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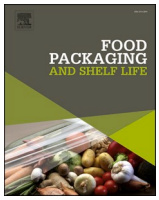

Antimicrobial activity of in-situ bacterial nanocellulose-zinc oxide composites for food packaging

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ABSTRACT

Active substances such as zinc oxide nanoparticles (ZnO) have been extensively explored due to their antimicrobial properties, low cost and scalability. Yet, their effectiveness is highly dependent on their morphology and specific surface area. Bacterial nanocellulose (BNC) is a suitable carrier due to its ability to transport and deliver active substances. In the case of nanocellulose-ZnO composites, conclusions drawn from antimicrobial studies are often based on only a few representatives of Gram-positive and Gram-negative bacteria. A more comprehensive study using different species and strains, and different methods to assess antimicrobial activity is required. Therefore, in this work, the antimicrobial activity of ZnO suspensions and BNC_{ZnO} films was assessed against a wide range of Gram-negative and Gram-positive bacteria using disc diffusion and viable cell count assays. Regarding the results of the disc diffusion assay, the increase of ZnO content (21–27% m_{Zn}/m_{BNCZnO}) (in both ZnO suspensions and BNC_{ZnO} films), increased antimicrobial activity against all Gram-negative bacteria tested and some Gram-positive bacteria. In the viable cell count assay, BNC_{ZnO} films were effective against *Escherichia coli* (3 log reduction) and *Listeria monocytogenes* (1–3 log reduction) after 24 h. Low temperatures reduced the antimicrobial activity of BNC_{ZnO}.

1. Introduction

Active packaging can provide new functionalities very useful for the food industry, with regards for instance to the extension of the shelf life, protecting from foodborne pathogens and preserving the organoleptic properties (Yildirim et al., 2018). Active agents may provide antimicrobial or antioxidant properties. Zinc oxide (ZnO) is a thermally stable metal oxide well-known for its antimicrobial and photocatalytic activities (Kim et al., 2022). ZnO has been recognized as safe by the European Food Safety Authority (EFSA), but only as an ultraviolet light absorber in unplasticized polymers up to 2.0% (m/m) (EFSA CEF Panel, 2017). Despite the excellent antimicrobial properties, the effectiveness of ZnO particles is highly dependent on their morphology, size (specific surface area) and applied dosage (Sirelkhatim et al., 2015). Most of the reported studies focus on optimising ZnO characteristics for maximum antimicrobial activity (Kumar et al., 2017; Yamamoto, 2001).

The incorporation of ZnO into different materials has also been studied to develop active packaging. One example is the incorporation of ZnO into nanocellulose (NC). This is one of the most abundant polymers in nature and, due to its mechanical performance, is highly versatile and can be used in various applications (de Amorim et al., 2020). Among all NCs, bacterial nanocellulose (BNC) stands out for its high degree of polymerization, high mechanical performance, long fibres and water holding capacity. This polymer is produced by *Komagataeibacter* species through fermentation (stirred or static), forming a 3D nanofibrillar gel-like membrane. The water holding capacity of BNC is an interesting feature for active food packaging applications, as suspensions comprising active substances (such as ZnO) can be easily incorporated into BNC.

Several authors reported the development of NC_{ZnO} and BNC_{ZnO}

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films to be used as alternatives to petroleum-based plastics (such as low density polyethylene and polypropylene) in active food packaging (Bastarrachea et al., 2015; Heidari et al., 2022; Janaki et al., 2015; Katepetch et al., 2013; Lefatshe et al., 2017; Mocanu et al., 2019; Shahmohammadi Jebel & Almasi, 2016; Ul-Islam et al., 2014; Wahid et al., 2019). Most of the literature concerns the optimization of ZnO synthesis in combination with nanocellulose to obtain an effective composite with antimicrobial activity (Heidari et al., 2022; Janaki et al., 2015; Katepetch et al., 2013; Lefatshe et al., 2017; Mocanu et al., 2019; Shahmohammadi Jebel & Almasi, 2016; Ul-Islam et al., 2014; Wahid et al., 2019). However, most of the studies have not explored the antimicrobial activity of NC_{ZnO} and BNC_{ZnO} against a wide range of bacterial species and strains but have selected one or two representatives of Gram-negative and Gram-positive bacteria. Most studies used *Escherichia coli* and *Staphylococcus aureus,* the bacteria recommended by the International Organisation for Standardisation (ISO 22196:2011) (Katepetch et al., 2013; Lefatshe et al., 2017; Shahmohammadi Jebel & Almasi, 2016; Wahid et al., 2019), as well as *Klebsiella pneumoniae* (Janaki et al., 2015) and *Bacillus subtilis* (Mocanu et al., 2019). The efficacy of other antimicrobial agents, in particular those derived from plant essential oils (specifically oregano or thyme) or, chitosan, ethanolic propolis extract, and nisin has been demonstrated considering a wider range of Gram-positive and Gram-negative bacteria (Carvalho et al., 2018; Gomes et al., 2021). Foodborne pathogenic bacteria such as *Listeria monocytogenes*, *Bacillus cereus*, *Salmonella* spp., *Yersinia enterocolitica* and *Campylobacter* spp., which may be present in several foods (fruits, vegetables, meat and fish derivatives) (Macieira et al., 2021) and have been responsible for several outbreaks of foodborne illness (Bintsis, 2017; Oliveira et al., 2020), should also be considered when developing active packaging for the food industry. In addition, to understand where nanocellulose-ZnO composites can act, a more comprehensive study using different species is needed. The antimicrobial activity against a pathogen when in contact with NC_{ZnO} and BNC_{ZnO}, should also be explored and confirmed using different strains of the same species, as reported by some authors (Carvalho et al., 2018; Gomes et al., 2021; Oliveira et al., 2020).

As protocolled by the World Organization for Animal Health (WOAH), there are several methods for determining bacterial susceptibility to antimicrobials, such as the disc diffusion method and viable cell count assays (World Organisation of Animal Health, 2019). The former is a widely used method for a preliminary assessment of the "qualitative" susceptibility (susceptible or resistant) of a bacteria to an antimicrobial agent (Cotton et al., 2019). The outcome is nominally observed through halos, which represent the inhibition effect. However, through the disc diffusion assay, only verification of bacterial growth inhibition is foreseen, meaning that bactericidal and bacteriostatic effects are not distinguished. Yet, further testing should be made for the minimum inhibitory concentration (MIC) and/or the minimum bactericidal concentration (MBC) determination, using agar or broth serial dilutions. For this purpose, the viable cell count assay may be used, which allows the quantification of the number of active growing cells in a sample (when in contact with an active agent). Hence, this work has the objective of demonstrating the efficiency of BNC_{ZnO} in inhibiting bacterial growth and its potential as an active food packaging. This is supported by conducting both disc diffusion and viable cell count assays, revealing the different susceptibility of various strains from a set of bacteria. It is also intended to understand the specific mechanisms of ZnO's impact on bacteria, particularly in relation to ROS and the release of Zn^{2+}. This body of information is important to define the specific application in food packaging.

In this work, a BNC_{ZnO} composite was developed, through *in situ* production of wet thin BNC membrane discs. The ZnO particles morphology and size (in suspensions and the BNC composite) were characterised. The ZnO concentration in the BNC composite was also determined. Antimicrobial studies of ZnO suspensions and BNC_{ZnO} (with different ZnO concentrations) were carried out using the disc diffusion assay for screening a wide variety of Gram-negative and Gram-positive bacteria and different strains thereof. The best performing BNC_{ZnO} composite was then further tested, using viable cell count assay method, for bactericidal assessment.

2. Materials and methods

2.1. BNC production and purification

Komagataebacter xylinus ATCC 700178 (from the American Type Culture Collection) cells were grown in 1 L conical flasks, containing 100 mL of Hestrin-Schramm culture medium (HS) (Hestrin & Schramm, 1954) with the following components (in mg mL^{-1}): 20.0 glucose (Fluka, Charlotte, US), 5.0 peptone (Himedia, Mumbai, India), 5.0 yeast extract (Fisher, Hampton, US), 3.39 disodium phosphate di-hydrated (Panreac, Barcelona, Spain) and 1.26 citric acid (Panreac). Hestrin-Schramm culture medium was autoclaved at 121 °C for 20 min. The inoculated fresh culture medium was incubated in cuvettes (10 ×20×10 cm) at 30 °C for 15 days (at a fixed culture medium depth of 1.5 cm). After 15 days of fermentation, BNC membranes were washed with NaOH 0.1 M (Panreac), at room temperature, to remove culture medium residues and trapped cells. Afterwards, the membranes were washed with distilled water, also at room temperature, until the pH became that of the distilled water.

2.2. BNCZnO production

The BNC membranes were sliced into thinner ones with established thickness of 2.0 mm. Discs with 9.0 and 13.0 mm diameter were prepared. In order to dry BNC while preserving its 3D structure, the discs were frozen in liquid nitrogen and lyophilized at − 99 °C and 0.0025 bar (Coolsafe 100–9 Pro, Labogene, Allerød, Denmark).

The ZnO NPs synthesis was performed as reported elsewhere (Jaber & Laânab, 2014), with slight modifications: a 20 mL solution of NaOH (1 M) was carefully added drop by drop (at a rate of 0.67 mL.min^{-1}) using a syringe pump (New Era Pump systems, New York, US) into a solution containing 100 mL of $Zn(CH_3COO)_2$ (Sigma, India) with varying concentrations (0.01 M, 0.03 M, and 0.05 M), along with polyvinyl alcohol (PVOH; Fluka, Germany) at a concentration of 5.0 mg mL^{-1} and the previously prepared BNC discs. A magnetic stirring at 200 rpm and a temperature of 50 °C was employed to the mixture. After complete addition of NaOH, the ZnO suspension was left standing overnight. The BNC discs were then washed with ultra-pure water to neutral pH and air dried at 37 °C. The average dry thickness of all films was 20 μm. Different concentrations of $Zn(CH_3COO)_2$ were used to achieve varying $[OH^-]/[Zn^{2+}]$ ratios, aiming to evaluate both the particle size of the resulting ZnO and the quantity of Zn incorporated into the BNC during *in situ* production.

For comparison purposes, ZnO NPs suspensions were also prepared, using the same procedure described above, but without BNC discs. The ZnO suspension was then washed with ultra-pure water by centrifugation (Eppendorf centrifuge 5430 R, Hamburg, Germany) at 4000 rpm until neutral pH, before being freeze-dried at − 99 °C and 0.025 mbar (Coolsafe 100–9 Pro). The freeze-dried material was then used to prepare ZnO NPs suspensions (by ultrasonication (Bandelin Sonoplus, Berlin, Germany) with 40% amplitude for 4 min) with different concentrations (10, 20, 30, 50, 75 and 100 mg mL^{-1}), for antimicrobial testing. This wide range of ZnO concentrations (in suspension) allows: i) assessing bacteria susceptibility at different ZnO concentration, and ii) comparing the antimicrobial effectiveness of ZnO when in suspension with the effectiveness when incorporated at similar concentrations into BC.

2.3. Physical characterization

BNC thickness - a digital thickness gauge (Adamel Lhomargy, France)

was used to measure the thickness of all the produced discs. For each sample, five measurements (at random positions) were taken.

Dynamic light scattering analysis - The size of ZnO NPs was estimated using a Zetasizer NanoZS (Malvern Instruments Ltd., Worcestershire, UK). Five measurements of each suspension were performed to obtain the average size (nm) and polydispersity (PdI). Before analysis, ZnO NPs suspensions were subjected to ultrasonication as previously described.

Microscopic morphology - BNC and BNC_{ZnO} discs were characterized using a desktop scanning electron microscope (SEM) coupled with energy-dispersive spectroscopy (EDS) analysis (Quanta 650). All results were acquired using the ProSuite software. The samples were placed in an aluminium pin stub with electrically conductive carbon adhesive tape (PELCO Tabs™). Samples were coated with 1 nm of Au (10 Angstrom) for improved conductivity. The analysis was conducted with reduced vacuum and a magnification scale of x5000. The size of ZnO NPs was measured through ImageJ software (version 1.8.0).

Zinc quantification - The concentration of Zn in BNC_{ZnO} was determined by atomic absorption spectroscopy (AAS) after digestion by microwave (based on EN 14084). Samples were prepared following the guidelines of EN 13804 (CEN, 2013): BNC_{ZnO} discs (ca 15 mg) were ground into small pieces and evenly weighed into the digestion vessel. Afterwards, 5 mL of nitric acid 65% (HNO_3) (Panreac) and 2 mL of hydrogen peroxide (H_2O_2) (Merck KGaA, Germany) were added, and submitted to microwave digestion (Speedwave MWS-3 +, Berghof, Eningen, Germany), as presented in Table 1.

2.4. Antimicrobial activity of ZnO and BNC_{ZnO}

The antimicrobial activity of the developed BNC_{ZnO} films was assessed by the disc diffusion method and viable cell counting. All tests were made in duplicate and performed twice for validation. For the disc diffusion assay, cocktails of *Salmonella* and of *Campylobacter* strains were prepared prior to testing. *Salmonella* cocktail comprised a blend of *Salmonella enterica* serovar Typhimurium SLM27C, *S. enterica* serovar Infantis M2016, *S. enterica* serovar Senftenberg 775 W, *S. enterica* serovar Enteritidis 545047 and *S.* Enteritidis 517536. *Campylobacter* spp. cocktail comprised a blend of *Campylobacter jejuni* DSM 4688, *C. jejuni* DFVF 1099, *C. jejuni* NCTC 11168, *C. jejuni* CJ305, *C. jejuni* C9, *C. jejuni* C21A, *Campylobacter coli* DSM4689, C. *coli* C3. Stock cultures of the strains were stored at − 80 °C. For inocula preparation, *Campylobacter* strains were streaked onto cefoperazone deoxycholate agar (MCCD agar; SARSTEDT) at 42 °C, in microaerophilic conditions (5.6% CO_2), for 48 h. *Salmonella* strains were streaked on Tryptic soy Agar (TSA, Biokar Diagnostics, Alonne, France) and incubated at 37 °C for 24 h. Subsequently, colonies were harvested with a sterile loop to prepare a cell suspension for each strain in 1/4 strength Ringer solution (Biokar Diagnostics) adjusting cell density to ca. 10^9 CFU mL^{-1}, using a 0.5 MacFarland scale as standard. Bacterial cocktails were prepared by mixing equal volumes of each strain from the same species.

2.4.1. Agar diffusion method

The bacteria selected for antimicrobial tests by the agar diffusion method are presented in Table 2. Except for *Campylobacter*, all the other species were grown on Müeller-Hinton Agar (MHA) (Biokar Diagnostics) at 30 °C for 24 h. *Campylobacter* strains were grown on MCCD agar at 42 °C, in microaerophilic conditions (5.6% CO_2) for 48 h. Isolated colonies were suspended in Ringer's solution to obtain a turbidity equivalent to 0.5 McFarland scale (10^9 CFU mL^{-1}). Afterwards, sterile swabs were immersed in each inoculum suspension and spread on previously prepared MHA plates. Then, 20 μL of the ZnO suspensions (from 10 to 100 mg mL^{-1}, as described in 2.2.) were directly inoculated on the contaminated plates. After 24 h of incubation at 30 °C, inhibition zones were measured using Interscience software (Interscience, Cantal, France).

Antimicrobial activity (target isolates in Table 5) was also evaluated for neat BNC and BNC_{ZnO} discs with increasing ZnO concentration (obtained using 0.01, 0.03 or 0.05 M of $Zn(CH_3COO)_2$). The discs were placed on top of the MHA contaminated plates and inhibition zones were measured using Interscience software after 24 h of incubation at 30 °C. Filter paper discs were used as negative control. The incubation of bacteria (in contact with ZnO suspensions and BNC_{ZnO} films) was carried out in an oven, with low light exposure (known to interfere with the ZnO mechanism of action against bacteria).

2.4.2. Viable cell count assay

Isolated colonies of *E. coli* ATCC 25922 and *L. monocytogenes* 2542 grown on TSA for 24 h at 30 °C were suspended in Ringer's solution to obtain an optical density (OD_{600}) of 1.0, equivalent to 10^9 CFU mL^{-1}. Then, 10 μL (10^9 CFU.mL^{-1}) of each suspension was inoculated onto BNC, BNC_{ZnO} and ZnO and incubated for 15 min (also referred as 0 h′), 4 h and 24 h at 4 °C (stored in a cold room with low light exposure;) and 22 °C (in an oven with low light exposure). After each incubation period, the contaminated discs were immersed into Ringer's solution (9 mL), vortexed for 1 min and decimal dilutions were made to 10^{-5}. Further automated dilutions and plating on TSA were performed using the automatic spiral method (easySpiral-Pro, Interscience). Viable cells were counted after 24 h of incubation at 30 °C. The loss of cell viability was determined as follows:

Table 1
Microwave digestion program.

Stage	1	2	3	4	5
T (°C)	130	170	200	100	100
Pressure (bar)	20	20	20	20	20
Time (min)	5	10	15	2	2
Ramp (min)	5	5	1	5	1
Power (watt)	30	40	50	30	20

Table 2
Bacterial strains and respective source used in the agar diffusion antimicrobial tests.

Microorganisms	Species	Sources
Gram-negative	*Escherichia coli* ATCC 25922	ATCC
	Escherichia coli DSM423 (K12)	DSMZ
	Escherichia coli DSM1576	
	Yersinia enterocolitica NCTC10406	NCTC
	Salmonella Infantis M2016	ESB culture collection
	Salmonella Typhimurium	
	Salmonella Enteritidis	
	Salmonella Typhimurium SLM27C	
	Salmonella Senftenberg 775 W	
	Salmonella Enteritidis 545047	
	Salmonella Enteritidis 517536	
	Campylobacter coli DSM4689	DSMZ
	Campylobacter jejuni DSM4688	
	Campylobacter jejuni DFVF 1099	DFVF
	Campylobacter jejuni NCTC 11168	NCTC
	Campylobacter jejuni CJ305	ESB culture collection
	Campylobacter jejuni C9	
	Campylobacter jejuni C21A	
	Campylobacter coli C3	
	Acinetobacter calcoaceticus S	
	Acinetobacter calcoaceticus R	
	Acinetobacter baumannii R	
	Acinetobacter baumannii 260	
Gram-positive	*Staphylococcus aureus* 18MRSA	ESB culture collection
	Staphylococcus aureus 6538	
	Bacillus cereus	
	Listeria monocytogenes 2542	
	Listeria monocytogenes FSL J1–117	
	Listeria monocytogenes FSL J031	
	Listeria monocytogenes FSL R2499	
	Enterococus faecium DSMZ 13590	DSMZ
	Enterococus flavescens DSMZ 7370	
	Enterococus faecalis ATCC 29212	ATCC

$$R(t) = \log\left(\frac{N}{N0}\right) \quad (1)$$

where R(t) stands to reduction over time; N corresponds to bacterial population (CFU mL^{-1}) after exposure for a certain time and N0 corresponds the initial bacterial population, before exposure (CFU mL^{-1}).

2.5. Statistical analysis

Statistical analysis was supported with Prism version 9.4.1 (GraphPad Software, La Jolla California USA), using one-way (and two-way) ANOVA and Tuckey's post-hoc analysis for pairwise comparison of more than two means. Mean differences were considered statistically non-significant (ns) when p-value was higher than 0.05 (95% of interval of confidence). The default statistical confidence level was considered to be 95% ($p < 0.05$) in all tests.

3. Results and discussion

3.1. BNC_{ZnO} film characterization

BNC_{ZnO} films were characterized concerning the ZnO particle size, polydispersity (PdI) and concentration. It is well known that antimicrobial efficiency of ZnO highly depends on particle size and dosage used (Sirelkhatim et al., 2015). By lowering the particle size of ZnO, specific surface area increases, which improves the activity against bacteria. On the other hand, the amount of ZnO incorporated in the matrix is also important since the minimum effective concentration should be determined for each bacterium.

As can be seen in Table 3, the method used for ZnO production provided a good range of ZnO particle sizes (between 160 and 230 nm) with a low polydispersity index (0.072–0.266) and provided homogeneous ZnO suspensions. The $Zn(CH_3COO)_2$ concentration influenced the ZnO particle size obtained, a maximum (230 nm) being observed for the intermediate concentration used (Table 3). Concerning polydispersity, all ZnO suspensions may be considered homogeneous, with PdI lower than 0.400 (Table 3). The increase of zinc acetate concentration during *in situ* ZnO production, led to higher Zn concentration on BNC, from 11.05% m_{Zn}/m_{BNCZnO} (BNC_{ZnO} 0.01 M) up to 27.10% m_{Zn}/m_{BNCZnO} (BNC_{ZnO} 0.05 M), under the conditions tested (Table 3). The ability to control the amount of ZnO incorporated in the BNC is of high importance since a minimum amount needs to be ensured, irrespective of the average particle size achieved.

SEM-EDS observations were carried out to analyse the morphology and distribution of ZnO within the BNC fibre network. These are shown in Fig. 1. ZnO particles were found attached to BNC fibres and well distributed.

From SEM images, the ZnO particle size was also determined, and compared with that obtained from DLS measurements presented in Table 3. The ZnO particle sizes obtained through SEM are moderately higher than those obtained by DLS for ZnO suspensions (Table 3; Fig. 1). According to SEM observations, the increase in $Zn(CH_3COO)_2$ concentration (0.01–0.05 M) led to an increase in ZnO particle size, from an average of 218 nm up to 380 nm. The most frequent ZnO particle size also increased with the Zn concentration used.

Despite the undesirable effect of increasing $Zn(CH_3COO)_2$ concentration in particle size and polydispersity, higher amount of ZnO incorporated may be important to achieve effective antimicrobial activity. The impact of ZnO concentration (and consequently of particle size) on the antimicrobial activity of BNC_{ZnO} against different bacteria was then studied.

Table 3
Properties of ZnO particles on BNCZnO films.

ZnO suspensions			BNC_{ZnO}
$Zn(CH_3COO)_2$	ZnO size (nm)	ZnO PdI	Zn (% m_{Zn}/m_{BNCZnO})
0.01 M*	158 ± 2	0.266 ± 0.017	11.05 ± 2.87
0.03 M	244 ± 9	0.072 ± 0.008	21.12 ± 2.23
0.05 M	219 ± 8	0.104 ± 0.037	27.10 ± 2.49

*ZnO suspension used for antimicrobial testing

3.2. Antimicrobial activity in the agar diffusion assay

Different concentrations of ZnO NPs (from 10 to 100 mg L^{-1}) were tested against Gram-negative (*S.* Enteritidis*, E. coli, Y. enterocolitica, A. baumannii* and *C. coli*) and Gram-positive (*Staph. aureus, B. cereus, L. monocytogenes, En. faecalis*) bacteria, through agar diffusion assay. Results of the tests performed using ZnO suspensions are presented in Table 4.

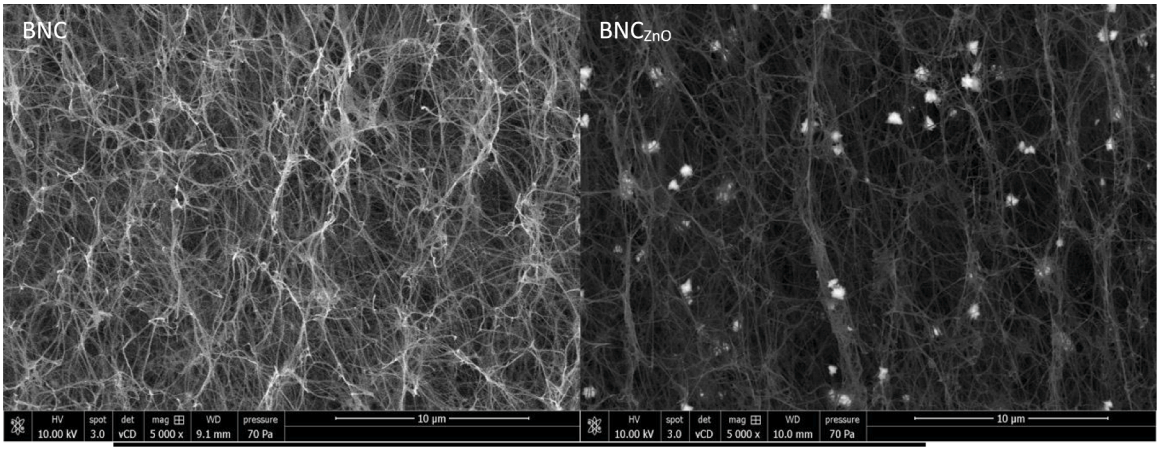
Overall, ZnO was effective against Gram-negative bacteria, with *S.* Enteritidis, *E. coli*, and *Y. enterocolitica* showing similar behaviour ($p > 0.05$) when in contact with ZnO (Table 4). The increase in ZnO concentration led to higher inhibition of all sensitive bacteria (Table 4; $p < 0.01$).

The Gram-positive bacteria *L. monocytogenes* and *En. faecalis* revealed to be resistant to ZnO at all the concentrations tested. *Staph. aureus* and *B. cereus* (both also Gram-positive) proved to be sensitive to ZnO at concentrations of 50 and 10 mg L^{-1}, respectively, or higher (Table 4). The Gram-negative bacteria *E. coli* and *Y. enterocolitica* were sensitive to a minimum ZnO concentration of 20 mg L^{-1}, *Salmonella* to 30 mg mL^{-1} and *Campylobacter* spp. to 10 mg mL^{-1} (Table 4).

ZnO was more effective against *C. coli*, when compared to the other tested bacteria (Table 4; $p < 0.01$). Only 10 mg mL^{-1} of ZnO was needed to obtain inhibition and the increase in ZnO concentration led to larger inhibition halos (up to 25 mm; see both Table 4 and Fig. 2). Concerning *B. cereus*, larger inhibition halos were obtained (higher than 20 mm); however, the halo formed corresponds to reduced bacterial growth and not total elimination of growth (Table 4). The same assay was carried out with BNC_{ZnO} discs, for the bacteria shown to be sensitive to ZnO (Table 4).

Only the BNC_{ZnO} discs with higher concentrations of ZnO (BNC_{ZnO} 0.03 M: 21% m_{Zn}/m_{BNCZnO} Zn and BNC_{ZnO} 0.05 M: 27% m_{Zn}/m_{BNCZnO}) were effective against all the tested bacteria (Table 4). The BNC_{ZnO} discs with the lower load of ZnO (BNC_{ZnO} 0.01 M: 11% of Zn), were only effective against *C. coli* and *B. cereus.* Concerning the dosage effect, higher concentrations (from 21% to 27% m_{Zn}/m_{BNCZnO}) led to significantly higher inhibitions of only *C. coli*. and *B. cereus* (Table 4; $p < 0.01$). Despite the larger ZnO particle size in the more loaded samples (as observed in Fig. 1), the ZnO dosage proved more important in achieving higher antimicrobial efficiency. Yet, the overall efficiency is lower when ZnO is encapsulated on BNC, as compared to the suspensions, as a higher minimum dosage was needed to ensure antimicrobial activity (Table 4). The diminished antimicrobial efficiency upon encapsulation may be explained by the higher particle sizes obtained on the BNC_{ZnO} (217 – 380 nm versus 160 nm for "free" ZnO). Also, the fact that ZnO particles were trapped in the BNC fibre matrix, may delay its antimicrobial effect, potentially promoting a prolonged effect over time.

In order to confirm the antimicrobial activity of ZnO (both encapsulated and in suspension) against each specie, additional strains were tested. Qualitative results are provided in Table 5. The effects were graded according to the diameters of the inhibition halos. Concerning *E. coli*, no differences were observed between strains, all showing similar inhibition halos. *Salmonella* Infantis and *S.* Typhimurium were slightly more sensitive than *S.* Enteritidis*,* being the minimum ZnO concentration 20 mg mL^{-1}. For the cocktail of *Salmonella* serovars, ZnO suspensions were only effective at a concentration of 30 mg mL^{-1}. Still, superior inhibition zones were observed at these levels of ZnO, as well as on the BNC_{ZnO} 0.05 M. For *Acinetobacter* species, only *A. calcoaceticus* (both sensitive and resistant to antibiotics) was inhibited when in contact with ZnO, although BNC_{ZnO} 0.05 M was only effective against the sensitive *A. calcoaceticus*. All *L. monocytogenes* and *Enterococcus* spp.



Particle size interval	BNC_{ZnO} 0.01M	BNC_{ZnO} 0.03M	BNC_{ZnO} 0.05M
<50 nm	1.3 %	nd	nd
50-150 nm	56.0 %	17.1 %	11.2 %
150-300 nm	38.7 %	47.5 %	50.0 %
300-450 nm	4.0 %	24.1 %	23.5 %
> 450 nm	nd	11.3 %	15.3 %
Average size (nm)	218 ± 85[a]	358 ± 146[a]	380 ± 175[a]

Fig. 1. SEM observations of BNC and BNC_{ZnO} and ZnO particle size distribution in BNC_{ZnO}; for the same row, the different superscript letters indicate statistically significant differences ($p < 0.05$).

strains revealed to be resistant to ZnO NPs. Interesting results were obtained with *Campylobacter* isolates, for which greater inhibitions were observed when in contact with ZnO NPs and BNC_{ZnO} than for other bacteria. ZnO nanoparticles have been shown to be quite effective against *Campylobacter* spp. in several studies (Duffy et al., 2018; Hakeem et al., 2020; Windiasti et al., 2019; Zhong et al., 2020). *Campylobacter* spp. are the most common cause of bacterial diarrhoea in developed countries (campylobacteriosis); 70–90% of raw chicken meat (across Europe and North America) was found to be contaminated with *Campylobacter* (Hakeem et al., 2020). Hence, there is extra interest in mitigating the presence of these bacteria in poultry, for example by using pads comprising active agents. The results obtained suggest that BNC_{ZnO} films may be used as active pads, to inhibit *Campylobacter* spp. in raw chicken meat. Another study was already conducted with the goal of assessing the antimicrobial activity on chicken skin, where BNC_{ZnO} was very effective against *Campylobacter*, indicating BNC_{ZnO} potential for active packaging applications in meat-based products (Silva et al., 2023).

Compared to previously published data, the current results are consistent when ZnO NPs were tested against *E. coli*, *Salmonella*, *Staph. aureus*, *Y. enterocolitica* and *Campylobacter* (Bacchu et al., 2021; Duffy et al., 2018; Hakeem et al., 2020; Jiang et al., 2016; Jin et al., 2009; Li et al., 2021; Windiasti et al., 2019; Xie et al., 2011). Despite of the good antimicrobial activity demonstrated by BNC_{ZnO}, some discrepancies were found in relation to previous studies reporting antimicrobial activity of ZnO NPs against *L. monocytogenes* (Abdollahzadeh et al., 2017; Olaimat et al., 2022), *Enterococcus* spp. (Leung et al., 2012; Narayanan et al., 2012) and *Acinetobacter* spp. (Ghasemi & Jalal, 2016; Shokrollahi et al., 2021; Yang et al., 2018). However, different ZnO NPs sources were used, either commercial (Abdollahzadeh et al., 2017; Leung et al., 2012; Olaimat et al., 2022; Yang et al., 2018) or synthesized using precipitation (Narayanan et al., 2012) and solvothermal (Ghasemi & Jalal, 2016; Shokrollahi et al., 2021) methods. Different ZnO production methods may lead to different characteristics such as particle size and morphology. Most of these studies yielded smaller ZnO particle sizes, in the range of 30–100 nm, which may enhance the antibacterial activity of ZnO (Babayevska et al., 2022; Stanković et al., 2013; Yamamoto, 2001), while in present work sizes of 160–220 nm were obtained (Table 3). Additionally, comparison between different studies should be careful and consider all relevant factors. For instance, light conditions during testing are another variable that affects the antibacterial activity of ZnO, since the presence of UV light triggers the synthesis of reactive oxygen species (ROS) (Adams et al., 2006; Sirelkhatim et al., 2015; Yang et al., 2018).

As reviewed by Kumar et al. (Kumar et al., 2017), ZnO affects Gram-positive and Gram-negative bacteria by different mechanisms. The generation of ROS (e.g., $O_2^{\bullet-}$, $HO\bullet$, H_2O_2) is responsible for damage to cells and cellular components through oxidative stress (Sirelkhatim et al., 2015), which affects more Gram-positive than Gram-negative bacteria. The latter have an extra outer plasma membrane with a thick lipopolysaccharide, thicker than the peptidoglycan layer of Gram-positive bacteria. These structural differences allow Gram-negative bacteria to resist lipid peroxidation in the presence of ROS (Kumar et al., 2017). Also, thinner peptidoglycan layers are less negatively charged, therefore less susceptible when in presence of positively charged ZnO NPs. However, according to the results obtained in this study, Gram-negative bacteria were more susceptible to ZnO NPs than Gram-positive bacteria. This difference may be explained by the fact that Gram-negative bacteria are more susceptible to releasing Zn^{2+} from ZnO (another main mechanism of action from ZnO) (Kumar et al., 2017). The thicker peptidoglycan layer of Gram-negative cells is more capable of trapping Zn^{2+} from ZnO NPs. However, the ability of a bacteria to resist ZnO NPs, should not rely only on the cell wall structure (Gram-positive vs Gram-negative) but rather on its specific composition. As stated above, Gram-negative bacteria appeared to be more susceptible to BNC_{ZnO}, suggesting that the mechanism of action seems to rely

Table 4
Antimicrobial activity of ZnO suspensions and BNC_{ZnO} discs determined by the agar diffusion method. Results expressed as inhibition halos (mm).

Bacteria strains		ZnO concentration, mg mL^{-1}					ZnO concentration in BNC			
		10	20	30	50	75	100	BNC (0.0 mg mL^{-1})	BNC_{ZnO} 0.01 M (27.5 mg mL^{-1})	BNC_{ZnO} 0.03 M (52.5 mg mL^{-1})	BNC_{ZnO} 0.05 M (67.5 mg mL^{-1})
Gram-negative	***S. Enteritidis*[1]**	0^{a}	0^{a}	**10.3 ± 0.8^{b}**	10.7 ± 0.4^{b}	12.1 ± 0.9^{bc}	13.7 ± 0.9^{c}	0^{a}	0^{a}	***13.2 ± 1.5*^{b}**	13.3 ± 0.6^{b}
	***E. coli*[1]ATCC 25922**	0^{a}	**9.8 ± 0.3^{b}**	10.2 ± 0.1^{b}	10.8 ± 0.3^{b}	11.6 ± 0.1^{bc}	13.9 ± 0.7^{c}	0^{a}	0^{a}	***13.4 ± 3.3*^{b}**	13.4 ± 0.1^{b}
	***Y. enterocolitica*[1] NCTC10406**	0^{a}	**9.8 ± 0.7^{b}**	10.7 ± 0.1^{bc}	11.4 ± 0.5^{bc}	12.4 ± 1.4^{c}	14.5 ± 1.0^{c}	0^{a}	0^{a}	***12.4 ± 0.6*^{b}**	14.8 ± 1.8^{b}
	***A. baumannii* 260 *I*[4]**	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	-	-	-	-
	***C. coli*[2] DSM4689**	**13.4 ± 1.9^{a}**	*16.1 ± 1.1^{b}*	19.1 ± 1.5^{c}	20.0 ± 1.8^{c}	22.4 ± 2.2^{d}	24.6 ± 2.1^{d}	0^{a}	***13.6 ± 0.8^{b}***	20.9 ± 4.0^{c}	27.3 ± 0.9^{d}
Gram-positive	***Staph. aureus*[1] 6538**	0^{a}	0^{a}	0^{a}	**10.8 ± 0.5^{b}**	12.6 ± 0.5^{bc}	14.1 ± 1.0^{c}	0^{a}	0^{a}	***11.5 ± 0.6*^{b}**	14.5 ± 1.3^{b}
	***B. cereus*[*3]**	**21.5 ± 0.5^{a}**	22.6 ± 0.5^{a}	23.2 ± 0.9^{ab}	25.0 ± 1.5^{b}	-	27.8 ± 0.5^{c}	0^{a}	***21.8 ± 1.3^{b}***	25.9 ± 0.8^{c}	28.3 ± 0.3^{d}
	L. monocytogenes[4] 2542	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	-	-	-	-
	En. faecalis[4] ATCC 29212	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	-	-	-	-

Letters in the same row with different superscripts are significantly different ($p < 0.05$)
Numbers in the same column with different superscripts are significantly different ($p < 0.05$)
*Inhibition halos with reduced bacteria growth (not total absence)
"-" not measured

more on the Zn^{2+} release rather than ROS generation. This is only increased by exposure to visible and UV light (Sirelkhatim et al., 2015). Our assays were performed under low light exposure; thus, ROS generation should not occur significantly (Figs. 2 and 3). Nonetheless, either Gram-positive or Gram-negative bacteria were sensitive/resistant to the developed ZnO NPs (Table 4), meaning that other mechanisms should be taken into account. Furthermore, resistance/susceptibility appears to be species and strain dependent (Table 5).

3.3. Antimicrobial activity - viable cell count assay

In order to study the bacterial inhibition under conditions mimetizing the direct contact of a food pathogen with a packaging material, e.g. the BNC_{ZnO} film, the activity against *E. coli* ATCC 25922 and *L. monocytogenes* 2542 was further tested over time (0, 4 and 24 h), at 4 °C and 22 °C by the viable cell count method. The log(N/N0) was used to express the reduction in cell viability (Eq. 1).

BNC_{ZnO} and ZnO suspensions showed antimicrobial activity against *E. coli* after 4 h of contact when stored at 22 °C ($p < 0.05$). After 24 h, ZnO was slightly more effective than BNC_{ZnO} (3 log reduction for ZnO and 2 log reduction for BNC_{ZnO}) ($p < 0.05$). At 4 °C, the ZnO antimicrobial activity from BNC_{ZnO} and suspension was significantly reduced ($p < 0.05$), with only a 2 log reduction achieved with BNC_{ZnO} and 1 log reduction with ZnO. However, at the lowest temperature, BNC_{ZnO} provided higher antimicrobial activity than the ZnO suspension ($p < 0.05$).

The cell counts of *L. monocytogenes* were not affected by the ZnO suspension at both temperatures tested ($p > 0.05$). However, cell viability decreased after 24 h in contact with BNC_{ZnO} (3 log reduction at 22 °C and 1 log reduction at 4 °C) ($p < 0.05$). The higher antimicrobial activity of BNC_{ZnO} (than ZnO itself) may be explained by the fact that the embedded ZnO (on BNC) remains intact, with no signs of aggregation/agglomeration, thus promoting a higher contact area of ZnO with the pathogen.

Overall, the results for *E. coli* are consistent with those obtained using the agar diffusion assay (Fig. 2 and Tables 4–5). Furthermore, BNC_{ZnO} had a bactericidal effect on *E. coli*, as the number of cells decreased over time. On the other hand, the viable cell count method demonstrated that BNC_{ZnO} inhibited *L. monocytogenes* (not observed in the agar diffusion tests), also with a bactericidal effect. The latter results (with BNC_{ZnO}) are consistent with those reporting inhibition of *L. monocytogenes* using ZnO NPs (Abdollahzadeh et al., 2017; Olaimat et al., 2022).

From a food packaging perspective, the results indicate that BNC_{ZnO} may prevent microbial growth under temperature abuse conditions, due to equipment failures and temperature fluctuations along the food chain, from production to consumer stages. Results show that the antimicrobial activity is higher at higher temperatures. ZnO NPs form a strong chemical bond with the hydroxyl groups of cellulose via hydrogen bonding, which hinders ZnO NPs migration into food (Azizan et al., 2023; Onyszko et al., 2022). Even if ZnO NPs migration occurs, its rate would be relatively low, as ZnO NPs are trapped (due to the high particle size obtained) in the fibre network of BNC (Table 3) (Kim et al., 2022). Hence, the main mechanism of action of BNC_{ZnO} material would be through Zn^{2+} migration. Zn^{2+} migration has a significant effect on the inhibition of active transport as well as in the amino acid metabolism and enzyme system disruption (Sirelkhatim et al., 2015). The effect of temperature on Zn transfer from materials to foods and simulants is well known. Higher levels of Zn migration previously observed at higher temperatures (Poças & Franz, 2018; Silva et al., 2023) were also demonstrated in the present study (Fig. 3) in parallel with higher antimicrobial activity.

The developed material exhibited high antimicrobial activity against *Campylobacter* species and therefore can be targeted towards meat and poultry-based foods since this bacterium is commonly found in such types of food (applied as pads). Additionally, as mentioned earlier, BNC_{ZnO} showed increased antimicrobial activity at higher temperatures,

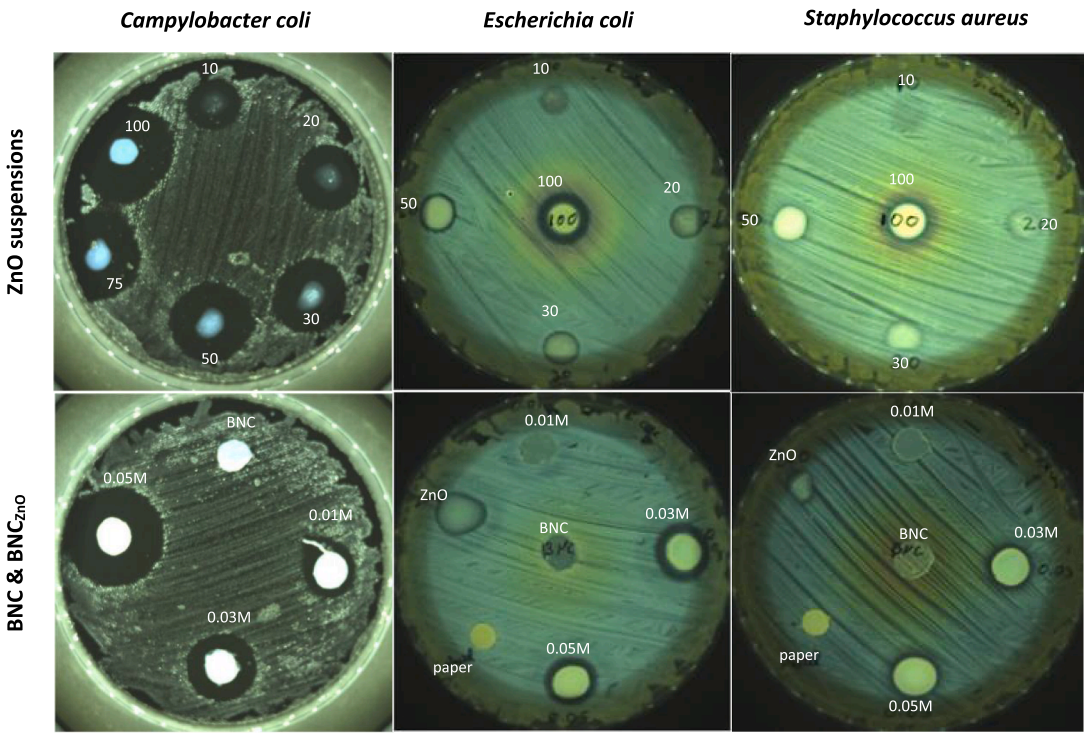


Fig. 2. Agar diffusion assays with ZnO suspensions (10–100 mg mL^{-1}), neat BNC and BNC_{ZnO} (0.01–0.05 M).

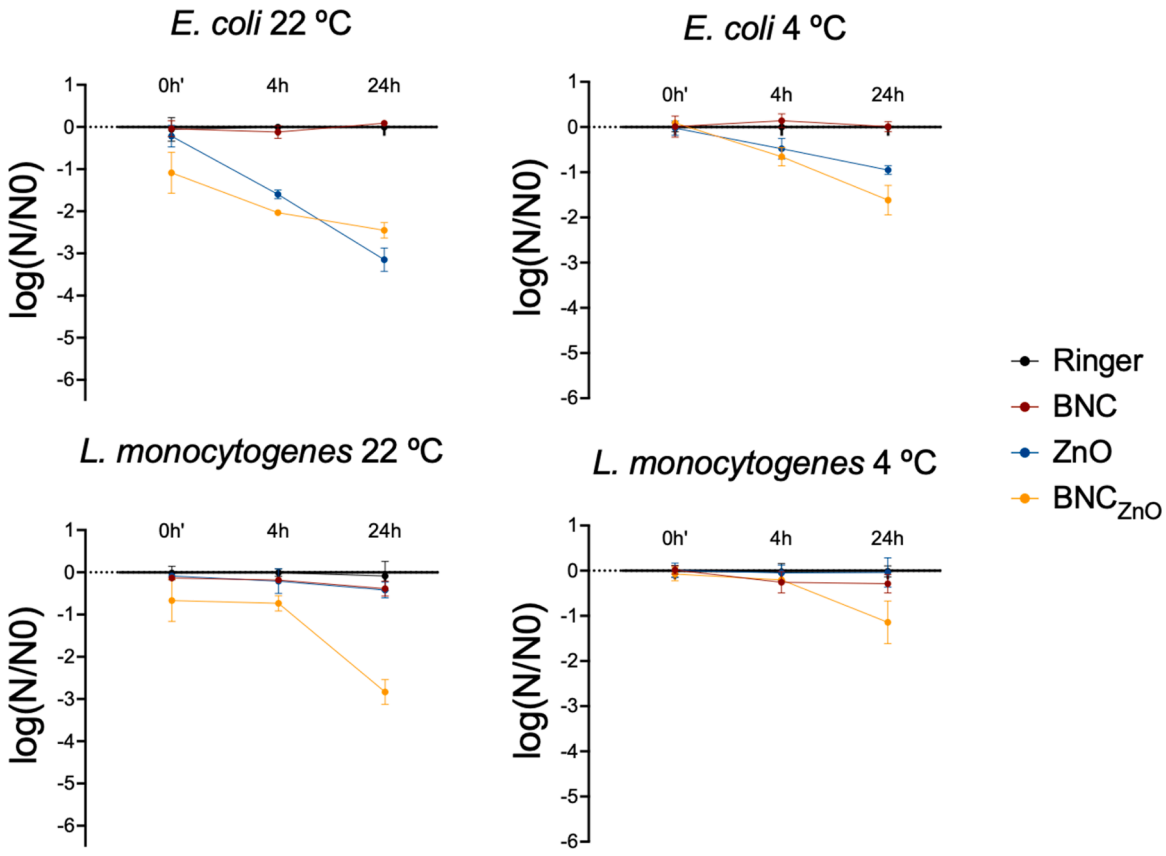


Fig. 3. Viable cell count assay for *E. coli* and *L. monocytogenes*; see text for statistical differences ($p < 0.05$).

enabling its application in foods subject to significant temperature fluctuations, preventing bacterial growth. However, to explore this application, Zn migration must be monitored, as it is important to prevent excessive migration and mitigate potential risks to consumers. In another study carried out by our research team (Silva et al., 2023), Zn^{2+} migration was assessed using BNC_{ZnO}. Migration onto food

Table 5
Screening of the antimicrobial activity of ZnO against different bacterial species and strains.

Bacterial strains*		ZnO (mg mL^{-1})					BNC films	
		0	10	20	30	50	BNC	$BNC_{ZnO\ 0.05\ M}$
Gram (-)	***E. coli* ATCC 25922**	-	-	+	+	+	-	+
	***E. coli* DSM423(K12)**	-	-	+	+	+	-	+
	***E. coli* DSM1576**	-	-	+	+	+	-	+
	***S.* Infantis M2016**	-	-	+	+	+	-	+
	***S.* Typhimurium**	-	-	+	+	+	-	+
	***S.* Enteritidis**	-	-	-	-	+	-	+
	***Salmonella* (cocktail)**	-	-	-	+ +	+ +	-	+ +
	***C. coli* DSM4689**	-	+ ++	+ ++	+ ++	+ ++	-	+ ++
	***C. jejuni* DSM4688**	-	+ ++	+ ++	+ ++	+ ++	-	+ ++
	***Campylobacter* (cocktail)**	-	+ ++	+ ++	+ ++	+ ++	-	+ ++
	***A. calcoaceticus* S**	-	+	+	+	+	-	+
	***A. calcoaceticus* R**	-	+	+	+	+	-	-
	***A. baumannii* R**	-	-	-	-	-	-	-
	***A. baumannii* 260**	-	-	-	-	-	-	-
Gram (+)	***Staph. aureus* 18MRSA**	-	-	+ +	+ +	+ +	-	+ +
	***Staph. aureus* 6538**	-	-	-	-	-	-	+
	***L. monocytogenes* 2542**	-	-	-	-	-	-	-
	***L. monocytogenes* J117**	-	-	-	-	-	-	-
	***L. monocytogenes* J031**	-	-	-	-	-	-	-
	***L. monocytogenes* R2499**	-	-	-	-	-	-	-
	***En. faecium* DSMZ 13590**	-	-	-	-	-	-	-
	***En. faecalis* ATCC 29212**	-	-	-	-	-	-	-
	***En. flavescens* DSMZ 7370**	-	-	-	-	-	-	-

*Each strain tested twice (duplicates)

The inhibition of bacteria growth is represented as (-;+;++;+++). The symbol "–" represents no inhibition, "+" refers to inhibition halos up to 12 mm, "++" refers to inhibition halos between 12 and 20 mm and "+++" refers to inhibition halos larger than 20 mm.

simulants was found to be minimal, below the specific migration limit (5.0 mg kg^{-1}). When using chicken skin as a food model, the Zn^{2+} migration was temperature dependent, with migration values surpassing the specific migration limit at temperatures higher than 10 °C (Silva et al., 2023). Therefore, it is crucial to assess the antimicrobial activity, migration levels, and the expected abusive temperatures to determine the suitability of employing BNC_{ZnO} for food packaging.

4. Conclusions

The present study aims to contribute to a better understanding of the antimicrobial activity of ZnO and its mechanism. A cellulosic composite, BNC_{ZnO}, was used to assess the effectiveness of ZnO against various bacterial species and strains. Two methods for antimicrobial activity were tested. Results for ZnO NPs and BNC_{ZnO} by the disc diffusion method revealed high antimicrobial activity against Gram-negative bacteria, with *S.* Enteritidis, *E. coli*, and *Y. enterocolitica* showing similar behaviour when in contact with ZnO. Inhibition increased with increasing ZnO concentration. The method based on the viable cell counts gave results for *E. coli* in agreement with those of the disc diffusion method, whereas for *L. monocytogenes* a reduction was observed only by viable cell counting. The screening of different bacterial species for the antimicrobial activity of ZnO, as well as the findings from the literature, suggest that the efficiency of ZnO depends on the strain/species tested and that the external factors (light exposure, temperature, ZnO particle size and morphology) impact the ZnO mechanism of action (Zn^{2+} release or ROS generation).

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CRediT authorship contribution statement

Francisco A.G. Soares Silva: Investigation, Methodology, Formal analysis, Writing – original draft, Writing – review & editing. **Marta Carvalho:** Investigation, Methodology, Writing – review & editing. **Teresa Bento de Carvalho:** Investigation, Methodology, Writing – review & editing. **Miguel Gama:** Conceptualization, Writing – review & editing. **Fátima Poças:** Supervision, Conceptualization, Writing – review & editing. **Paula Teixeira:** Conceptualization, Supervision, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data will be made available on request.

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