35 mg N 100 g^{-1}; therefore, *patin* fillets in all treatments were still considered acceptable at this stage of storage.

After 16 days of storage, a similar trend was observed in samples treated with 250 IU ml^{-1} nisin and 1% chitosan (30.57 mg N 100 g^{-1}), 500 IU ml^{-1} nisin and 0.5% chitosan (25.04 mg N 100 g^{-1}), and 1,000 IU ml^{-1} nisin alone (30.58 mg N 100 g^{-1}), all of which remained within the acceptable limit. In contrast, samples treated with 2% chitosan alone (82.57 mg N 100 g^{-1}) and with the combination of 125 IU ml^{-1} nisin and 1.5% chitosan (51.02 mg N 100 g^{-1}) exceeded the recommended freshness threshold, indicating spoilage and rendering them unsuitable for consumption.

TBA value

At day 0 of observation, the TBA value of *patin* fillets ranged from 0.09 to 0.25 mg malondialdehyde (MDA) per kg fillet, indicating that the initial fillets were all in the fresh condition (Suárez-Medina et al., 2024). According to Laksmanan (2000), the acceptable limit for fish is 1 to 2 mg MDA kg^{-1}, indicating that, in this study, *patin* fillets treated with chitosan, nisin, or their combination were still of good quality. The TBA values at day 0 in this study were also consistent with the findings of Wu et al. (2017), who reported a TBA value of 0.5 mg MDA kg^{-1} on day 0 in small yellow croaker (*Pseudosciaena crocea*) treated with 1% chitosan microcapsules containing 0.26% nisin.

Table 4 shows that the TBA values of all samples increased during the storage period. The ANOVA results at the 5% significance level

Table 4. TBA value of *patin* fillet during refrigeration storage (4±1 °C)

Nisin (IU ml^{-1}); Chitosan (%)	TBA (mg MDA kg^{-1})				
	Day-0	Day-4	Day-8	Day-12	Day-16
0; 2	$0.10\pm0.00^{a}{}_{A}$	$0.17\pm0.00^{b}{}_{A}$	$0.18\pm0.10^{b}{}_{A}$	$0.32\pm0.01^{c}{}_{A}$	$0.65\pm0.01^{d}{}_{B}$
125; 1.5	$0.25\pm0.02^{a}{}_{C}$	$0.34\pm0.02^{b}{}_{B}$	$0.40\pm0.01^{c}{}_{D}$	$0.41\pm0.03^{c}{}_{B}$	$0.47\pm0.01^{d}{}_{A}$
250; 1	$0.09\pm0.00^{a}{}_{A}$	$0.18\pm0.01^{b}{}_{A}$	$0.27\pm0.02^{c}{}_{B}$	$0.40\pm0.01^{d}{}_{B}$	$0.87\pm0.06^{e}{}_{C}$
500; 0.5	$0.12\pm0.00^{a}{}_{A}$	$0.18\pm0.02^{b}{}_{A}$	$0.35\pm0.03^{c}{}_{C}$	$0.42\pm0.00^{d}{}_{B}$	$0.43\pm0.06^{d}{}_{A}$
1,000; 0	$0.19\pm0.02^{a}{}_{B}$	$0.20\pm0.01^{a}{}_{A}$	$0.23\pm0.02^{a}{}_{B}$	$0.31\pm0.18^{b}{}_{A}$	$0.98\pm0.05^{c}{}_{D}$

Note: The same lowercase letters in the same row and the same uppercase letters in the same column indicate no significant difference ($\alpha = 0.05$)

indicate that storage time significantly affected the TBA values of *patin* fillets over 16 days of storage. The increase in TBA values is presumed to result from oxidation reactions occurring during storage. The rise in TBA values during storage indicates progressive lipid oxidation, which is affected by oxygen exposure, temperature, and the absence of effective antioxidants (Olatunde and Benjakul, 2022). Lipid oxidation can occur through two mechanisms: enzymatic and non-enzymatic. In the enzymatic process of lipolysis, lipase hydrolyzes glycerides, forming free fatty acids that can degrade oil quality, lead to an undesirable flavor, and increase rancidity. Meanwhile, non-enzymatic oxidative deterioration occurs when unsaturated fatty acids react with atmospheric oxygen. Free oxygen oxidizes double bonds in unsaturated fatty acids, and free radicals and oxygen can form active peroxides that can produce highly unstable hydroperoxides. These hydroperoxides can oxidize intact or decomposed fatty acids, producing aldehydes, ketones, alcohols, polymers, and other low-molecular-weight compounds (Adilla et al., 2017).

Based on the ANOVA analysis at the 5% significance level, samples treated with either the nisin-chitosan combination or without it showed a significant effect on the TBA values of *patin* fillets during 16 days of storage. The results were further analyzed using DMRT. According to the DMRT results, on day 0, fillets treated with 2% chitosan, 250 IU ml^{-1} nisin + 1% chitosan, and 500 IU ml^{-1} nisin + 0.5% chitosan showed significantly lower TBA values than other treatments. On day 4, samples treated with 2% chitosan, 250 IU ml^{-1} nisin + 1% chitosan, 500 IU ml^{-1} nisin + 0.5% chitosan, and 1,000 IU ml^{-1} nisin exhibited significantly lower TBA values than the other treatments. On day 8, the *patin* fillets treated with 2% chitosan had significantly lower MDA levels than those in the other treatments. By day 12, samples treated with 1,000 IU ml^{-1} nisin exhibited the lowest significant TBA value. Meanwhile, on day 16 of storage, samples treated with a combination of nisin activity 500 IU ml^{-1} and 0.5% chitosan, as well as those treated with a combination of nisin activity 125 IU ml^{-1} and 1.5% chitosan, showed significantly lower TBA values than the other treatments.

The inhibition of lipid oxidation observed in this study is likely attributed to the synergistic antioxidant activity of nisin and chitosan. As reported by Wei et al. (2017), chitosan exhibits a notable free radical-scavenging ability, primarily due to the presence of nitrogen groups at the C-2 position, which can effectively neutralize various reactive radicals. The mechanism of chitosan in scavenging free radicals involves the hydrogen atoms from the ammonium ion group (NH_3^+) in chitosan binding to hydroxyl radicals (OH^-) generated during lipid oxidation, forming more stable antioxidant molecules (Nursyamsiah, 2017). Similarly, Wu et al. (2017) reported that yellow croaker fillets coated with chitosan microparticles (1%) combined with nisin (0.26%) exhibited MDA levels below 4 mg MDA kg^{-1} after 9 days of storage, compared to treatments with nisin (0.26%) or chitosan (1%) alone, which showed MDA levels exceeding 4 mg MDA kg^{-1} fillet.

On the final day of storage (day 16), the MDA content of *patin* fillets treated with 2% chitosan, 125 IU ml^{-1} nisin + 1.5% chitosan, 250 IU ml^{-1} nisin + 1% chitosan, 500 IU ml^{-1} nisin + 0.5% chitosan, and 1,000 IU ml^{-1} nisin were 0.65, 0.47, 0.87, 0.43, and 0.98 mg MDA kg^{-1} fillet, respectively. These results indicate that the TBA values of *patin* fillets treated with 2% chitosan, 1,000 IU ml^{-1} nisin, and the combinations of nisin and chitosan remained within the acceptable range of 1 to 2 mg MDA kg^{-1} fillet (Laksmanan, 2000), suggesting that lipid oxidation had not yet reached the level associated with spoilage by the end of the 16-day storage period. This result is similar to Olatunde and Benjakul (2022) and Muñoz-Tebar et al. (2023), who state that coating-based chitosan efficiently lowers lipid oxidation and bacterial populations, achieving a 50% reduction in TBARs values compared to uncoated samples and keeping TBARS values below the standard value (≈1 to 1.5 mg MDA kg^{-1}). Other research on Pangasius fish products (Jeyakumari et al., 2016) and trout fillets (Jasour et al., 2015) also shows that the chitosan-treated samples had lower values than the controls.

CONCLUSIONS

Both the combined treatments of nisin and chitosan, as well as the individual applications of 1,000 IU ml^{-1} nisin and 2% chitosan, slowed the increase in TVB-N and TBA values. However, these treatments did not significantly affect pH or TPC. The combination treatment effectively delayed chemical spoilage (TVB-N, TBA) during

cold storage; however, microbial limits (TPC) were reached by day 12, indicating that the overall shelf life of *patin* fillets could not be extended beyond this period.

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