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DESENVOLVIMENTO DE NANOCOMPÓSITOS CONTENDO NISINA COMO EMBALAGENS PARA ALIMENTOS E AVALIAÇÃO DA SUA TOXICIDADE AGUDA

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Tese apresentada ao Programa de Pós-Graduação em Ciência e Tecnologia de Alimentos da Universidade Federal do Rio Grande do Sul como requisito parcial para a obtenção do título de doutora em Ciência e Tecnologia de Alimentos.

Orientador: Prof. Dr. Adriano Brandelli

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“Life is like riding a bicycle. To keep your balance, you must keep moving”

Albert Einstein

RESUMO

Novas estruturas nanométricas vêm sendo desenvolvidas e estudadas para aplicação em várias áreas, inclusive na indústria de alimentos. No presente estudo, foram desenvolvidos lipossomas de fosfatidilcolina purificada (Phospholipon®) e semi-purificada a partir de lecitina de soja, contendo o peptídeo antimicrobiano nisina. Esses lipossomas foram caracterizados através de seu tamanho, polidispersividade, potencial zeta, eficiência de encapsulação e estabilidade. Lipossomas com 1,0 mg/ml de nisina, feitos de Phospholipon® apresentaram maior estabilidade e eficiência de encapsulação, sendo selecionados para incorporação em filmes nanocompósitos contendo 0,5g/l da nanoargila haloisita e biopolímeros (gelatina e caseína). Esses filmes foram caracterizados quanto a propriedades antimicrobianas, estruturais, mecânicas, térmicas e ópticas. Os filmes inibiram as bactérias Gram-positivas *Listeria monocytogenes*, *Clostridium perfringens* e *Bacillus cereus. A* adição de lipossomas e haloisita aumentou a rugosidade dos filmes e diminuiu a temperatura de transição vítrea. A espectroscopia infravermelho evidenciou que não houve interações químicas entre os lipossomas e haloisita com a matriz protéica. Os filmes de caseína se mostraram mais finos e amarelados, mais rígidos e menos elásticos quando comparados aos filmes de gelatina. Para realizar uma avaliação da toxicidade aguda dos componentes nanométricos presentes nos filmes, foi utilizado o nemátodo *Caenorhabditis elegans* como modelo biológico. Foram avaliados os parâmetros dose letal mediana (DL_{50}), desenvolvimento dos vermes, peroxidação lipídica, produção de espécies reativas de oxigênio (ERO) e produção das enzimas catalase e superóxido dismutase. A nisina livre apresentou a menor DL_{50} entre os componentes do presente estudo (0,239 mg/ml) e promoveu a diminuição do tamanho corporal dos vermes nessa concentração. A taxa de sobrevivência em lipossomas controle (contendo apenas tampão fosfato) e lipossomas contendo nisina foi de 94,3 e 73, 6%, respectivamente, sugerindo que os lipossomas podem ser considerados sistemas não-tóxicos para aplicação em alimentos. Foram também testadas as nanorgilas haloisita, bentonita e montmorilonita modificada com octadecilamina, puras e com nisina adsorvida. A haloisita foi a argila menos tóxica, com DL_{50} 8,38 mg/ml, ao passo que a montmorilonita modificada com octadecilamina foi a mais tóxica, com DL_{50} 0,35 mg/ml. A adsorção de nisina à haloisita e bentonita provocou uma grande diminuição na sobrevivência dos vermes, o que não ocorreu com a montmorilonita modificada com octadecilamina. Todas as argilas alteraram em algum grau o desenvolvimento dos vermes, sendo mais pronunciado no tratamento com bentonita. Todos os componentes testados provocaram aumento nos níveis de espécies reativas de oxigênio e alterações nas quantidades de CAT e SOD. A peroxidação lipídica foi detectada apenas em vermes expostos à montmorillonita modificada com octadecilamina, em doses maiores que a DL_{50}. O teste de Spearman demonstrou que a taxa de morte e o nível de ERO estão positivamente correlacionados, sugerindo que o provável mecanismo de toxicidade é o estresse oxidativo.

ABSTRACT

New nanoscale structures have been developed and studied for use in many areas, including food industry. In the present study, were developed liposomes made of purified(Phospholipon®) and semi-purified phosphatidylcholine, containing the antimicrobial peptide nisin. These liposomes were characterized by their size, polydispersity, zeta potential, encapsulation efficiency and stability. Liposomes with 1.0 mg/ml of nisin, made of Phospholipon® showed greater stability and encapsulation efficiency and were selected for incorporation in nanocomposite films containing 0.5 g/ l of halloysite nanoclay and biopolymers (gelatin and casein). These films were characterized for antimicrobial properties, structural, mechanical, thermal and optical. The films inhibited Gram-positive bacteria *Listeria monocytogenes, Clostridium perfringens* and *Bacillus cereus*. The addition of liposomes and halloysite increased roughness of the films and decreased the glass transition temperature. The infrared spectroscopy showed that no chemical interactions occurred between the liposomes and halloysite with the protein matrix. The casein films were thinner, yellowish, more rigid and less elastic compared to gelatin films. To evaluate the acute toxicity of nanometric components present in the films, we used the nematode *Caenorhabditis elegans* as biological model. Were evaluated the parameters median lethal dose (LD_{50}), development of worms, lipid peroxidation, production of reactive oxygen species (ROS) and production of enzymes catalase and superoxide dismutase. The free nisin showed the lowest LD_{50} among the components of this study (0.239 mg/ml) and promoted decrease in body size of the worms at this concentration. The survival rate in control liposomes (containing only phosphate buffer) and liposomes containing nisin was 94.3 and 73 6%, respectively, suggesting that the liposomes may be considered non-toxic for use in food systems. The nanoclays halloysite, bentonite and montmorillonite modified with octadecylamine, pure and with adsorbed nisin were also tested. Halloysite was less toxic nanoclay, with LD_{50} 8.38 mg/ml, whereas the montmorillonite modified with octadecylamine was more toxic with LD_{50} 0.35 mg/ml. The adsorption of nisin to halloysite and bentonite caused a large decrease in the survival of worms, which did not occur with the montmorillonite modified with octadecylamine. All nanoclays altered in some degree the development of the worms, but it was more pronounced in treatment with bentonite. All tested components caused increased levels of reactive oxygen species and changes in amounts of CAT and SOD. Lipid peroxidation was detected only in worms exposed to montmorillonite modified with octadecylamine, at doses higher than the LD_{50}. Spearman's test showed that the death rate and the level of ROS are positively correlated, suggesting that the probable mechanism of toxicity is oxidative stress.

SUMÁRIO

1 INTRODUÇÃO

A exigência dos consumidores por alimentos mais naturais que, no entanto, possuam vida de prateleira estendida sem adição de conservantes químicos vem aumentando, desafiando a indústria e as instituições de pesquisa a desenvolver novas tecnologias de conservação. Para suprir essa necessidade, a bioconservação surge como uma alternativa interessante, na qual uma microbiota protetora ou seus metabólitos antimicrobianos são aplicados no alimento com o objetivo de controlar o desenvolvimento de microrganismos patogênicos e deteriorantes.

O surgimento e a proliferação de bactérias resistentes a antibióticos tem se tornado um grave problema de saúde em todo o mundo, fazendo com que muitas infecções bacterianas não possam ser tratadas com os antimicrobianos convencionais. Aliado a isso, há a crescente escalada da incidência das doenças de origem alimentar provocadas por bactérias. Nesse contexto, a descoberta de novos antimicrobianos e o estudo de seus usos potenciais são extremamente importantes.

Os peptídeos antimicrobianos produzidos por bactérias, chamados de bacteriocinas, representam uma alternativa promissora na bioconservação de alimentos. As bacteriocinas são reconhecidas como substâncias antimicrobianas naturais seguras e podem ser utilizadas como parte da tecnologia de barreiras. A nisina, produzida por *Lactococcus lactis* subsp. l*actis,* foi a primeira bacteriocina aprovada para uso em alimentos. Atualmente é o bioconservante mais utilizado no mundo. No Brasil, seu uso é permitido apenas em produtos lácteos, visando principalmente a inibição da *Listeria monocytogenes.*

Contudo, na aplicação direta de bacteriocinas nos alimentos pode haver um comprometimento da atividade antimicrobiana devido à degradação proteolítica e interações indesejáveis com os demais componentes da matriz alimentícia. Para proteger as bacteriocinas, essas podem ser encapsuladas em lipossomas. Os lipossomas são estruturas coloidais que possuem um núcleo aquoso envolvido por uma bicamada fosfolipídica. Assim, podem ser utilizados tanto para encapsular substâncias hidrofílicas no seu interior aquoso quanto para encapsular substâncias hidrofóbicas em sua membrana lipídica, o que permite uma ampla aplicação em alimentos. A encapsulação pode melhorar a atividade biológica ao liberar o peptídeo gradualmente, possibilitando a inibição de microrganismos indesejáveis ao longo da vida útil do produto.

A conscientização ecológica dos consumidores nos últimos anos afeta também o setor de embalagens alimentícias, impulsionando a procura por materiais biodegradáveis e não-tóxicos, obtidos a partir de recursos renováveis de maneira sustentável. Biopolímeros tradicionais, como o amido e a quitosana, podem, no entanto, apresentar propriedades mecânicas, térmicas e de barreira insatisfatórias. Para melhorar essas características, nanopartículas como as nanoargilas podem ser adicionadas.

Chamamos de nanocompósitos os materiais que são formados por híbridos em que pelo menos um componente possui dimensão nanométrica (escala do bilionésimo de metro). O uso de nanotecnologia para o desenvolvimento de filmes antimicrobianos é particularmente promissor, devido à melhora das propriedades dos materiais, ao mesmo tempo em que protege o alimento e aumenta sua vida útil. Além disso, são considerados ambientalmente favoráveis ao reduzir a necessidade de plásticos para embalagem. Assim, a incorporação de bacteriocinas e nanocompósitos em embalagens ativas para alimentos representa uma potencial inovação tecnológica na área de conservação de alimentos.

Por outro lado, os nanomateriais possuem propriedades químicas e físicas diferentes dos materiais em escala macro, mesmo que possuam composição semelhante ou mesmo idêntica a esses. Assim, a exposição a nanomateriais pode resultar em diferentes respostas biológicas, tornando imprescindível uma avaliação toxicológica para que alimentos contendo essa tecnologia não representem um risco ambiental e para a saúde humana.

Assim, o objetivo do presente trabalho foi desenvolver filmes antimicrobianos para uso em alimentos a partir de biopolímeros com a adição de peptídeos antimicrobianos nanoencapsulados e nanoargila e avaliar a toxicidade desses nanocompósitos no modelo *Caenorhabditis elegans.*

Os objetivos específicos do estudo foram:

- Desenvolver lipossomas contendo o peptídeo antimicrobiano nisina;
- Caracterizar os lipossomas contendo nisina através de seu tamanho, polidispersividade, potencial zeta, eficiência de encapsulação e estabilidade;
- Incorporar os lipossomas contendo nisina em nanocompósitos de polímeros biodegradáveis (caseína e gelatina) e nanoargilas;
- Avaliar as propriedades estruturais, mecânicas, ópticas e térmicas dos filmes;
- Avaliar a atividade antimicrobiana *in vitro* dos nanocompósitos;
- Avaliar a toxicidade dos lipossomas e das nanoargilas após exposição aguda em *Caenorhabditis elegans,* através dos ensaios de sobrevivência e desenvolvimento;

- Avaliar o envolvimento de estresse oxidativo na possível toxicidade de lipossomas e nanoargilas, através dos ensaios de geração de espécies reativas de oxigênio, quantificação das enzimas catalase e superóxido dismutase e peroxidação lipídica.

2 REVISÃO BIBLIOGRÁFICA

2.1 Peptídeos antimicrobianos

Peptídeos antimicrobianos são fragmentos de proteína produzidos em resposta ao ataque microbiano, atuando como uma barreira química primária. São produzidos por seres vivos de todos os Reinos (ESPITIA *et al.*, 2012). Esses compostos podem ter estrutura variável, mas em sua maioria são catiônicos e anfifílicos, contendo menos de 100 resíduos de aminoácidos, em arranjo simples ou linear (HWANG, VOGEL, 1998; OYSTON *et al.,* 2009; DE SIMONE, SOUZA, 2002). São divididos em vários grupos, de acordo com sua massa molecular, estruturas secundárias e terciárias, presença ou ausência de pontes dissulfeto (BRANDELLI, 2012).

Quanto à biossíntese, podem ser produzidos de duas formas: ribossomicamente ou não ribossomicamente. A síntese não ribossomal ocorre por meio de uma enzima ou complexo enzimático e inclui peptídeos amplamente modificados, que podem conter uma grande variedade de substratos, como aminoácidos não protéicos, hidroxiácidos e substâncias policetídicas, especialmente elaboradas para serem incorporadas na estrutura desses peptídeos. Exemplos desse tipo de peptídeo são as gramicidinas, polimixinas, glicolipídeos, peptídeos cíclicos, lipopeptídeos, entre outros (HANCOCK, CHAPPLE, 1999; MOFFITT, NEILAN, 2000). A síntese ribossomal envolve a produção de peptídeos por várias formas de vida, como plantas, insetos, mamíferos, bactérias e até mesmo vírus, fazendo parte do sistema de defesa do hospedeiro (KOLTER, MORENO, 1992; HANCOCK, CHAPPLE, 1999). Dentre esses peptídeos estão as bacteriocinas, que são sintetizadas no ribossomo e liberadas no meio extracelular, apresentando ação bactericida ou bacteriostática sobre outros microrganismos (COTTER, HILLS, ROSS, 2005).

Para fins comerciais, a obtenção de peptídeos antimicrobianos produzidos por bactérias é a alternativa mais viável (BRANDELLI, 2012). As bactérias produzem esses peptídeos como metabólitos secundários, durante sua fase estacionária ou no final da fase de crescimento (DEMAIN, 1998). Na indústria de alimentos, as bacteriocinas têm sido amplamente investigadas devido ao potencial uso como conservantes naturais (MOTTA, BRANDELLI, 2002; PAPAGIANNI, 2003; ESPITIA *et al*., 2012; MALHEIROS *et al.* 2012).

2.2 Bacteriocinas

Bacteriocinas são geralmente definidas como peptídeos antimicrobianos de natureza protéica, sintetizados ribossomicamente, que inibem ou matam outros microrganismos, relacionados ou não à cepa produtora (BALCIUNAS *et al.,* 2013). A bactéria produtora não é afetada pela ação da bacteriocina por possuir um mecanismo de imunidade específica (COTTER, HILLS, ROSS, 2005).

Em 1925, André Gratia publicou um dos primeiros estudos sobre bacteriocinas, onde observou o antagonismo de uma linhagem de *Escherichia coli* sobre outras linhagens da mesma espécie. As substâncias responsáveis por esse efeito foram chamadas de "colicinas". Em 1953, Jacob e colaboradores descobriram que a produção desses compostos não se limitava ao grupo dos coliformes e propuseram o termo "bacteriocina" para as proteínas antimicrobianas produzidas por bactérias Gram-positivas e Gram-negativas.

As bacteriocinas são encontradas em vários grupos de bactérias, e estas diferem no modo de ação, espectro de atividade, peso molecular, propriedades bioquímicas e origem genética (DIEP, NES, 2002). A maioria das bacteriocinas descritas até o momento apresenta baixa massa molecular, variando de 30 a 60 aminoácidos e são estáveis ao calor.

A sensibilidade dos microrganismos às bacteriocinas baseia-se na composição química da parede celular, sendo portanto diferente para as bactérias Gram-positivas e Gram-negativas. Adicionalmente, as bactérias Gram-negativas geralmente precisam ser desestabilizadas por estresse físico e/ou químico químico para tornarem-se suscetíveis às bacteriocinas (COTTER, HILLS, ROSS, 2005; NES, DIEP, HOLO, 2007). O modo de ação desses peptídeos é variável devido à diversidade de estruturas químicas possíveis, mas a maioria atua através da formação de canais ou poros na membrana, levando a destruição do potencial energético das células sensíveis (OSCÁRIS, PISABARRO, 2001). As bacteriocinas precisam interagir com a membrana citoplasmática da célula alvo para que consiga formar os poros. Duas fases estão envolvidas nesta formação de poros. A primeira envolve as interações eletrostáticas entre a carga positiva e os resíduos polares da bacteriocina com os fosfolipídios aniônicos presentes na bicamada lipídica da membrana alvo (ABEE, KROCKEL, HILL, 1995). A segunda fase envolve mudanças letais em cepas sensíveis à bacteriocina, sendo por isso irreversível (DESMAZEAUD, 1997).

A classificação das bacteriocinas ainda não está bem estabelecida e ainda é objeto de debate. As classificações vêm sendo modificadas com a descoberta de novas bacteriocinas, devendo ser necessário algum tempo até que um sistema de classificação definitivo seja obtido (DE MARTINIS, ALVES, FRANCO, 2002). Geralmente são utilizados vários critérios, como a família bacteriana produtora, massa molecular, sequência de aminoácidos homólogos e/ou organização do grupo de genes.

A classificação mais citada foi proposta por Klaenhammer (1993) e divide as bacteriocinas em quatro classes, conforme a diversidade de estruturas químicas e suas características.

A classe I (lantibióticos) é constituída por peptídeos termoestáveis que sofrem mudanças pós-traducionais e possuem de baixo peso molecular (< 5 kDa), diferenciados dos demais pela presença de aminoácido não usuais como a lentionina e derivados (NAGAO, 2009). De acordo com suas características estruturais, carga e modo de ação, os lantibióticos são subdivididos em dois grupos: A e B. Os lantibióticos tipo A inibem as células sensíveis por despolarização da membrana citoplasmática, consistem de peptídeos catiônicos e hidrofóbicos que formam poros em membranas alvo e tem uma estrutura flexível comparado com o grupo B. A nisina, bacteriocina melhor caracterizada, está incluída nesta classe bem como a subtilina e a epidermina. Os lantibióticos tipo B têm uma estrutura secundária globular, agem através de inibição enzimática e consistem de peptídeos aniônicos ou neutros. Um exemplo é a mersacidina, a qual interfere na biossíntese da parede celular.

A classe II é composta por peptídeos pequenos (< 10 kDa) termoestáveis, que são subdivido em três classes. A subclasse IIa abrange peptídeos com uma seqüência de aminoácidos N-terminal comum (-Tyr-Gly-Asn-Gly-Val-Xaa-Cys) como a pedicina e a enterocina. A subclasse IIb contém as bacteriocinas lactococcinas G, M e lactacina F, as quais requerem dois peptídeos diferentes para a sua atividade. Estes dois peptídeos podem ser tanto individualmente ativos quanto sinergísticos ao agirem juntos (enterocinas L50A e L50B) ou ambos podem ser necessários para a atividade antimicrobiana (lactococinas M e N, plantaricinas EF e JK). A subclasse IIc correspondente a peptídeos ativados por tiol, dependentes de baixas concentrações de cisteína para sua ação, como a lactococcina B. Nesta classe também estão incluídas bacteriocinas sem cisteína, já que esta classe inclui todas as bacteriocinas da classe II que não se enquadram nas sub-classes IIa e IIb, como as cereínas 7 e 8.

A classe III é representada por peptídeos termolábeis de elevada massa molecular (>30 kDa), como helveticinas J e V, produzidas pelos *Lactobacillus helveticus*, lactacinas A e B e enterolisina.

Na classe IV estão os complexos peptídicos contendo carboidrato ou lipídio. A composição e função dessas porções não-protéicas são desconhecidas. Nesta classe encontram-se leuconocina S, lactocina 27 e pediocina SJ-1. No entanto, Cleveland e colaboradores (2001) acreditam que este tipo de bacteriocina é um artefato resultante da purificação parcial que decorre das propriedades catiônicas e hidrofóbicas das bacteriocinas, o que resulta em complexação com outras macromoléculas em extrato bruto.

Uma modificação desta classificação foi proposta por Cotter, Hills e Ross (2005), separando as bacteriocinas produzidas por bactérias ácido láticas em duas categorias: os lantibióticos contendo lantionina (classe I) e os não lantibióticos (classe II). As bacteriocinas da classe III de Klaenhammer (1993) foram reclassificadas como "bacteriolisinas", e a classe IV extinta

Uma nova organização foi proposta por Drider et al. (2006), distribuindo as bacteriocinas de acordo com suas características bioquímicas e genéticas em três classes. A classe I (lantibióticos) é composta pelos peptídeos contendo lantionina ou β-lantionina e foi dividida em duas subcategorias: subcategoria A, onde encontram-se as moléculas lineares, como a nisina e a subtilina, e a subcategoria B, que engloba as moléculas globulares, como a mersacidina e a mutacina. Já a classe II é composta por peptídeos de até 10 kDa heterogêneos e estáveis, enquanto que a classe III é composta por proteínas maiores que 30 kDa termolábeis.

Na classificação sugerida por Nes, Diep e Holo (2007), a classe II foi dividida em quatro subclasses: IIa composta por bacteriocinas com atividade contra *Listeria*, como a pediocina; a IIb corresponde às bacteriocinas duplo-peptídeo; IIc compreendendo as bacteriocinas que não apresentam peptídeo guia; e por último a classe IId composta pelas bacteriocinas circulares, modificadas pós-traducionalmente. Contudo, já foi proposta a separação das bacteriocinas circulares para uma classe própria (MAQUEDA *et al*., 2008).

A classificação mais recente de bacteriocinas produzidas por bactérias Gram-positivas foi proposta por Zouhir e colaboradores (2010). Nessa classificação, as bacteriocinas conhecidas até o momento são divididas em 12 grupos, de acordo com as seqüências de consenso dos aminoácidos que fazem parte de sua composição.

Além do uso como bioconservantes, as bacteriocinas tem sido estudadas nos campos médico e veterinário, contra mastite, cáries, acne e eczema, como contraceptivo, como viricida na prevenção da AIDS e herpes simplex e no combate a certos tipos de tumores de cabeça e pescoço (ARANHA, GUPTA, REDDY, 2008; NAGAO, 2009; JOO *et al.,* 2012; TONG, NI, LING, 2014).

2.3 Nisina

A nisina é um peptídeo antimicrobiano anfifílico e catiônico de 3,5 kDa, composto por 34 aminoácidos, produzido por cepas de *Lactococus lactis* subsp. *lactis*. (BALCIUNAS *et al.,* 2013) (Figura 1). Sua biossíntese ocorre durante a fase exponencial de crescimento, cessando completamente quando as células entram na fase estacionária (PONGTHARANGKUL, DEMIRCI, 2004). Foi descoberta por Rogers em 1928, mas somente em 1947 foi parcialmente purificada e analisada por Mattick & Hirsh (NASCIMENTO, MORENO, KUAYE, 2008). A primeira preparação comercial de nisina (Nisaplin®) foi produzida na Inglaterra em 1953 pela Aplin & Barrett Ltda, com o intuito de prevenir a deterioração causada por clostrídios em queijos processados (THOMAS, CLARKSON, DELVES-BROUGHTON, 2000). Em 1969 foi considerada segura para uso em alimentos pelo Joint FAO/WHO Expert Committee on Food Additives (JECFA), por ser produzida por *L. lactis*, que tem um longo histórico de uso como cultura starter (HAGIWARA *et al.,* 2010). Na Europa, em 1983, foi adicionada à lista de aditivos alimentares como E234 e em 1988, nos EUA, o FDA (Food and Drug Administration) concedeu o status de GRAS – *Generally Regarded as Safe* e autorizou seu uso em queijos processados (COTTER, HILLS, ROSS, 2005). Atualmente é aprovada como conservante de alimentos em mais de 50 países. É utilizada em produtos lácteos, enlatados e carne curada (TONG, NI, LING, 2014). No Brasil (INS 234), é permitida desde 1996 para uso em queijos pasteurizados no limite máximo de 12,5 mg/kg (BRASIL, 1996).

Alguns estudos apontam que a nisina não possui qualquer efeito tóxico crônico ou subcrônico, toxicidade reprodutiva, genotoxicidade ou efeitos carcinogênicos e teratogênicos (ARANHA, GUPTA, REDDY, 2008; HAGIWARA *et al.,* 2010). Seu uso como aditivo alimentar baseia-se ainda na sua alta estabilidade térmica, no fato de ser insípida e inodora e ser hidrolisada por enzimas digestivas, influenciando minimamente a microbiota humana (NAGAO, 2009).

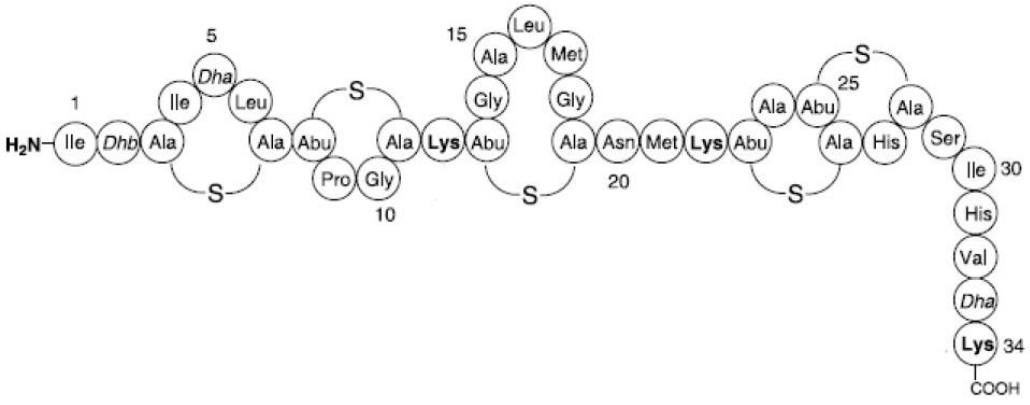

Figura 1. Representação esquemática da nisina. (Fonte: De Vuyst e Vandamme, 1994).

Esta bacteriocina tem recebido grande atenção por causa do seu amplo espectro inibitório contra bactérias Gram-positivas (como *Listeria* e *Staphylococcus*), assim como pela inibição efetiva de esporos das espécies de *Bacillus* e *Clostridium* (ARAUZ *et al*., 2009), sendo amplamente utilizada na indústria de laticínios para prevenir o estufamento tardio em queijos. A ação contra esporos é causada pela ligação aos grupos sulfidril dos resíduos protéicos. Os esporos se tornam mais sensíveis à nisina quanto maior for o dano provocado pelo calor, o que é um importante fator relacionado ao uso da nisina como conservante em alimentos processados termicamente (DELVES-BROUGHTON *et al.*, 1996; MONTVILLE, CHEN, 1998).

A nisina pertence à classe dos peptídeos lantibióticos por conter os aminoácidos raros *meso*-lantionina e 3-metil-lantionina, possivelmente responsáveis por importantes propriedades funcionais, como tolerância a acidez, termoestabilidade a baixo pH e modo de ação bactericida especifico (DE VUYST, VANDAMME, 1994).

A nisina possui duas variantes naturais, de estrutura e espectro de ação semelhantes, diferindo apenas no aminoácido na posição 27 (histidina na nisina A e asparagina na nisina Z). Cada cepa é capaz de produzir apenas uma das variantes (THOMAS, CLARKSON, DELVES-BROUGHTON, 2000). A nisina Z possui melhores propriedades de difusão no ágar quando comparada à nisina A, característica esta importante para aplicação em alimentos (DE VOS *et al*., 1993). Porém, apenas a nisina A é comercializada, estando disponível na forma de pó, o qual não é completamente solúvel. O produto comercial (Nisaplin®) apresenta 2,5% de nisina pura, sal (77,5%) e sólidos do leite (12% de proteínas e 6% de carboidratos). As proteínas do leite se ligam à

nisina e diminuem sua atividade antimicrobiana, quando comparada a uma preparação purificada ou parcialmente purificada (CLEVELAND *et al*., 2002).

A atividade antimicrobiana da nisina em células vegetativas ocorre sobre a membrana plasmática através da formação de poros que destroem a integridade da membrana, alterando o pH de equilíbrio, causando a perda de íons e hidrólise de ATP, levando à morte celular. A natureza flexível e as propriedades anfifílicas da molécula de nisina são importantes para esse modo de ação (ABEE, KROCKEL, HILL, 1995). Outro mecanismo de ação relatado é a capacidade da nisina ligar-se ao lipídeo II, o principal transportador das subunidades de peptideoglicano do citoplasma para a parede celular, impedindo desse modo a síntese da parede celular, ou ainda usando o lipídeo II como molécula âncora para facilitar sua inserção na membrana celular, o que levaria à formação de poros e, consequentemente, à morte da célula (COTTER, HILLS, ROSS, 2005; DEEGAN *et al*., 2006). O envolvimento do lipídeo II na sensibilidade a nisina também sugere um possível mecanismo de aparecimento de resistência contra esse peptídeo antimicrobiano. Adicionalmente, algumas bactérias Gram-positivas demonstraram ser resistentes à nisina devido à habilidade em sintetizar uma enzima, a nisinase, que inativa a bacteriocina (ABBE 1995; LIANG *et al*., 2010; KAUR *et al*., 2011).

2.4 Aplicação de bacteriocinas em alimentos

Na produção de alimentos, é importante que tanto a indústria quanto as pesquisas na área estejam em consonância com a demanda dos consumidores. Atualmente, os consumidores requerem alimentos mais naturais, de alta qualidade nutricional e organoléptica, sem conservantes químicos e com longa vida útil. Para atender essas exigências, estão sendo produzidos alimentos mais frescos e novos produtos estão sendo desenvolvidos, como alimentos com pouco ou nenhum conservante químico, mas que necessitam ser mantidos em baixas temperaturas ou atmosferas modificadas para garantir sua segurança microbiológica (GÁLVEZ *et al.,* 2007). Isso evidencia a necessidade de novas e complementares tecnologias de preservação de alimentos, considerando o risco de contaminação, em paralelo à emergência de patógenos psicrotróficos, como *Listeria monocytogenes*.

Embora as bacteriocinas tenham potencial como bioconservantes efetivos, sua melhor eficiência é obtida através da tecnologia de barreiras (CLEVELAND *et al.,* 2001). A tecnologia de barreiras se refere à manipulação de múltiplos fatores, intrínsecos e/ou extrínsecos, a fim de prevenir e controlar a contaminação bacteriana, uma vez que a combinação de métodos promove maior segurança e qualidade dos alimentos. (DEEGAN *et al.*, 2006). A sinergia com outros compostos ou tratamentos físicos permite a redução da adição de antimicrobianos sintéticos e a aplicação de tratamentos térmicos menos intensos, além de possibilitar o uso de tratamentos não-térmicos. Isso permite a preservação das características sensoriais e nutricionais dos alimentos, podendo ainda minimizar a resistência microbiana (GÁLVEZ *et al*., 2007).

As bacteriocinas podem ser incorporadas nos alimentos através da adição direta de bactérias produtoras de bacteriocinas, pelo uso de ingredientes preparados através da fermentação com bactérias produtoras de bacteriocinas e através do uso de uma preparação purificada ou semi-purificada de bacteriocina como ingrediente (COTTER, HILLS, ROSS, 2005; GÁLVEZ *et al*., 2008). As bacteriocinas produzidas por bactérias ácido láticas (BAL) são as mais estudadas atualmente, devido a tradicional associação destas a alimentos considerados seguros (ABRIOUEL *et al*., 2011). É o caso das formas comerciais da nisina (Nisaplin®) e da pediocina PA-1 (ALTA 2431®) (COTTER, HILLS, ROSS, 2005).

A eficiência das bacteriocinas em alimentos depende de inúmeros fatores, como as condições de processamento, temperatura de estocagem, pH do alimento e instabilidade da bacteriocina a mudanças de pH, inativação por enzimas, interação com aditivos/ingredientes, adsorção da bacteriocina a componentes alimentares (como gordura e proteínas), baixa solubilidade, distribuição das moléculas de bacteriocina na matriz alimentar e estabilidade limitada da bacteriocina durante a vida útil do produto. A microbiota do alimento é outro fator limitante devido à carga e à diversidade microbiana, sensibilidade da bacteriocina e interações microbianas do sistema alimentar. Além disso, a bactéria alvo pode influenciar a ação da bacteriocina considerando a carga microbiana, a sensibilidade da bactéria à bacteriocina (tipo de Gram, gênero, espécie, linhagem), o estado fisiológico (crescimento, fase estacionária, células viáveis mas não cultiváveis, estressadas ou injuriadas por tratamento sub-letal, na forma de endósporos), a proteção da célula por barreiras físico-químicas (biofilme) ou ainda desenvolvimento de resistência/adaptação (GALVÉZ *et al*., 2007).

O uso como bioconservantes já foi considerado eficiente em diversos alimentos, como carnes, produtos lácteos, enlatados, peixes, bebidas alcoólicas, saladas, ovos, produtos de confeitaria e vegetais fermentados, tanto sozinhos como em combinação com outros métodos de preservação ou através de sua incorporação em embalagens e superfícies de alimentos (CLEVELAND *et al.*, 2001; COTTER, HILLS, ROSSl, 2005; DEEGAN *et al*, 2006; MALHEIROS *et al*., 2010, BALCIUNAS *et al*., 2013). Além disso, as bacteriocinas poder ser utilizadas para melhorar a qualidade e os atributos sensoriais de certos alimentos, como queijos (DEEGAN *et al*., 2006).

No entanto, muitos trabalhos indicam que a aplicação direta de bacteriocinas em alimentos pode levar à diminuição ou perda da atividade antimicrobiana da mesma, devido à interação com os componentes dos alimentos (JUNG, BODYFELT, DAESCHEL, 1992; BRANEN, DAVISON, 2004; CHOLLET *et al*., 2008). A encapsulação das bacteriocinas em lipossomas pode representar uma alternativa tecnológica promissora, assim como sistemas de embalagem de alimentos podem ser explorados (MALHEIROS *et al.*, 2012).

2.5 Lipossomas

Lipossomas são estruturas coloidais formadas pela absorção de energia para uma combinação adequada de moléculas constituintes em uma solução aquosa. Quando moléculas anfifílicas, como fosfolipídios, são colocados em um ambiente aquoso, formam complexos agregados para tentar proteger suas seções hidrofóbicas das moléculas de água, mantendo contato com a fase aquosa através de grupos hidrofílicos. Se uma quantidade suficiente de energia é fornecida aos fosfolipídeos agregados, eles podem arranjar-se de forma organizada em bicamadas vesiculares fechadas (lipossomas ou nanoliposomas/nanovesículas) (JESORKA, ORWAR, 2008). Durante esse processo, os lipossomas podem aprisionar solutos hidrofílicos que estão presentes nos meios de hidratação. Assim, devido à presença da presença de fases aquosa e lipídica na estrutura das vesículas, estas podem ser utilizadas na encapsulação e liberação de substâncias hidrossolúveis, lipossolúveis e anfifílicas (KHOSRAVI-DARANI *et al*., 2007; MOZAFARI *et al*., 2008a) (Figura 2).

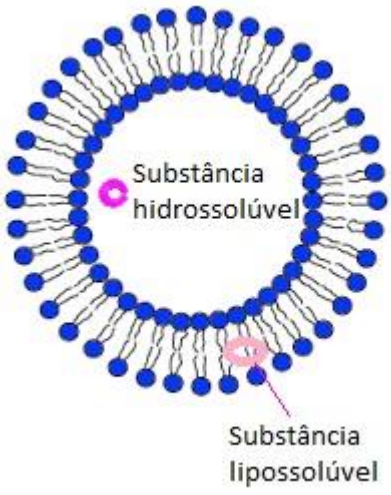

Figura 2. Representação esquemática de um lipossoma

Os lipossomas têm sido investigados como eficientes carreadores de substâncias, tanto na área clínica, carreando genes e drogas, como na área de alimentos, usados para promover a liberação controlada de proteínas, enzimas, vitaminas, antioxidantes, *flavors*, prebióticos e probióticos (TAYLOR *et al*., 2005; MOZAFARI *et al*.,2008b; SOZER, KOKINI, 2009). Os lipossomas podem proteger o material encapsulado contra muitas mudanças ambientais e químicas, como variações de pH e temperatura (MOZAFARI *et al*., 2008b).

A encapsulação em nanovesículas promove uma melhora nas propriedades dos compostos bioativos, pois protege os compostos da degradação e de interações com compostos indesejáveis, além de aumentar sua solubilidade aparente, eficácia e estabilidade (SOZER, KOKINI, 2009; BRANDELLI, 2012).

A encapsulação de peptídeos em nanovesículas lipídicas pode ser obtida através de vários métodos, embora o mais utilizado seja o de hidratação do filme fosfolipídico (BRANDELLI, 2012). Nesse método, o lipídio é solubilizado em solvente que depois é evaporado, originando um fino filme na superfície interna de um balão de fundo redondo. Este filme é então hidratado com uma solução tampão contendo o peptídeo antimicrobiano, a uma temperatura acima da temperatura de transição de fase dos lipídios, originando vesículas de diâmetro elevado e multilamelares. Para diminuir e homogeneizar o tamanho dos lipossomas deve-se fornecer energia, como ultra-som, extrusão ou microfluidificação (SHARMA, SHARMA, 1997; JESORKA, ORWAR, 2008). O objetivo é a obtenção de vesículas com tamanho adequado, polidispersidade aceitável e alta eficiência de encapsulação (MOZAFARI *et al*., 2008a).

A concentração de composto que pode ser encapsulado depende da estrutura lipídica e pode ser atribuída a interações eletrostáticas e hidrofóbicas entre os peptídeos antimicrobianos e os fosfolipídios (WERE *et al*., 2003). A nisina é um peptídeo antimicrobiano anfifílico catiônico e por isso pode ser encapsulada tanto no interior da fase aquosa quanto ser imobilizada nas membranas dos lipossomas (LARIDI *et al*., 2003). Uma maior eficiência de encapsulação da nisina é geralmente obtida com a utilização de fosfolipídios neutros (zwitterionicos), como a fosfatidilcolina, quando comparada com lipossomas contendo lipídios aniônicos, como o fosfatidilglicerol (WERE *et al*., 2003; TAYLOR *et al*., 2007).

A composição lipídica assim como a incorporação de diferentes peptídeos em lipossomas pode resultar diferenças no diâmetro (tamanho), potencial zeta e na morfologia dos lipossomas (SILVA *et al*., 2008). O potencial zeta é uma medida que permite inferir a estabilidade dos lipossomas através da carga superficial dos lipossomas. Quanto maior o potencial zeta, tanto positivo quanto negativamente, maior também serão as interações repulsivas, reduzindo portanto a frequência das colisões entre os lipossomas (MALHEIROS *et al.*, 2010). A carga do lipossoma também pode interferir na interação entre o peptídeo encapsulado e a bactéria. A célula bacteriana tem carga negativa e se houver repulsão entre os lipossomas e a superfície da célula do patógeno pode não ocorrer a fusão com a membrana plasmática, sendo este o principal mecanismo sugerido para interação entre os lipossomas e as bactérias (WERE *et al*., 2004; MUGABE *et al*., 2006; COLAS *et al*., 2007). Além disso, o potencial zeta tem sido citado como uma propriedade crítica para modular os efeitos citotóxicos de nanopartículas (SAYES, IVANOV, 2010), sendo os lipossomas catiônicos mais envolvidos na geração de fatores tóxicos (MURA *et al.*, 2011; KNUDSEN *et al.*, 2014).

O pH é outro fator que influencia a encapsulação dos peptídeos nas nanovesículas. Variações no pH podem alterar o estado de ionização de certos grupos, tanto do peptídeo quanto dos lipídios, interferindo na solubilidade e nas interações entre esses componentes (BRANDELLI, 2012). Além disso, foi demonstrado que a quantidade de nisina encapsulada é maior para soluções mais ácidas, uma vez que a nisina é mais solúvel e tem maior atividade antimicrobiana em pHs mais baixos (LARIDI *et al*., 2003).

Vários estudos vêm sendo feitos com diferentes bacteriocinas e substâncias semelhantes à bacteriocinas (“bacteriocin-like”), mas a maioria das pesquisas avaliando a aplicação em sistemas modelos ou alimentos de bacteriocinas encapsuladas foi desenvolvida com nisina Z, por ser um peptídeo aprovado para uso em alimentos e

devido às suas propriedades de difusão em ágar. Os resultados tem se mostrado positivos, indicando que a encapsulação de peptídeos antimicrobianos em nanolipossomas é uma alternativa potencial para superar os problemas relacionados com a aplicação direta de peptídeos antimicrobianos em alimentos, sendo uma ferramenta valiosa para a tecnologia de barreiras.

2.6 Embalagens antimicrobianas

As embalagens são um fator importante na indústria alimentícia, pois ajudam a controlar a proliferação microbiana, a degradação dos componentes do alimento e as alterações organolépticas que levam à rejeição pelo consumidor (KUREK *et al.*, 2013). A maioria dos materiais tradicionais usados para embalagem não é degradável, polui o ambiente e consome combustíveis fósseis para a sua produção. Uma alternativa biocompatível é o uso de polímeros biológicos, como amido, quitosana e gelatina (AZEREDO, 2009). Esses biopolímeros formam filmes e revestimentos (*coatings*) comestíveis que para serem competitivos com os termoplásticos tradicionais precisam ter suas propriedades mecânicas e de barreira melhoradas com a adição de plastificantes, sais ou outros compostos (ABDOLLAHI, REZAEI, FARZI, 2012). Nanocompósitos de polímero-argila tem recebido atenção por melhorar essas propriedades, já que estruturas nanométricas apresentam propriedades funcionais diferentes das propriedades encontradas na escala macro, devido à elevada área superficial que possuem, resultando numa maior interação entre a matriz e as nanopartículas (AVÉORUS, POLLET, 2011; ASSIS *et al*., 2012).

Recentemente, embalagens antimicrobianas/bioativas têm chamado atenção, uma vez que doenças transmitidas por alimentos causadas por microrganismos ainda são um grande problema de saúde pública em todo o mundo. Essas embalagens deliberadamente interagem com o alimento, retardando o crescimento microbiano na superfície, sendo capazes de estender a vida útil e promover segurança (APPENDINI, HOTCHKISS, 2002; DAINELLI *et al*., 2008). Essas embalagens também têm sido propostas como uma alternativa às operações de pós-acondicionamento para melhorar a segurança de alimentos prontos para o consumo (MARCOS *et al.*, 2013). As exigências do consumidor para produtos minimamente processados e isentos de conservantes e a preocupação relacionadas com o uso de aditivos químicos faz com que o uso de aditivos naturais como

bacteriocinas sejam uma alternativa promissora para a indústria alimentar (KUREK *et al*., 2013).

A adição direta de agentes antimicrobianos pode resultar em perda de atividade devido à lixiviação na matriz alimentícia e reações com seus componentes, como lipídios ou proteínas. O uso dos antimicrobianos aplicados nas embalagens é mais eficiente, devido à migração dos compostos para o alimento permitindo não só a inibição inicial de microrganismos indesejáveis, mas também a atividade residual ao longo do tempo, podendo agir durante o transporte e armazenamento do alimento (MAURIELLO *et al.*, 2005). As bacteriocinas são ideais para aplicação em embalagens antimicrobianas porque elas interagem com a superfície externa do microrganismo (parede celular e membrana plasmática) e não necessitam ser internalizadas para exibirem o efeito antimicrobiano (MILLS *et al*., 2011).

As bacteriocinas podem ser aplicadas na superfície interna de embalagens plásticas ou de papel, ou incorporados diretamente nos filmes poliméricos. Neste último caso, os peptídeos devem ser capazes de difundir para a superfície da embalagem ao longo do tempo para serem efetivos. A nisina incorporada em filmes de caseinato plastificados com sorbitol a uma concentração de 1000 UI/cm^2 resultou em redução de 1,1 log na contagem de *Listeria innocua* em queijo inoculado na superfície. Entretanto, o efeito antimicrobiano parece ter sido dependente da distância entre a superfície de contato dos filmes contendo nisina e a matriz do queijo, tal como observado pela profundidade de *Listeria* inoculada no queijo (CAO-HOANG *et al.*, 2010).

Filmes poliméricos biodegradáveis de ácido polilático (PLA) incorporados com nisina (0,04 mg/cm^2 de filme) pelo método de evaporação do solvente inibiram significativamente *Listeria monocytogenes* em meio de cultura e em clara de ovo líquida, reduziram a população celular de *Escherichia coli* O157:H7 em suco de laranja e diminuíram os níveis *Salmonella enteritidis* em clara de ovo líquida (JIN, ZHANG, 2008).

Filmes de acetato de celulose contendo pediocina comercial ALTA® 2351, dispostos entre fatias de presunto, reduziram as contagens de *Listeria innocua* em 2 log e de *Salmonella* sp. em 0,5 log após 15 dias de estocagem a 12ºC (SANTIAGO-SILVA *et al.*, 2009). Em salsichas fermentadas, filmes de álcool polivinílico com nisina foram mais efetivos do que tratamento com alta pressão na redução de *L. monocytogenes* (MARCOS *et al.*, 2013). Em carne de porco crua fatiada, filmes de ácido poli-láctico com serragem impregnados de pediocina PA-1/AcH reduziram em 2 log a contagem de *L. monocytogenes* após 14 dias (WORAPRAYOTE *et al.*, 2013).

Espitia (2013) obteve filmes de meticelulose com pediocina e nanopartículas de óxido de zinco com bons resultados na inibição de *Staphylococcu aureus* e *L. monocytogenes.*

Uma comparação entre filmes de hidroxipropil metilcelulose contendo nisina livre, nisina encapsulada e uma emulsão contendo a mistura desses dois tipos de aplicação foi feita por Imran e colaboradores (2012). Os filmes contendo a emulsão foram mais efetivos na inibição de *L. monocytogenes*. Em 2014, esse mesmo grupo de estudos comparou a migração da nisina livre dos filmes de hidroxipropil metil celulose com filmes de ácido poliláctico, caseinato de sódio e quitosana. Concluíram que o melhor efeito contra *S. aureus* e *L. monocytogenes* foi obtido com os filmes de hidroxipropil metilcelulose e de caseinato de sódio.

Os antimicrobianos presentes nas embalagens capazes de migrar para o alimento são considerados aditivos alimentares e devem obedecer à legislação pertinente. No Brasil, a Resolução-RDC nº 51, de 26 de novembro de 2010, estabelece os critérios gerais para a determinação de migrações total e específicas de materiais, embalagens e equipamentos plásticos destinados a entrar em contato com alimentos, dispondo sobre os ensaios a serem realizados por meio do contato dos materiais plásticos como soluções simulantes de alimentos (BRASIL, 2010).

2.7 Caseína

As proteínas são substâncias adequadas para a preparação de filmes devido a sua alta plasticidade e elasticidade, além de serem abundantes na natureza. As caseínas são uma família de fosfoproteínas sintetizadas pelas glândulas mamárias e secretadas como agregados coloidais grandes chamados micelas, que são responsáveis por muitas das propriedades do leite (PITKOWSKI, DURAND, NICOLAI, 2008), representando em torno de 80% do total das proteínas lácteas (FOX, BRODKORB, 2008).

Os caseinatos são polímeros hidrossolúveis obtidos através da precipitação ácida da caseína, a principal proteína do leite (AUDIC, CHAUFER, 2005). Podem ser preparados por subsequente elevação do pH e dissolução das caseínas precipitadas. São chamados caseinato de sódio ou caseinato de cálcio, dependendo da solução utilizada na neutralização: hidróxido de sódio ou de cálcio, respectivamente (ALVAREZ *et al*., 2008). Caseínas e caseinatos são extensivamente usados na indústria alimentícia devido a suas propriedades físico-químicas, nutricionais e funcionais (PITKOWSKI, DURAND,

NICOLAI, 2008), sendo ingredientes utilizados na formulação de produtos cárneos, produtos lácteos, produtos de panificação, chocolates e confeitos, coberturas comestíveis, bebidas lácteas e achocolatados, salgadinhos e snacks, filmes comestíveis e impermeabilizantes (ROMAN, SGARBIERI, 2005). O caseinato de cálcio não é tão utilizado quanto o de sódio, devido à sua limitada solubilidade (PITKOWSKI, DURAND, NICOLAI, 2008).

Os caseinatos têm sido considerados atrativos para o uso em embalagens, devido a suas numerosas propriedades funcionais como solubilidade em água e poder emulsificante, além de conferir valor nutricional ao produto final (ARRIETA *et al.*, 2013; MATSAKIDOU, BILLADERIS, KIOSSEOGLOU, 2013).

Os caseinatos possuem propriedades termoplásticas e formadoras de filme devido à sua habilidade para formar interações intermoleculares fracas, como pontes de hidrogênio, interações eletrostáticas e hidrofóbicas (AUDIC, CHAUFER, 2005). Além disso, por possuírem grande número de grupos polares em sua estrutura, os caseinatos apresentam boa adesão a diferentes substratos, fazendo com que sejam excelentes barreiras a substâncias apolares, como o oxigênio, dióxido de carbono e aromas (ARRIETA *et al.*, 2013). O uso de filmes de caseinato é adequado para embalar diversos alimentos, como queijos, vegetais e frutas, devido à sua transparência e flexibilidade (HELAL *et al.*, 2012). Os filmes de caseinato são sensíveis à água, de fraca barreira mecânica, mas boa barreira contra gases. Devido à sua fragilidade inerente, é necessário usar plastificantes para melhorar suas propriedades dúcteis. Para essa mesma função, pode-se adicionar também nanoargilas (POJANAVARAPHAN *et al.*, 2010).

2.8 Gelatina

A gelatina é um composto solúvel obtido pela hidrólise parcial do colágeno, a principal proteína dos ossos, cartilagens, peles e couros. A fonte, idade do animal e tipo de colágeno são fatores que influenciam as propriedades da gelatina. As propriedades mais importantes das gelatinas podem ser divididas em dois grupos: as associadas com o comportamento gelificante (formação de gel, textura e capacidade de ligação à água) e propriedades relacionadas ao comportamento de superfície (formação de emulsão e espuma, estabilização, adesão, coesão, função colóide e capacidade de formação de filme) (GÓMEZ-GUILLÉN *et al.*, 2011).

O uso de gelatina para a elaboração de coberturas e filmes comestíveis tem sido estudado desde os anos 60, por ser um produto de fácil obtenção e baixo custo (SOBRAL *et al.*, 2001). Além disso, esses filmes são transparentes, homogêneos e flexíveis. Uma desvantagem é sua alta higroscopicidade, fazendo com que os filmes inchem ou dissolvam em contato com a superfície dos alimentos com alta umidade. Por isso, o desenvolvimento de filmes a base de gelatina tem focado na melhoria de suas propriedades mecânicas e de resistência água, combinando a gelatina com outros polímeros (GÓMEZ-GUILLÉN *et al*, 2011).

2.9 Nanopartículas e nanocompósitos

Nanocompósitos são materiais formados por híbridos de materiais orgânicos e inorgânicos, onde a fase inorgânica está dispersa em nível nanométrico em uma matriz polimérica (PAIVA, MORALES, GUIMARÃES, 2006). Essas estruturas nanométricas podem atuar como nanopreenchedores, e de acordo com sua morfologia podem ser classificados em esféricos (como a sílica), aciculares (como nanotubos de carbono) ou estratificados (como as argilas) (AVÉORUS, POLLET, 2011). As dimensões geométricas afetam as propriedades do produto final, já que os nanocompostos têm área de superfície maior do que seus homólogos em microescala, favorecendo as interações com a matriz e o desempenho do material resultante. Além de atuarem como reforços, as nanopartículas podem ter outras funções quando adicionado a um polímero, como atividade antimicrobiana, imobilização de enzimas e biosensoriamento (AZEREDO, 2009).

As nanoargilas são nanopartículas bastante utilizadas, devido a sua disponibilidade, baixo custo, melhorias significativas e processamento relativamente simples. As argilas formam barreiras à água e a gases, já que os forçam a seguir sinuosamente ao redor das camadas de argila, dificultando a difusão no filme. Além disso, as argilas aumentam a biodegradabilidade dos filmes e causam o aumento da transição vítrea e das temperaturas de degradação térmica (AZEREDO, 2009; SOZER, KOKINI, 2009).

Os argilominerais são os principais constituintes da fração argilosa do solo. Os mais freqüentes são minerais dos grupos da caulinita, da montmorilonita e das ilitas. Além destes, encontram-se também em razoável proporção, em certos solos, minerais de outros grupos: cloritas, vermiculitas e minerais de camada mista (SILVA, 2007).

A montmorilonita (Figura 3) é um dos minerais argilosos mais abundantes e investigados (SILVA, 2007). Possui partículas de tamanhos que variam de 2μm a 0,1μm em diâmetro. Essa argila pertence ao grupo estrutural dos filossilicatos 2:1, é composta por duas folhas tetraédricas de sílica e uma folha central octaédrica de alumina, com formato de placas ou lâminas, que se mantêm unidas por átomos de oxigênio comuns a ambas as folhas (PAIVA, MORALES, GUIMARÃES, 2006). As placas desse argilomineral apresentam perfil irregular, são muito finas, possuem tendência a agregação no processo de secagem, e apresentam boa capacidade de delaminação quando colocadas em contato com água, já que são hidrofílicas (SILVA, FERREIRA, 2008). Sua esfoliação é difícil em matriz polimérica hidrofóbica. Por isso, o tratamento superficial das camadas de silicato objetiva promover maior hidrofobicidade à argila, sendo que a argila modificada (ou organoargila) tende a apresentar uma melhor compatibilidade com polímeros orgânicos (AZEREDO, 2009).

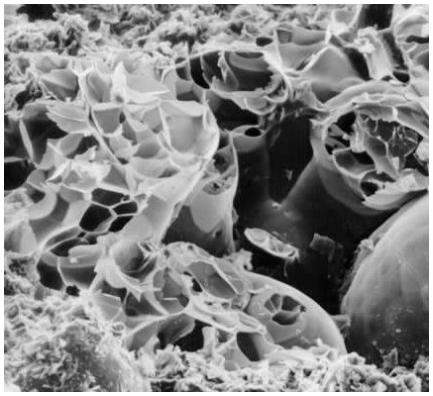

Figura 3. Montmorilonita em microscopia eletrônica de varredura – aumento de 15000 x (Fonte: Nanoshel LLC©, Dellaware, EUA, disponível em http://www.nanoshel.com/product/montmorillonite-bentonite-clay/)

A haloisita é um argilomineral do grupo das caulinitas, formada a partir do intemperismo das rochas vulcânicas. Apresenta-se sob a forma de tubos ocos (Figura 4), compostos internamente por duas camadas de folhas tetraédricas (silício e oxigênio) e uma folha partilhada octaédrica (alumínio e oxigênio). A sua superfície externa é composta de grupos siloxano (Si-O-Si), enquanto a superfície interna consiste de uma matriz de aluminol (Al-OH), e realizando a separação dessas camadas encontram-se as monocamadas de moléculas de água. Sua fórmula química é $Al_2Si_2O_5\ (OH)_4$. n H_2O (GORRASI *et al.*, 2014). Os nanotubos de haloisita tem comprimento de 0,5 a 1μm,

diâmetro externo de 50 a 80nm e lúmen de 15 nm (CAVALLARO, LAZZARA, MILIOTO, 2013). Devido ao seu formato e aos poucos grupos hidroxil na sua superfície, a haloisita pode ser dispersa em polímeros sem a necessidade de esfoliação. Os nanotubos de haloisitas são mais rígidos do que as placas de montmorilonita e a alta razão entre comprimento e diâmetro dos tubos podem promover nanoreforço em nanocompósitos poliméricos (CARLI *et al*, 2011). Além de melhorarem as propriedades mecânicas e térmicas dos polímeros, seus tubos ocos permitem que sejam bons carreadores de várias moléculas, liberando-as paulatinamente (GORRASI *et al*. 2014).

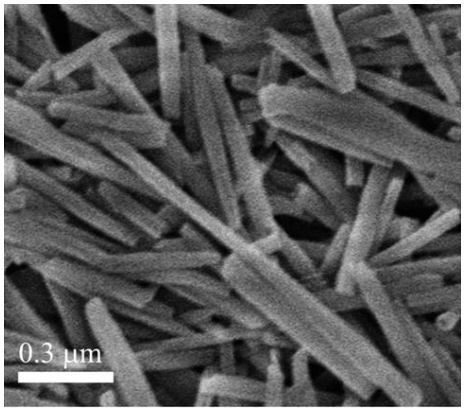

Figura 4. Haloisita em microscopia eletrônica de varredura (Fonte: Wei *et al.*, 2013)

A eficiência de argilas na modificação das propriedades do polímero depende principalmente do seu grau de dispersão na matriz polimérica (AYRES, ORÉFICE, 2007). De acordo com sua morfologia, os compósitos de argila-polímeros são divididos em três categorias (Figura 5), que são fortemente dependentes das condições do processo:

a) microcompósito - as partículas encontram-se aglomeradas, com separação entre as fases, ou seja, ocorre quando o polímero não é capaz de difundir-se entre as camadas da argila, impossibilitando a intercalação da mesma. Isto ocorre quando há incompatibilidade entre matriz e nanocarga.

b) nanocompósito intercalado - as cadeias poliméricas conseguem difundir-se entre as galerias da argila, promovendo separação das camadas, produzindo um material com morfologia de camadas alternadas de polímero/argila; suas propriedades são superiores às de um compósito convencional.

c) nanocompósito esfoliado - as camadas individuais da argila estão separadas em uma matriz polimérica contínua por uma distância média que depende da carga da argila, ou seja, este tipo de material é obtido quando as camadas da argila são separadas. Estes

nanocompósitos são considerados como os de melhores propriedades devido à melhor interação entre argila e polímero (PAIVA, MORALES, GUIMARÃES, 2006; AZEREDO, 2009).

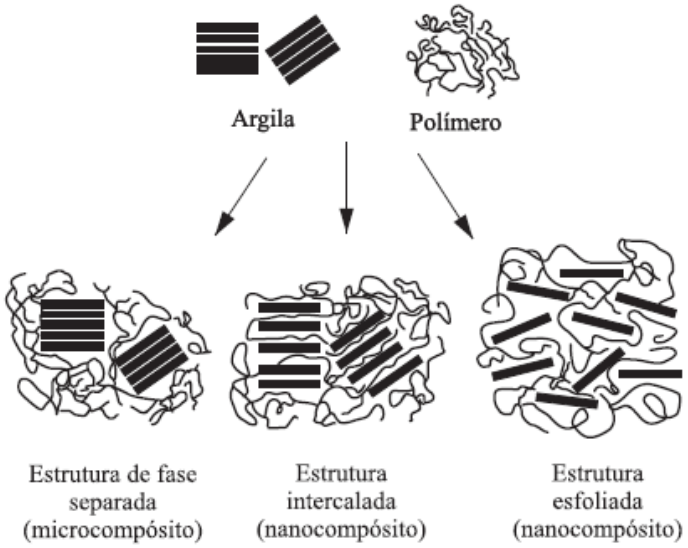

Figura 5. Estrutura dos nanocompósitos (Fonte: Paiva, Morales, Guimarães, 2006)

Estas estruturas podem ser caracterizadas por duas técnicas analíticas complementares: difração de raios-X (XRD) e microscopia eletrônica de transmissão (MET). Os difratogramas permitem determinar se ocorreu ou não intercalação das cadeias poliméricas através da determinação da distância interplanar. De acordo com a lei de Bragg, a intercalação deverá provocar uma mudança do pico de difração para um menor ângulo. Se o espaçamento entre as camadas aumenta muito, esses picos desaparecem nos difratogramas, o que implica completa esfoliação das lamelas na matriz polimérica. Neste caso, a técnica de MET é utilizada para identificar as camadas de argila esfoliada (RAY *et* al., 2006).

A utilização de nanomateriais em embalagens possibilita o desenvolvimento de embalagens bioativas, pois estes são capazes de manter os compostos bioativos - como prebióticos, probióticos, vitaminas encapsuladas ou flavonoides biodisponíveis - em ótimas condições, até que sejam liberados de forma controlada para o produto alimentício (SOZER, KOKINI, 2009). Tunç e Duman (2011) desenvolveram filmes de hidroximetil celulose com carvacrol e nanoargila e observaram que a quantidade de carvacrol liberada foi inversamente proporcional à concentração da nanoargila, demonstrando a capacidade desse composto em controlar a migração do antimicrobiano.

2.10 Nanotoxicologia

Segundo Delgado (2010), a nanotecnologia, junto com a biotecnologia, e as tecnologias de energia e informação são as quatro áreas tecnológicas mais importantes do início deste século e prometem mudanças nos processos de produção e nas relações sociais. Os nanomateriais vêm sendo utilizados em diversos setores, desde materiais esportivos a aeronáuticos, passando pelas indústrias eletrônica, farmacêutica e alimentícia e até no desenvolvimento de armas mais leves e potentes. Marano e Guadagnini (2013) acreditam que a principal razão para o desenvolvimento da nanotecnologia seja a mudança nas propriedades adquiridas pelas partículas em escala nanométrica, que interagem diferentemente de partículas de maiores dimensões com mesma composição química. Por isso, não é possível prever sua atividade biológica através da extrapolação das propriedades químicas e físicas dos componentes que os constituem.

Na área de alimentos, os nanomateriais são utilizados em embalagens para permitir uma melhor conservação dos produtos, como sensores para o controle da sua vida útil, como anticoagulantes e na clarificação de sucos. Nanoemulsões são utilizadas para encapsular, proteger e liberar aditivos, como os carotenóides, fitoesteróis, flavonóides, vitaminas e antimicrobianos. Estes nanomateriais em alimentos serão ingeridos e passam pelo sistema digestivo. Aqueles que são incorporados na embalagem de alimentos podem também ser liberados acidentalmente e alcançar o trato gastrointestinal. Por isso, é importante fazer uma avaliação dos riscos dos nanomateriais para o consumidor (MARANO, GUADAGNINI, 2013).

O risco potencial de qualquer substância está relacionado ao organismo exposto, à duração e à magnitude da exposição, à persistência do material e sua toxicidade inerente e à suscetibilidade do indivíduo (DELGADO, 2010) No caso dos nanomateriais, outras características tornam-se importantes e, por isso, o primeiro passo é defini-los e caracterizá-los em relação a tamanho, forma, carga superficial, tendência à agregação e aglomeração e sua solubilidade (MARANO, GUADAGNINI, 2013).

O tamanho tem um papel importante não só na biodistribuição de materiais, mas também na sua toxicidade. O aumento da área superficial promove um maior contato entre as partículas nanométricas e o meio que as circundam. Estudos *in vitro* demonstram que área superficial determina a resposta pró-inflamatória em células epiteliais (MONTEILLER *et al.*, 2007).

O potencial zeta representa a carga total do plano de cisalhamento, ou seja, a nuvem de íons que circunda a superfície das partículas, que é influenciada pela composição da superfície da nanopartícula. Esse parâmetro é importante para modular os efeitos citotóxicos das nanopartículas, controlando a interação eletrostática entre a partícula e a célula-alvo (SAYES, IVANOV, 2010; WINCLER *et al.*, 2012). Usando parâmetros histopatológicos, hematológicos e bioquímicos, Knudsen e colaboradores (2014) não observaram efeitos adversos de micelas e lipossomas catiônicos em ratos. No teste cometa, que investiga efeitos genotóxicos, foram observados efeitos adversos em todas as concentrações de lipossoma, indicando que o teste cometa pode ser um método sensível que deve ser incluído na bateria de testes para nanocarreadores. Ou seja, houveram efeitos genotóxicos mesmo em concentrações não citotóxicas e o aumento da carga estava relacionado com o aumento da genotoxicidade. Dokka e colaboradores (2000) observaram que lipossomas catiônicos induziram a geração de espécies reativas de oxigênio em células pulmonares, causando inflamação e toxicidade devido à parte catiônica e não ao lipossoma *per se*.

Existem ainda poucas informações sobre a toxicidade de nanopartículas em relação às suas características específicas, principalmente em alimentos, onde interações com matrizes alimentares e alterações dessas nanopartículas no trato digestivo desempenham um papel chave na avaliação do risco para o consumidor. As principais questões estão relacionadas com a sua reatividade biológica, sua capacidade de atravessar barreiras biológicas e se acumularem nos órgãos-alvo, sua biopersistência e como se comportam as respostas biológicas em relação à dose. No entanto, devido à complexidade das matrizes alimentares, é importante que essas caracterizações sejam realizadas sob as condições em que irão ser absorvidos. Na verdade, as suas propriedades serão modificados, dependendo do ambiente, especialmente o seu estado de agregação (interações fracas) ou aglomeração (interações fortes) (MARANO, GUADAGNINI, 2013).

É preciso, então, que sejam realizados estudos toxicológicos, para que se avalie a real possibilidade de aplicação dos nanomateriais sem causar efeitos adversos ao consumidor. Para isso, modelos *in vitro* e *in vivo* devem ser utilizados.

2.11 O modelo alternativo *Caenorhabditis elegans*

O uso de animais, especialmente mamíferos, em pesquisas científicas tem sido motivo de diversas discussões, principalmente de caráter ético, devido ao grande número de indivíduos requeridos e do sofrimento causado durante alguns tipos de experimentos. A isso soma-se o elevado custo de manutenção desses animais em laboratório. Nesse contexto, surgem os métodos ou modelos alternativos, que representam um esforço da comunidade científica mundial para reavaliar o uso de mamíferos em experimentos, através de um programa conhecido como 3Rs (do inglês "Reduction, Refinement, Replacement"), que objetiva a redução do número de animais, que eles sofram o menos possível e que novos métodos possam ser desenvolvidos para substituir a experimentação animal (CAZARIN, CORRÊA, ZAMBRONE, 2004).

Caenorhabditis elegans é um pequeno (cerca de 1mm de comprimento quando adulto) e transparente verme nematóide de vida livre que habita o solo e se alimenta de fungos e bactérias (Figura 6). Seu ciclo de vida é curto, sendo necessárias cerca de 80 horas a 20°C para a passagem de ovo a adulto (SALGUEIRO *et al*, 2014).

Figura 6. *Caenorhabditis elegans* hermafrodita adulto. (Fonte: Hyman Lab, disponível em http://hymanlab.mpi-cbg.de/hyman_lab/c-elegans/)

O *C. elegans e*xiste nas formas hermafrodita e macho. Entretanto, a linhagem N2 selvagem apresenta maioria de hermafroditas, com apenas 0,1 % de machos na população. Os machos produzem somente esperma e devem cruzar com hermafroditas para se reproduzirem. Já os hermafroditas produzem tanto esperma como oócitos e podem se autofecundar. Através da autofecundação, um hermafrodita produz, aproximadamente, 300 descendentes. Caso ocorra fecundação cruzada com um macho, o número de descentes pode ser superior a mil. *C. elegans* possui seis pares de cromossomos, sendo cinco pares de cromossomos autossomos. Hermafroditas possuem um par de cromossomos sexuais (XX) e machos têm apenas um cromossomo sexual (X0)

(HODGKIN, HORVITZ, BRENNER, 1979). O hermafrodita grávido expele os ovos, o qual, após a eclosão, passa por quatro estágios larvais (L1, L2, L3 e L4) até chegar à fase adulta (Figura 7).

C. elegans vem sendo utilizado como modelo biológico em diversas áreas, como genética, biologia do desenvolvimento e toxicologia, pois foi o primeiro organismo multicelular a ter seu genoma completamente seqüenciado, e este mostrou um alto nível de conservação com o genoma de vertebrados (LI *et al.*, 2014). Além disso, sua manutenção em laboratório é relativamente fácil e barata, baseada em sua dieta de *Escherichia coli* e cultivo em placas de petri. Tem sido considerado uma alternativa importante para estudos toxicológicos (WILLIAMS *et al*., 2000) em que uma série de parâmetros, como desenvolvimento, reprodução, longevidade, função e desenvolvimento neuronal, resposta ao estresse e dano oxidativo podem ser avaliados *in vivo*.

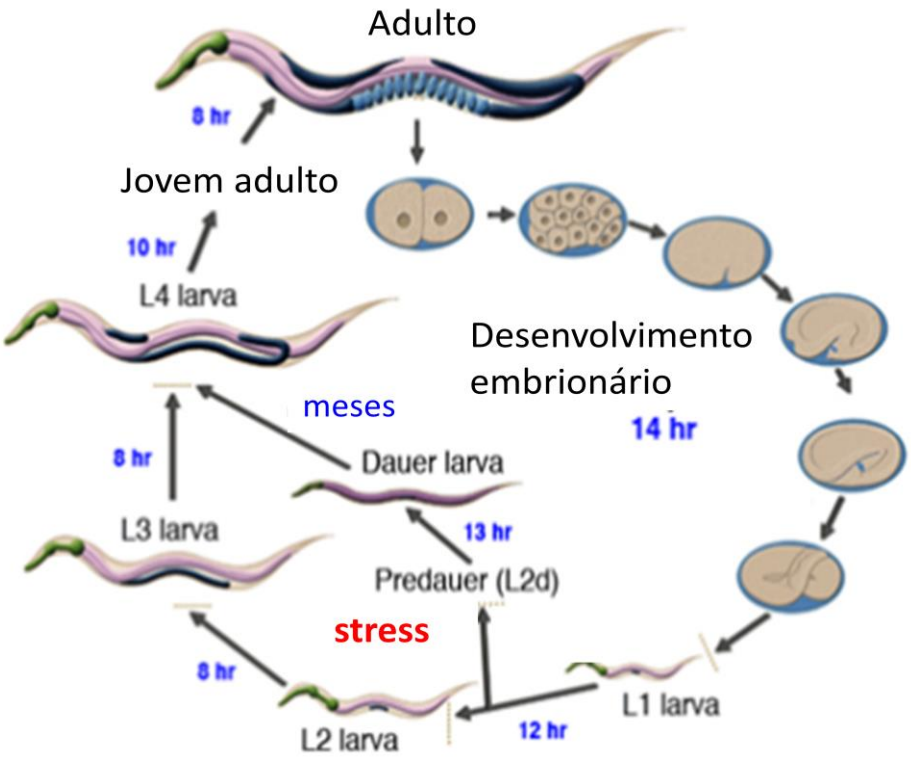

Figura 7. Ciclo de vida do verme *C. elegans.* (Modificado a partir de Worm Atlas. Original em: http://www.wormatlas.org/hermaphrodite/introduction/Introframeset.html)

2.12 Estresse oxidativo

A respiração aeróbia representou um grande salto na história evolutiva da Terra. Mas o mesmo oxigênio que permite uma geração de energia química muito mais eficaz também é capaz de danificar biomoléculas, principalmente proteínas e lipídios prejudicando o metabolismo intracelular (HALLIWELL, GUTTERIDGE, 2015). Essas substâncias originadas a partir de processos oxidativos são chamadas de radicais livres ou espécies reativas e são consideradas moléculas ubíquas em diversos processos fisiológicos e patológicos (COOPER *et al*., 2002; MURPHY *et al*., 2011).

Espécies reativas (ERs) são moléculas ou elementos químicos altamente reativos com capacidade de interagir com outros elementos, alterando sua estrutura e carga. Os radicais derivados do oxigênio representam a classe mais importante de espécies reativas geradas em sistemas vivos, embora também existam espécies reativas de nitrogênio (ERNs), cloro (ERC), bromo (ERB) e enxofre (ERS). As ERs podem ser classificadas como radicalares e não radicalares. A espécie reativa radicalar, ou radical livre, é qualquer espécie química (átomo, íon ou molécula) que possui um ou mais elétrons desemparelhados, o que lhes confere a capacidade de reagir mais rapidamente com outras moléculas para emparelhar os elétrons em seus orbitais. As não radicalares não apresentam elétron desemparelhado, sendo, portanto, menos reativas. Contudo, espécies não radicalares podem reagir com metais de transição, como o ferro e o cobre, gerando mais radicais livres (HALLIWELL, GUTTERIDGE, 2015).

Durante a respiração mitocondrial, uma pequena parte do oxigênio consumido é transformado em vários subprodutos reativos, especialmente superóxido ($O_2^{\bullet -}$). As EROs mais importantes biologicamente são o ânion superóxido ($O_2^{\bullet -}$), o radical hidroxil ($OH^{\bullet}$), peroxil ($ROO^{\bullet}$), alcoxil ($RO^{\bullet}$) e hidroperoxil ($HOO^{\bullet}$) (RIZZO *et al*., 2012).

O superóxido possui baixa reatividade com a maioria das moléculas biológicas, mas apresenta uma reatividade maior com grupamentos tiol e metais, como ferro, cobre e manganês (ABREU, CABELLI, 2010). O superóxido reage com grupamentos ferro-enxofre ou é convertido em peróxido de hidrogênio (H_2O_2), pela ação da enzima superóxido dismutase (SOD). Por sua vez, o peróxido de hidrogênio tem a propriedade de difundir-se facilmente pelos tecidos, reagindo com metais, principalmente com o ferro livre, produzindo radicais hidroxil ($OH^{\bullet}$). O $OH^{\bullet}$ é o radical mais reativo em sistemas biológicos, pois facilmente liga-se a metais, outros radicais ou qualquer molécula biológica (RIZZO *et al*., 2012)

Em concentrações baixas a moderadas, as espécies reativas podem ser benéficas em diversos processos biológicos como fagocitose, regulação do crescimento celular, sinalização intra e intercelular, além de aumentar a tolerância a diversos tipos de estresse ambiental (VALKO *et al*., 2006; ZHOU *et al*, 2011; HALLIWELL, GUTTERIDGE, 2015).

Quando a produção de EROs supera as defesas antioxidantes ou mecanismos de reparo do organismo, resultando em aumento dos níveis de dano oxidativo e perda da função e homeostase redox celular dizemos que há estresse oxidativo. Considera-se dano oxidativo qualquer modificação molecular, reversível ou não, com efeito deletério, causado por espécies reativas a moléculas biológicas, como proteínas, lipídeos e DNA (HALLIWELL, GUTTERIDGE, 2015). Para se protegerem dos danos, as organismos desenvolveram mecanismos enzimáticos e não enzimáticos de defesa antioxidante. A principais defesas enzimáticas são a superóxido dismutase (SOD), que catalisa a dismutação do $O_2^{\bullet -}$ a H_2O_2 e O_2, a catalase (CAT), que catalisa a decomposição do H_2O_2 a água e O_2 e glutationaperoxidase (GPx) que atua sobre peróxidos em geral, utilizando a glutationa reduzida (GSH) como cofator, formando adissulfetoglutationa oxidada (GSSG) (GOLDEN *et al*, 2002).

3 RESULTADOS E DISCUSSÃO

Os resultados obtidos neste trabalho estão apresentados na forma de manuscritos. Além dos artigos científicos, elaborou-se uma revisão bibliográfica a respeito de aplicação de antimicrobianos nanoestruturados em alimentos, abrangendo os nanocompósitos para embalagens (filmes e *coatings),* considerações sobre nanotoxicologia e métodos de avaliação toxicológica para nanomateriais, a qual fará parte de um capítulo no livro Nanotechnology in Food Industry a ser publicado pela Editora Elsevier.

São apresentados três artigos técnico-científicos que contemplam os estudos realizados ao longo deste doutorado. O primeiro artigo foi publicado na revista Colloids and surfaces B: Biointerfaces e refere-se ao desenvolvimento e caracterização de lipossomas, a partir de duas fontes diferentes de fosfatidilcolina, e sua incorporação junto a nanoargilas em nanocompósitos de gelatina e caseína. O segundo artigo será submetido ao periódico Food and Chemical Toxycology e trata da avaliação da toxicidade dos lipossomas após exposição aguda em *Caenorhabditis elegans*. O terceiro artigo foi submetido ao periódico Toxicology in Vitro e trata da avaliação da toxicidade aguda das nanoargilas utilizadas ao longo do trabalho, adsorvidas ou não com nisina.

Manuscrito 1 – Innovative bionanocomposite films of edible proteins containing liposome-encapsulated nisin and halloysite nanoclay

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Innovative bionanocomposite films of edible proteins containing liposome-encapsulated nisin and halloysite nanoclay

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ABSTRACT

Films and coatings based on natural polymers have gained increased interest for food packaging applications. In this work, halloysite and phosphatidylcholine liposomes encapsulating nisin were used to develop nanocomposite films of gelatin and casein. Liposomes prepared with either soybean lecithin or Phospholipon® showed particle size ranging from 124 to 178 nm and high entrapment efficiency (94–100%). Considering their stability, Phospholipon® liposomes with 1.0 mg/ml nisin were selected for incorporation into nanocomposite films containing 0.5 g/l halloysite. The films presented antimicrobial activity against Listeria monocytogenes, Clostridium perfringens and Bacillus cereus. Scanning electron microscopy revealed that the films had a smooth surface, but showed increased roughness with addition of liposomes and halloysite. Casein films were thinner and slightly yellowish, less rigid and very elastic as compared with gelatin films. Thermogravimetric analysis showed a decrease of the degradation temperature for casein films added with liposomes. The glass transition temperature decreased with addition of liposomes and halloysite. Gelatin and casein films containing nisin-loaded liposomes and halloysite represent an interesting alternative for development of active food packaging.

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1. Introduction

The increasing consumer demand for natural foods, with extended shelf life but without the addition of chemical preservatives, motivate the development of new conservation technologies. Biopreservation emerges as an alternative, in which a protective microbiota or their metabolites are employed aiming the control of pathogenic and spoilage microorganisms in food products [1,2].

Bacteriocins are recognized as safe and natural antimicrobial substances, especially useful when applied in hurdle technology [3]. Nisin is a bacteriocin known since the 1920s and currently approved as biopreservative in more than 50 countries [4]. This bacteriocin has received great attention because of its broad inhibition spectrum against Gram-positive bacteria, such as *Listeria* and *Staphylococcus*, and the effective control of *Bacillus* and *Clostridium* spores [5]. However, the application of nisin in its free form can result in undesirable interactions with the food matrix, leading to loss of activity [6,7].

Encapsulation of bioactive compounds into nanovesicles may promote a number of beneficial effects by protecting them against degradation and undesirable interactions, and increasing their stability, apparent solubility and efficiency [8,9]. Besides, the amount of encapsulated bioactive required for a specific effect is often much less than the amount required when non-encapsulated [10]. Liposomes have been used as an interesting platform to deliver bioactive compounds, such as antimicrobials, antioxidants, vitamins in food systems [7,11].

Most traditional materials used for packaging are not biodegradable, pollute the environment and consume fossil fuels for their production. A biocompatible alternative is the use of natural polymers such as starch, gelatin and chitosan [12,13]. Despite the intensive research performed in the last decade on development of biopolymer-based food packaging, this topic still needs significant advances [14,15]. To be competitive with traditional thermoplastics, biopolymers may have its mechanical and barrier properties improved with the addition of plasticizers, salts or other compounds. Nanoclays have been successfully used as reinforcement

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in biopolymer-based composites, frequently providing better characteristics to the films [16,17].

Previous investigations on edible films containing nisin have been reported [18–20]. However, the combination of liposome-encapsulated nisin with nanoclays in protein-based films has not been investigated yet. Thus, the objective of the present study was to develop edible antimicrobial films of gelatin and casein incorporating nanoclays as reinforcer and phosphatidylcholine liposomes containing nisin. Phosphatidylcholine liposomes made of Phospholipon® 90G were compared with liposomes made with soy lecithin, and then used to prepare gelatin and casein films in the presence of bentonite, surface-modified montmorillonite or halloysite. The antimicrobial, mechanical, thermal and morphological properties of the films were evaluated.

2. Materials and methods

2.1. Materials

Phospholipon®90G (pure phosphatidylcholine stabilized with 0.1% ascorbyl palmitate) was supplied by Lipoid GMBH (Ludwigshafen, Germany). Soybean lecithin was provided by Solae S.A. (Esteio, Brazil). Gelatin was from Oetker Brasil (Cotia, Brazil) and casein was obtained from Synth (Diadema, Brazil). Ethyl acetate used for purification of crude soybean lecithin, chloroform used in liposome production and glycerol used as a plasticizer in films were purchased from Merck (Darmstadt, Germany). Nylon membrane filters (0.22 μm) were obtained from TPP (Trasadingen, Switzerland). The nanoclays hydrophilic bentonite (Nanomer—PGV), montmorillonite (MMT) surface modified with 25–30% octadecylamine (Nanomer—I.30E) and halloysite (HNT), were from Sigma-Aldrich (St. Louis, MO, USA).

Commercial nisin (Nisaplin®) was obtained from Danisco Brasil Ltda (Cotia, Brazil). According to the manufacturer, the formulation contains NaCl and denatured milk solids as fillers, and 2.5% (w/w) pure nisin. The stock solution was prepared by solubilizing Nisaplin with 0.01 M HCl to obtain a nisin concentration of 2.5 mg/ml. This solution was filter sterilized through 0.22 μm membranes, and further diluted in 10 mM phosphate buffer pH 7.0 to reach working concentrations. Nisin is expressed as levels of pure nisin (mg/ml). To convert these units to International Units (IU/ml) or to equivalent Nisaplin® levels (mg/ml), the levels of pure nisin should be multiplied by 40 [21].

2.2. Purification of crude soybean lecithin

Crude soybean lecithin (5 g) was dissolved in 25 ml ethyl acetate. Then, distilled water (1 ml) was slowly added under gentle agitation, resulting in the formation of two phases. The upper phase was discarded. The lower phase, having a gel aspect, was dispersed in 30 ml acetone, forming clusters that were crushed using a glass stick. Then, the acetone was separated by decanting and a new aliquot of 30 ml acetone was added, repeating the shredding process. The precipitate was vacuum filtered and dried in a desiccator.

2.3. Liposome production by film hydration

Liposome production was performed according to the film hydration method [22], with some modifications. Phosphatidylcholine (76 mg) was dissolved in chloroform (15 ml) in a round-bottom flask and the organic solvent was removed using a rotary evaporator until a thin film was formed on the walls. Traces of organic solvents were removed by storage in desiccator under vacuum for 18 h. The nisin stock solution was diluted in phosphate buffer to reach 0.5, 1.0 and 1.5 mg/ml and 5 ml were added to disperse the resulting dried lipid film. These mixtures were then homogenized above their phase transition temperature (60 °C) to produce multilamellar lipid vesicles (MLVs). In order to reduce the size and homogenize the liposomes, sonication of the MLVs was carried out in an ultrasonic cell disrupter (Unique, Brazil) by five cycles of 1 min at intervals of 1 min, during which the samples were kept in ice. Then, the solution was filtered through 0.22 μm membranes.

2.4. Liposome characterization

Liposome size and polydispersity index (PDI) were determined by dynamic light scattering (DLS) in a particle size analyzer (BI–200 M goniometer, BI-9000AT digital correlator, Brookhaven Instruments, Holtsville, NY, USA) essentially as described elsewhere [23]. DLS measurements were performed immediately after the liposome preparation. Samples (300 μl) were diluted in 10 ml of 10 mM phosphate buffer pH 7.0 prior analysis. The zeta potential of the nanovesicles was determined after dilution of the formulations in 1 mM NaCl using a Zetasizer Nano-ZS ZEN 3600 equipment (Malvern Instruments, Herrenberg, Germany).

2.5. Antimicrobial activity

The antimicrobial activity was detected by agar diffusion assay as described previously [24]. Aliquots (10 μl) of free and liposome-encapsulated nisin were applied onto BHI agar plates previously inoculated with a swab submerged in a suspension to a final OD_{600}nm of 0.15 of *Listeria monocytogenes* ATCC 7644 (approximately 10^7 UFC ml^{-1}). After incubation at 37 °C for 24 h, the plates were checked for zones of inhibition. The reciprocal value of the highest dilution that produced a definite inhibition zone was taken as the activity units (AU). Antimicrobial activity, in AU/ml, was calculated as D/V (ml), where D is the dilution factor and V is the sample volume. Antimicrobial activity was evaluated immediately after the liposome preparation and aliquots were stored at 4 °C and at room temperature (25 ± 2 °C) to analyze the activity and stability of liposomes over time.

2.6. Entrapment efficiency

The entrapment efficiency (EE) of liposomes was determined by evaluation of nisin antimicrobial activity [22]. The liposome suspension was subjected to ultrafiltration (Amicon Ultracel YM-10, Millipore, Billerica, MA, USA) at 10,000*g* for 20 min. The antimicrobial activities of encapsulated nisin (retentate) and free nisin (filtrate) were measured as described above, and EE was calculated using the following equation [6]:

$$EE\ (\%) = \frac{encapsulated\ nisin\ (AU/ml)}{encapsulated\ nisin\ (AU/ml) + free\ nisin\ (AU/ml)} \times 100$$

2.7. Film preparation

Film formulations were tested for each polymer (casein and gelatin) with the addition of plasticizer (glycerol), liposomes and nanoclays (montmorillonite, known as hydrophilic bentonite (MMT), montmorillonite modified with octadecylamine and halloysite, tested at 0.5 and 1.0 g/l).

Casein films were obtained from a 30 g/l casein solution in distilled water. As casein is poorly soluble in distilled water, the pH solution was adjusted to 7.0 with the addition of 1 M NaOH and then heated to 70 °C under stirring. This process was repeated and the solution was centrifuged at 6000*g* for 5 min to remove insoluble material. The supernatant was then diluted in distilled water at a 3:1 ratio. Gelatin films were made of a 40 g/l commercial colorless

gelatin solution heated to 60 °C and maintained under magnetic stirring until total dissolution.

After the incorporation of nanoclays, the protein solutions were sonicated for 2 min at 20 kHz on ultrasonic device (Unique OF S500, Unique, Brazil). Glycerol (1%, v/v) and liposomes were added under stirring. The filmogenic solutions were poured into sterile polystyrene plates and allowed to dry inside an exhaust hood for 48 h at room temperature.

Casein and gelatin films were prepared incorporating nisin-encapsulated liposomes and nanoclays. Considering their stability, Phospholipon® liposomes prepared with 1.0 mg/ml nisin were chosen for incorporation into films. The amount of liposome suspension added to the film forming solution was optimized as the lowest amount that showed a clear inhibition zone in the antimicrobial test. This amount corresponded to 6.5% (v/v).

Preliminary tests showed that the montmorillonite modified with octadecylamine produced opaque films with dotted appearance due to lumps of undispersed nanoclay. The addition of hydrophilic montmorillonite (bentonite) produced rigid, breakable and off-white films. Although not easily dissolvable, halloysite was chosen because it allowed to obtain more flexible and transparent films.

2.8. Film characterization

2.8.1. Antimicrobial properties

The antimicrobial activity was tested using the inhibition zone assay in agar plates. Pieces with 4 cm^2 were cut from films and placed on Brain Heart Infusion (BHI, Oxoid, Basingstoke, UK) agar plates inoculated with *L. monocytogenes* ATCC 7644 (10^7 CFU ml^{-1}). Petri dishes were stored at 4 °C during 5 h to initiate nisin desorption and after incubated at 37 °C for 24 h. The antimicrobial activity is evidenced by clear zones (no microorganism growth or survival) surrounding film pieces.

To simulate a food matrix, the films were also tested in skimmed milk agar [25]. *L. monocytogenes* ATCC 7644, *Bacillus cereus* ATCC 14579, *Staphylococcus aureus* ATCC 1901, and *Clostridium perfringens* ATCC 3624 were used as indicator strains. The bacteria were inoculated (10^7 CFU ml^{-1}) in skimmed milk agar plates, which were stored at 4 °C for 5 h and then incubated at 37 °C for 24 h. Inhibition zones around the film pieces were measured in millimeters.

2.8.2. Structural properties

The thickness of films was measured in millimeters using a digital micrometer (Digimess, model IP40, São Paulo, Brazil) with an accuracy of 0.001 mm. The measurement was done in five random points in four different samples of each film.

The evaluation of morphology and dispersibility of the nanoclays and nanovesicles in the polymeric matrix was performed by scanning electron microscopy. The film surfaces were analyzed using a JEOL microscope (model JSM-5800, Tokyo, Japan) operated at a voltage of 10 kV. Samples were coated with a gold layer prior to analysis in order to increase their electrical conductivity.

2.8.3. Infrared spectroscopy

Fourier Transform Infrared (FTIR) spectroscopy was used to explore the interaction of biopolymers with liposomes and halloysite. The spectra were recorded on a Bruker Alpha FTIR spectrometer (Bruker Banner Lane, Coventry, Germany) in attenuated total reflectance (ATR) mode with a ZnSe cell/crystal. The scans were collected between 400 and 4000 cm^{-1}, with a resolution of 4 cm^{-1} and accumulation of 32 scans.

2.8.4. Mechanical properties

Tensile tests were carried out using films with 25 mm × 60 mm of size under crosshead speed of 50 mm/min in a DL 10000 universal testing machine (EMIC, São José dos Pinhais, Brazil) equipped with a 50 N load cell, according to ASTM D-638 standard. Maximum strength (MS, in N), maximum pressure (MP, in MPa), tensile strength (TS, in MPa), elongation at break (EAB, in%) were determined from five replicates for each formulation. The Young's modulus (YM) was obtained from the slope of the elastic phase in the relationship between stress and strain. All values were generated by the Bluehill 3 software (Instron, Norwood, MA, USA).

2.8.5. Thermal properties

The thermal properties were analyzed by thermogravimetry and differential scanning calorimetry (DSC). Thermogravimetric analysis (TGA) was performed using a QA50 analyzer (TA Instruments, New Castle, DE, USA). The samples were heated from 25 to 800 °C at the rate 20 °C min^{-1} under nitrogen atmosphere (50 ml min^{-1}). The characteristic temperatures $T_{10\%}$ and $T_{50\%}$ were determined as the initial decomposition temperature (10% degradation) and the temperature where 50% of sample was decomposed, respectively. DSC was performed using a Thermal Analyst 2100 (TA Instruments, New Castle, DE, USA), where linear heating and cooling experiments were performed at 20 °C min^{-1} under a dry nitrogen atmosphere (50 ml min^{-1}). All samples (10 mg) were heated from ambient temperature to 200 °C and kept for 2 min to erase the thermal history. The samples were then cooled down to −50 °C and heated again until 200 °C. The glass transition temperature (T_g) is indicated by a change in DSC thermogram baseline due to the change in heat capacity between the glassy and rubbery states, and was determined by the Q2000 software (TA Instruments, New Castle, DE, USA).

2.8.6. Optical properties

The optical properties of the films were expressed as color measurements determined in five random points of each film by a colorimeter (Minolta CR-400, Osaka, Japan). The following parameters were measured: L^* (luminosity, ranging from 0 to 100, from dark to light), a^* (red on positive direction and green in negative direction) and b^* (yellow in the positive direction and blue in negative direction). The colorimeter was calibrated against a standard white background.

Total color difference (ΔE) and whiteness index (WI) of the composite films were calculated by the following equations [26]:

$$\Delta E = \sqrt{(\Delta L*^2) + (\Delta a*^2) + (\Delta b*^2)}$$

$$WI = 100 - \sqrt{(100 - L*)^2 + a*^2 + b*^2}$$

2.9. Statistical analysis

The results were subjected to variance analysis (ANOVA) and means were compared by the Tukey test at a level of 5% significance, using the Prism 5.0 software (GraphPad Software Inc., La Jolla, CA, USA).

3. Results and discussion

3.1. Liposome characterization

Liposomes were prepared with 0.5, 1.0 and 1.5 mg/ml nisin from two sources of phosphatidylcholine, namely Phospholipon 90G® and soybean lecithin. Liposome solutions containing 1.5 mg/ml nisin were difficult to filter and in a few days became biphasic and were excluded from the study. Liposomes loaded with phosphate buffer were used as control. The liposomes showed mean diameters ranging from 124.5 to 173.0 nm, excepting for those prepared with soybean lecithin and 1.0 mg/ml nisin (Table 1). These values are in

Table 1
Size, polydispersity index (PDI) and zeta potential of liposomes.

Lipid source	Nisin (mg/ml)	Size (nm)	PDI	Zeta Potential
Phospholipon®	0	124.5 ± 6.2 [a]	0.196 ± 0.08 [a]	2.96 ± 13.3[d]
Soybean lecithin	0	173.0 ± 53.5 [a]	0.201 ± 0.10 [a]	−16.98 ± 25.7 [bc]
Phospholipon®	0.5	162.3 ± 26.8 [ab]	0.264 ± 0.03 [a]	−5.95 ± 1.98 [c]
Soybean lecithin	0.5	140.4 ± 35.3 [ab]	0.271 ± 0.03 [a]	−39.48 ± 1.56 [a]
Phospholipon®	1.0	168.5 ± 31.0 [b]	0.247 ± 0.04 [a]	−4.82 ± 4.95 [c]
Soybean lecithin	1.0	516.1 ± 23.3 [c]	0.139 ± 0.03 [a]	−27.74 ± 3.00 [b]

Values represent means ± standard deviation of three independent experiments. Treatments with different letter in the same column are significantly different ($P < 0.05$).

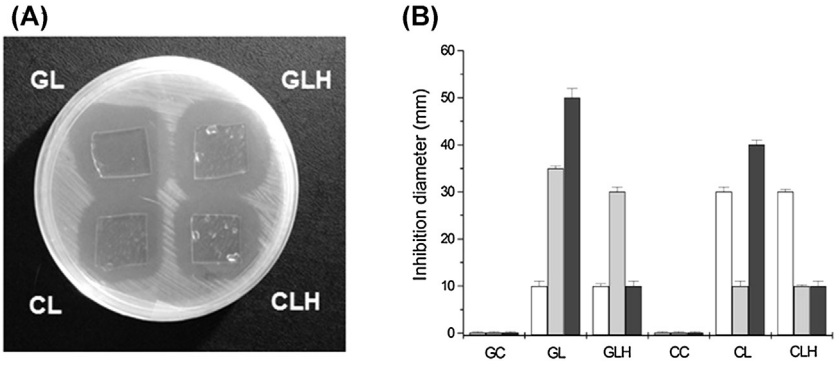

Fig. 1. Antimicrobial activity of casein and gelatin films. (A) Inhibition zones (halos) of gelatin and casein films against *L. monocytogenes* in BHI medium. Film samples were placed onto agar surface and the inhibitory zones were observed after 24 h incubation at 37 °C. (B) Antimicrobial activity of casein and gelatin films against *B. cereus* (white bars), *C. perfringens* (grey bars) and *L. monocytogenes* (black bars) in skimmed milk agar. Values represent means ± standard deviation of three independent experiments. GC, control gelatin films, GL, gelatin films with liposomes, GLH, gelatin films with liposomes and halloysite, CC, control casein films, CL, casein films with liposomes, CLH, casein films with liposomes and halloysite.

agreement with those reported by other authors for phosphatidylcholine liposomes loaded with nisin, ranging from 145 to 181 nm [3,22,27]. The size of control liposomes without nisin were similar to those encapsulating nisin. The polydispersity index (PDI) is an indicator of the width of particle size distribution and ranges from 0 (monodispersed) to 1 (very broad distribution). The PDI values were lower than 0.3, indicating the homogeneity of the nanoparticles.

There was no difference in size and polydispersity between liposomes containing 0.5 mg/ml nisin entrapped with different sources of phosphatidylcholine, but soybean lecithin liposomes showed higher surface charge, as evidenced by zeta potential values (Table 1). The addition of increased amount of nisin did not cause significant changes in size, polydispersity or zeta potential of Phospholipon® liposomes. However, soybean lecithin liposomes with 1.0 mg/ml nisin probably aggregated, forming particles much larger than the others, with more than 500 nm.

An elevated entrapment efficiency was obtained for both Phospholipon® liposomes (EE 100%) and soybean lecithin liposomes (EE 94%). Other studies using phosphatidylcholine also reported high entrapment efficiency for nisin, frequently reaching values above 90% [22,26,28].

The antimicrobial activity of the liposomes was monitored for 30 days at room temperature (25 °C) and refrigeration temperature (4 °C) as shown in Fig. S1 (see Supplementary material Fig. S1 in the online version at DOI: 10.1016/j.colsurfb.2016.05.080). The initial activity of free nisin solutions at 0.5 mg/ml and 1.0 mg/ml reached 6400 AU/ml and 9600 AU/ml, respectively. Liposomes loaded with the same concentration of nisin had the same initial antimicrobial activity, which decreased with time and was more pronounced at room temperature (Fig. S1). Phospholipon® liposomes maintained its activity for a longer time, especially under refrigeration, keeping the homogeneous appearance of the solution. After two months the liposomes prepared with soybean lecithin begin to become viscous and biphasic. The liposomes produced with Phospholipon® and 1.0 mg/ml nisin were maintained refrigerated and tested after four, five and six months, with activities of 400, 200 and 100 AU/ml respectively.

3.2. Film characterization

3.2.1. Antimicrobial properties

A clear inhibitory zone was observed when 0.5 g/l halloysite was used in film formulation, but not with 1.0 g/l halloysite (data not shown). The improved barrier properties of polymer-clay nanocomposites is associated to an increased tortuosity of the diffusive path for a molecule, forcing it to travel a longer path to diffuse through the film [12]. Thus, it is conceivable that halloysite is impairing nisin migration, delaying the release of the antimicrobial activity at 0.5 g/l, but obstructing it at 1.0 g/l. Based on this result, antimicrobial films were prepared with 0.5 g/l halloysite.

Both gelatin and casein films showed antimicrobial activity as evidenced by formation of clear inhibition zones in agar plates (Fig. 1A). The inhibitory halos were 43 ± 2 mm and 41 ± 2 mm for gelatin films containing liposomes and those containing liposomes + halloysite, respectively. Casein films containing liposomes showed inhibitory halos of 38 ± 3 mm, whereas casein films with liposomes + halloysite exhibited halos of 37 ± 2 mm. In skimmed milk agar, antimicrobial activity was observed against *L. monocytogenes*, *C. perfringens* and *B. cereus* (Fig. 1B). However, none of the films presented inhibitory zones against *S. aureus*. For both biopolymers, films with halloysite presented lower inhibition diameters, indicating the controlled release role of the nanoclay. This effect could be interesting for application of the active film in products subjected to long-term storage, enhancing the shelf life of the active film.

These results suggest that incorporation of nanoencapsulated antimicrobial substances can be a promising method to obtain active films. However, there is limited information on this approach. Nisin was encapsulated using soy lecithin liposomes and incorporated into hydroxypropyl methylcellulose to form antimi-

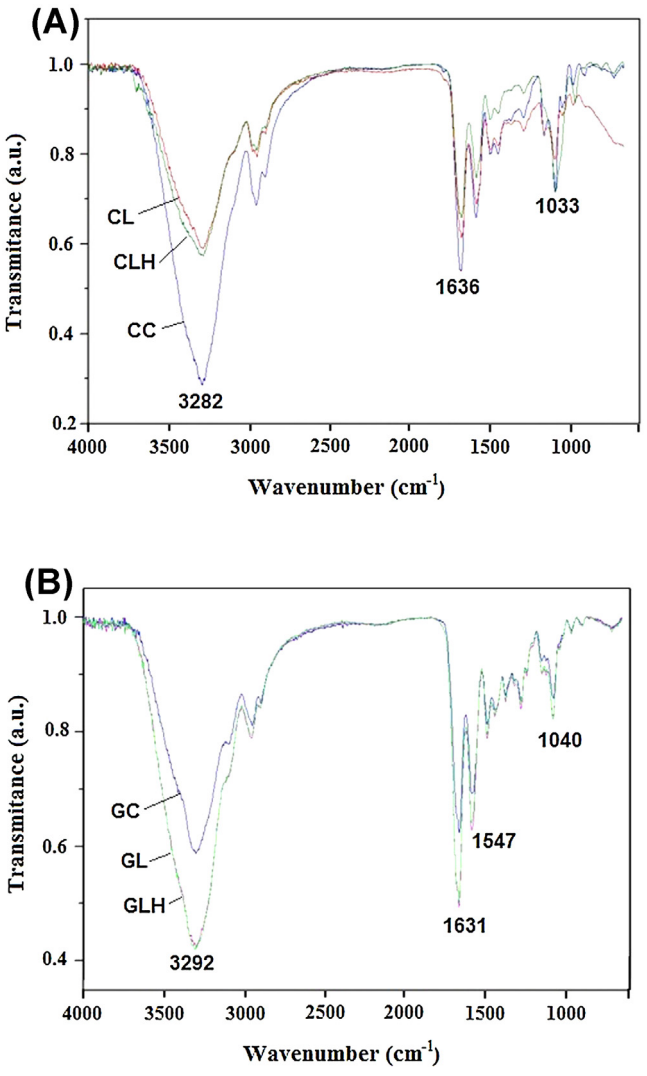

Fig. 2. FTIR spectra of casein (A) and gelatin films (B). Film samples were analyzed by attenuated total reflectance (ATR) method with a ZnSe crystal. CC, control casein films; CL, casein films with liposomes; CLH, casein films with liposomes and halloysite; GC, control gelatin films; GL, gelatin films with liposomes; GLH, gelatin films with liposomes and halloysite.

crobial films that were effective against *L. monocytogenes* [18]. Nanoemulsions containing thyme, lemongrass or sage essential oils as dispersed phase and sodium alginate solution as continuous phase have been also described. Edible films containing thyme showed the highest antimicrobial effect against *E. coli*, achieving up to 4.7 log reductions after 12 h [29].

3.2.2. Infrared spectroscopy

The FTIR technique was performed to investigate possible chemical interactions among the components during the film preparation process. The FTIR spectra of the films are shown in Fig. 2. The main absorption bands were similar in casein and gelatin films: 1033–1040 cm^{-1} (C—N), 1631–1636 cm^{-1} (H—N) and 3282–3292 cm^{-1} (O—H). Such bands are typical of the protein matrix. Some differences in band intensity can be noticed, but no displaced or additional bands were observed in nanocomposite films. The absence of significant changes indicates that no chemical interactions occurred between the liposomes and halloysite with the protein matrix of the films. As nisin also has peptide nature, its absorption bands should be coincident with the bands of the protein matrix. Minor bands observed at 1220–1250 cm^{-1} (P═O) and 2920–2980 cm^{-1} (aliphatic C—H stretching) could be assigned to phosphatidylcholine liposomes. The FTIR spectrum of halloysite includes the presence of typical bands around 3620 cm^{-1} (—OH stretching of structural OH groups), 1028 cm^{-1} (Si—O stretching) and isolated Si-O groups at 910 cm^{-1} [30]. However, these bands are not prominent in films with liposomes and halloysite because they are probably overwritten by bands of the protein matrix.

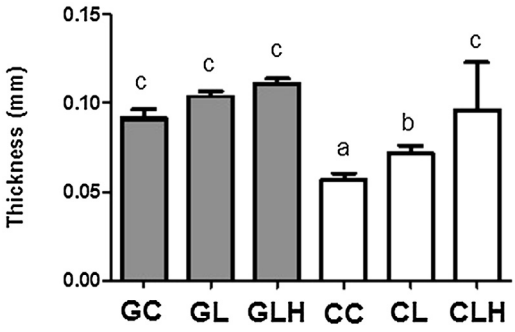

Fig. 3. Thickness of gelatin and casein films. Values represent means ± standard deviation of five measurements done in four different samples of each film. Treatments with different letters are significantly different ($P < 0.05$). GC, control gelatin films; GL, gelatin films with liposomes; GLH, gelatin films with liposomes and halloysite; CC, control casein films; CL, casein films with liposomes; CLH, casein films with liposomes and halloysite.

3.2.3. Morphological and structural properties

The thickness of the films ranged from 0.057 to 0.111 mm. The gelatin films are significantly thicker than casein films, excepting for casein film with liposomes and halloysite (Fig. 3). The addition of both halloysite and liposomes to casein films increases almost twice their thickness. The nature and concentration of the biopolymer and type of the additives incorporated (e.g. antimicrobial, glycerol, clay) are primarily factors influencing the film thickness [31]. The increased the film thickness observed by the incorporation of halloysite in casein films may be due to the amorphous characteristics of casein [32]. It is possible that the halloysite could not form an organized structure with the casein polymer in the course of drying, resulting less structured films.

The surface morphology of the films was evaluated by SEM and the images are depicted in Fig. 4. The casein and gelatin films showed a smooth appearance, although some fibrous structures could be observed in gelatin films (Fig. 4A and D). The morphological aspect of control films resemble those observed by Chambi and Grosso [33], although they obtained gelatin films with a more prevalent fibrous structure. The casein films with liposomes (Fig. 4B) have a dotted appearance, while the film with further addition of halloysite has a flatter bottom and it can be seen the nanoclay granules (Fig. 4C). The gelatin films added with liposomes (Fig. 4E) showed an irregular and rough appearance when compared to the control film and the films containing liposomes and halloysite were more wrinkled, presented fibrous structures, and cracks arise in the surface (Fig. 4F). Kadam et al. [13] also obtained gelatin and sodium caseinate films with smooth surfaces that become with a rough appearance after the incorporation of *Ascophyllum nodosum* extracts. It seems that the incorporation of additional substances into the biopolymer matrix results in films with a more irregular appearance. This fact could be associated to the phase separation of the additives in the polymeric matrix, which usually occurs during the film drying when the substance is not fully compatible with the polymer used.

3.2.4. Mechanical properties

The mechanical behavior of gelatin and casein films was quite different and strongly dependent on the film thickness. The addi-

Table 2
Mechanical properties of casein and gelatin films.

Film	Maximum strength (N)	Maximum pressure (MPa)	Elongation at break (%)	Tensile strength (MPa)	Young's modulus
GC	18.1 ± 2.5^{c}	7.2 ± 1.0^{c}	64.4 ± 12.2^{b}	8.6 ± 0.8^{c}	234.3 ± 38.0^{c}
GL	12.4 ± 0.9^{b}	4.9 ± 0.3^{b}	63.3 ± 7.8^{b}	4.9 ± 0.3^{b}	95.7 ± 1.7^{b}
GLH	21.5 ± 2.0^{c}	8.6 ± 0.8^{c}	22.8 ± 4.3^{a}	7.2 ± 1.0^{c}	146.1 ± 11.7^{b}
CC	1.6 ± 0.6^{a}	1.1 ± 0.4^{a}	132.0 ± 13.5^{c}	1.1 ± 0.5^{a}	4.3 ± 0.8^{a}
CL	1.5 ± 0.3^{a}	1.0 ± 0.2^{a}	143.7 ± 8.9^{c}	1.0 ± 0.2^{a}	5.3 ± 1.3^{a}
CLH	1.3 ± 0.6^{a}	1.1 ± 0.5^{a}	148.3 ± 24.1^{c}	1.1 ± 0.4^{a}	5.1 ± 2.5^{a}

GC, control gelatin films; GL, gelatin films with liposomes; GLH, gelatin films with liposomes and halloysite; CC, control casein films; CL, casein films with liposomes; CLH, casein films with liposomes and halloysite.
Values are means ± standard deviation of three independent experiments. Treatments with different letter in the same column are significantly different ($P < 0.05$).

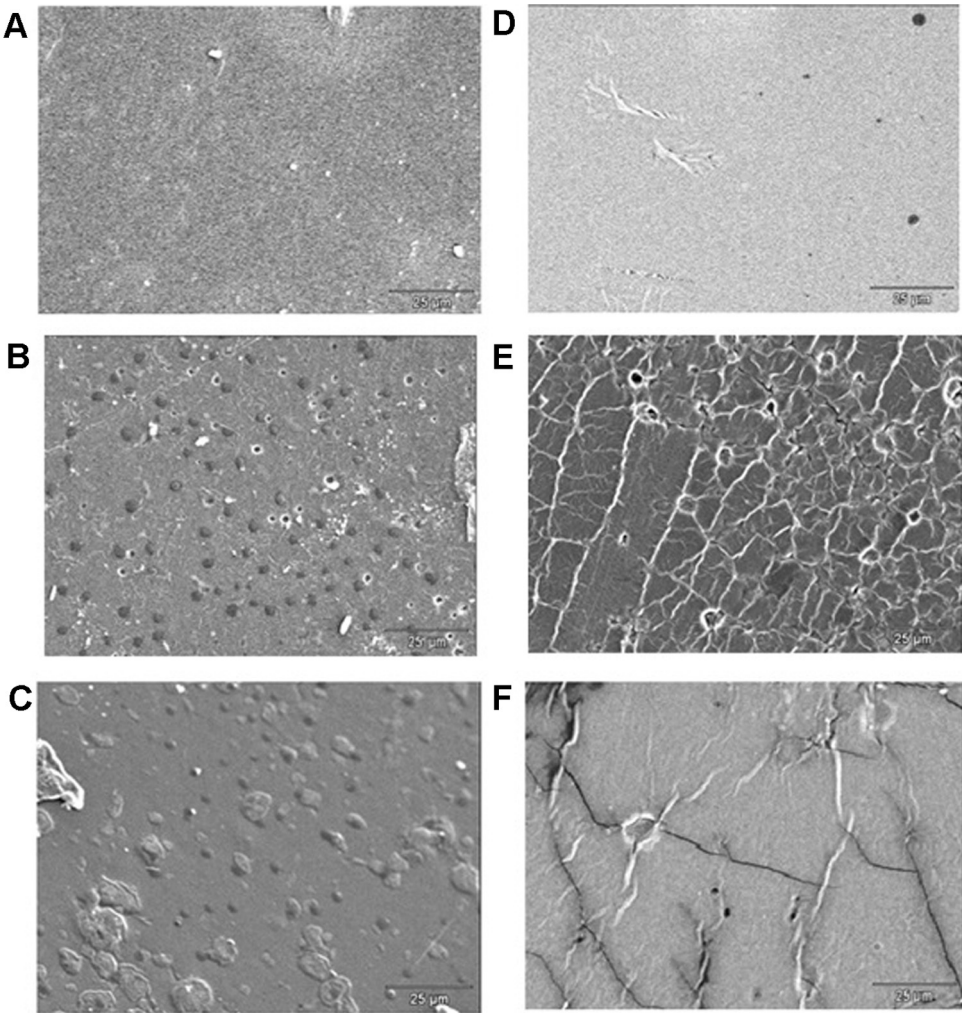

Fig. 4. Scanning electron microscopy images of casein and gelatin films. Samples were coated with a gold layer and then film surfaces were analyzed using a scanning electron microscope operated at a voltage of 10 kV. A, control casein film; B, casein film with liposomes; C, casein film with liposomes and halloysite; D, control gelatin film; B, gelatin film with liposomes; C, gelatin film with liposomes and halloysite. Bar = 25 μm.

tion of halloysite and liposomes did not alter the behavior of casein films in any of the evaluated parameters (Table 2).

The gelatin films supported a higher strength, but were much less elastic. The addition of liposomes in gelatin films resulted lower maximum strength and maximum pressure, but with halloysite these films have similar values to control films. In the case of casein films, no significant differences between formulations were observed. In relation to the elongation at break, addition of halloysite made gelatin films less elastic, but did not affect the casein films, which elongate at least twice more than gelatin films (Table 2). The values of Young's modulus of gelatin films were at least 20 times greater than casein films, indicating that gelatin films are much more rigid. In gelatin films, the addition of liposomes causes a sharp drop in this parameter.

A previous investigation on control and cross-linked gelatin and casein films also revealed that gelatin films present greater tensile strength than those produced from casein [33]. These results seem to be related to the organizational level of the protein network. Caseins and caseinates are generally classified as non-ordinate proteins (low level of α-helical or β-sheet structural conformations) [32]. Unlike the casein, gelatin can renature during the gelling and film forming process, reacquiring part of the triple helix structure of the collagen, a protein with a high degree of organization [34].

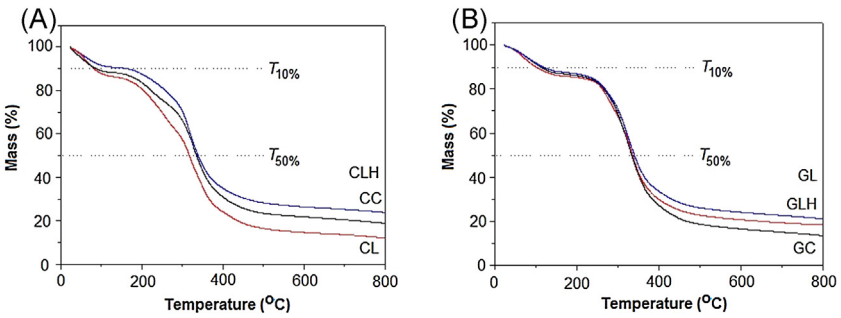

Fig. 5. TGA curves of casein (A) and gelatin (B) films. Samples were heated from 25 to 800 °C at the rate 20 °C min^{-1} under nitrogen atmosphere. The points indicated as $T_{10\%}$ and $T_{50\%}$ correspond to the initial decomposition temperature (10% degradation) and the temperature where 50% of sample was decomposed, respectively. The CC, control casein films; CL, casein films with liposomes; CLH, casein films with liposomes and halloysite; GC, control gelatin films; GL, gelatin films with liposomes; GLH, gelatin films with liposomes and halloysite.

Table 3
Thermal parameters obtained for gelatin and casein films.

Film	$T_{10\%}$ (°C)	$T_{50\%}$ (°C)	Tg (°C)
GC	114 ± 2 [c]	335 ± 5 [a]	62.4 ± 0.2 [a]
GL	102 ± 2 [b]	337 ± 7 [a]	60.3 ± 0.2 [b]
GLH	121 ± 2 [d]	342 ± 7 [a]	12.0 ± 0.2 [c]
CC	147 ± [e]	339 ± 7 [a]	–
CL	88 ± 2 [a]	317 ± 6 [b]	–
CLH	104 ± 2 [b]	334 ± 6 [a]	–

GC, control gelatin films; GL, gelatin films with liposomes; GLH, gelatin films with liposomes and halloysite; CC, control casein films; CL, casein films with liposomes; CLH, casein films with liposomes and halloysite.
Values are means ± standard deviation of three independent experiments. Treatments with different letter in the same column are significantly different ($P < 0.05$). (–) Not determined.

Thus, the films produced from gelatin are expected to have a more organized network compared to those made from casein.

Modification of mechanical properties are expected when additives are included in film formulation. Incorporation of *Zataraia multiflora* essential oil [35] or citric pectin [36] to casein films caused a significant decrease in Young's modulus. The values obtained by those authors, ranging from 0.31 to 2.0, were considerably lower as compared to the values observed in this work. The addition of nanocelullose as reinforcer decreased the tensile strength of gelatin films [37], while the addition of MTT to gelatin films increased tensile strength but leads to more rigid films, which results in the decrease of elongation at break parameter [38]. An increase in tensile strength and a decreased elongation at break in films of fish skin gelatin with ZnO nanoparticles was also described [39]. In this work, similar decrease in elongation at break was observed by halloysite addition to gelatin films, but not to casein films (Table 2). For packaging application, high values for elongation can be an advantage.

3.2.5. Thermal properties

Thermogravimetric analysis (TGA) was performed to determine the thermal behavior of the samples and the influence of halloysite and liposomes and in thermal stability of the films. Fig. 5 shows TGA curves for films prepared with gelatin and casein. The samples of gelatin films suffer a small loss of mass at the beginning of heating, indicating the presence of water (Fig. 5A). Gelatin begins its degradation/decomposition at around 200 °C and ends around 600 °C, with nearly 80% mass loss. The samples of casein films show a very similar behavior to films with gelatin since they suffer a small loss of mass at the beginning of heating, indicating the presence of water, but casein (major component) begins its degradation/decomposition at around 140 °C (Fig. 5B).

Table 3 shows the decomposition temperature parameters $T_{10\%}$ and $T_{50\%}$, corresponding to initial decomposition temperature and a maximum degradation temperature, respectively. It can be observed that the films showed similar decomposition temperatures, indicating that the addition of liposome and halloysite had little influence on thermal stability. Other works reported higher thermal stability of starch films after addition of halloysite, but such differences can be explained by the smaller amount used in our films [16,40].

The DSC thermograms of gelatin and casein films did not show definite peaks, similar to that described for amorphous coacervates of gelatin and carboxymethyl sago pulp, a semisynthetic polymer from sago palm that typically contains carboxymethyl hemicellulose and carboxymethyl alpha cellulose [41]. Control gelatin films presented a flat transition around 62 °C (data not shown), which was assigned to the glass transition temperature (*Tg*). In general, this behavior was similar to that reported for pure gelatin or films based on gelatin, because of cryogenic cooling after the first scan, the microcrystalline interactions of the macromolecules did not have enough time to occur resulting in a completely amorphous material [42]. The addition of liposomes and both liposomes and halloysite decrease *Tg* (Table 3), indicating that the material becomes more brittle. According to Broumand et al. [35], the presence of lipid material can produce a relatively weak thermoplastic material. The effect of clay on *Tg* of a polymer is quite complex, and both increased and decreased *Tg* values have been reported [43,44]. Addition of nanoclay to wheat gluten caused the *Tg* transition to start at lower temperatures for the matrix, although it only enlarged the peaks but did not shift the maximum of these peaks significantly [45]. Multiple components such as proteins, lipids and plasticizers co-exist in the films and their interactions with the nanoclay would be different. Thus, it seems that the presence of complicated phase structures in combination with their different interactions with clay nanoparticles influence the behavior of the resulting films.

3.2.6. Optical properties

Color is an important parameter for both edible and non-edible films, since it is related to product acceptance by consumers. In general, films intended for food wrapping must be as neutral as possible to reveal the authentic food color. The films produced in this work were all clear and transparent. The CIELAB parameters obtained for the casein and gelatin films are listed in Table S1 (see Supplementary material Table. S1 in the online version at DOI: 10.1016/j.colsurfb.2016.05.080). The casein films with liposomes (CL) and with liposomes and halloysite (CLH) were more yellowish than the other films, as evidenced by higher values of b* parameter. Significant differences in the total color difference (ΔE) were also observed among casein films. On contrary, addition of liposomes and halloysite did not affect the color parameters of gelatin films. Likewise, the ΔE parameter of gelatin-based films was not affected

by the concentration and type of plasticizer [42]. The whiteness index (WI) was not modified with addition of liposomes and halloysite (Table S1). Overall, the results of color parameters indicate that gelatin and casein films are compatible for food packaging applications.

4. Conclusions

The phosphatidylcholine liposomes loaded with 25 μg/ml nisin produced in this study showed excellent physicochemical characteristics as demonstrated by high encapsulation efficiency and stability during storage. The addition of such liposomes to protein-based films revealed a great potential for antimicrobial food packaging to reduce post-process growth of food pathogens. The barrier properties of halloysite seems interesting for controlled release of nisin, although improvement of the mechanical properties of the films was not observed. Further studies should be conducted to test film formulations in real food systems.

Conflicts of interest

None to declare.

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Manuscrito 2 - Acute toxicity evaluation of phosphatidylcholine liposomes containing nisin in the nematode *Caenorhabditis elegans*

Abstract: Liposomes are among the most studied nanostructures, being effective carriers of active substances, both in the clinical area, delivering genes and drugs, as in the food industry, promoting the controlled release of several substances, including food preservatives. Nisin is one of the most used biopreservative worldwide and its encapsulation can overcome problems related to the direct application in a food matrix. However, to ensure consumer's safety, toxicological screenings must be performed. In this study, we used the nematode *Caenorhabditis elegans* as an alternative model to investigate the potential in vivo toxicity. We evaluated the toxic effects of liposomes containing nisin, drug-unloaded liposomes (control liposomes) and free nisin through classical toxicity assays in this nematode, such as letal dose, development of worms and reactive oxygen species (ROS) levels. Due to its low toxicity, it was not possible to experimentally determine the LD_{50} of both liposomes. The survival rate for control liposomes and nisin-loaded liposomes was 94.3 and 73.6%, respectively. The LD_{50} found to free nisin was 0.239 mg mL^{-1}. Highest concentrations of free nisin significantly affected the development of worms, which were 25% smaller than control and liposomes samples. The exposure to liposomes and to free nisin caused a significant increase in reactive oxygen species (ROS). There was a significant increase in CAT levels at highest concentration of free nisin and liposomes containing nisin. Only free nisin induced an increase in SOD levels. The treatments did not induce lipid peroxidation. Liposomes can be considered a non-toxic delivery system for nisin in food applications.

Keywords: nanoencapsulation, nanotoxicology, acute exposure, nisin, *C. elegans.*

1. INTRODUCTION

In this century, nanotechnology has emerged as a new and promising science, revolutionizing the production process of many materials, from sports equipments to aircraft, through the electronics, pharmaceutical and food industries (Delgado, 2010). However, materials on nanoscale have different properties compared with the same material on bulk state and it is not possible to predict its biological activity by

extrapolating the physical and chemical properties of the components which constitute them (Dhawan *et al*, 2011).

Nanostructures – biodegradable materials such as natural and synthetic polymers, lipids and metallic particles – hold enormous potential as effective drug delivery systems (Brandelli, 2012). Liposomes are one of these nanostructures, being among the most applied organic nanoparticles, since they can be produced using natural ingredients on an industrial scale and can encapsulate compounds with different solubilities. Liposomes can be defined as colloidal structures formed by absorption of energy for a suitable combination of constituent molecules in an aqueous solution (Mozafari *et al*, 2008). When amphiphilic molecules such as phospholipids, are placed in an aqueous environment, they form complex aggregates to protect their hydrophobic sections from the water molecules, maintaining contact with the aqueous phase by hydrophilic groups. If sufficient energy is provided to the phospholipid aggregates, they can arrange themselves in an orderly manner in closed vesicular bilayer (Jesorka and Orwar, 2008). Encapsulation in nanovesicles promotes an improvement in properties of bioactive compounds because it protects the compounds from degradation and interactions with undesirable compounds, increases their apparent solubility, stability and efficiency (Sozer and Kokini, 2009). In food applications, liposomes can be used to disperse hydrophobic β-carotene in beverages, to entrap enzymes for cheese production, delivery of food preservatives, and to fortify products with vitamins to increase their nutritional quality (Peters *et al*., 2011).

Many studies have used food antimicrobials incorporated in liposomes (Malheiros *et al*, 2010; Imran *et al*, 2012, Silva *et al*., 2014) but there is still little information about the toxicity of nanoparticles for food application. These nanomaterials present in food are ingested and pass through the digestive system. Those who are incorporated into the food package may also be accidentally freed and end up in the gastrointestinal tract. Since a material can be benefic or deleterious to an organism, it is important to know its behavior in biological environment and its mechanism of action and to make a risk assessment of nanomaterials to the consumer (Alves et al, 2014).

Besides the effectiveness, a new food or food component must be safe to be approved before industrialization, and to ensure the safety of engineered nanomaterials to human health and the environment is one of the the greatest challenges in the nanotoxicological area (Brandelli et al, 2016) The great number of new nanomaterials requires the use of in vivo models to provide the toxicological screening. However, the

traditional models used in scientific researches, specially mammals, has been the subject of many discussions because of the number of required individuals and suffering caused during some kind of experiments (Andrade *et al*, 2002). Added to this, there is the high cost of maintaining these laboratory animals. In this context, methods or alternative models emerge, representing an effort by the world scientific community to reassess the use of mammalian experiments, through a program known as the 3Rs ("Reduction, Refinement, Replacement"), which aims to reduce the number of animals, making they suffer as little as possible and that new methods can be developed to replace animal experimentation (Cazarin, Correa, Zambrone, 2004).

In this study, we employed the free-living nematode *Caenorhabditis elegans*, which has been shown to be a useful model for testing effects of many kinds of chemicals, including metals (Höss *et al*. 2003; Wang and Xing 2008), persistent organic pollutants (Sochova *et al*. 2007), pesticides (Cole *et al*. 2004), and nanomaterials (Roh *et al.* 2010; Avila *et al.*, 2012; Charão *et al* 2015,). *C elegans* is a small and transparent worm that lives mainly in the liquid phase of soil and feeds on soil microorganisms. These animals have short life-cycle and easy and inexpensive maintenance in laboratory (Peterson *et al*, 2008)

C. elegans was the first multicellular organism to have its genome completely sequenced and it is one of the best characterized animals at the genetic, physiological, molecular, and developmental levels (Zhang *et al*, 2013). Besides, the worm genome shows about 70% of homology with humans (Brenner, 1994). Currently there are knockout strains available for genes of interest and of transgenic worms expressing green fluorescent protein (GFP) tagged proteins make it an ideal model for expression or protein localization studies (Helmcke *et al.*, 2010)

The objective of this study was to evaluate the acute toxicity of phosphatidylcholine liposomes encapsulating the antimicrobial peptide nisin, used for food conservation, using C. elegans as an alternative in vivo model. Additionally, the potential involvement of oxidative stress mechanisms was investigated.

2. MATERIAL AND METHODS

2.1 Materials

Phospholipon®90G (pure phosphatidylcholine stabilized with 0.1% ascorbylpalmitate) was supplied by Lipoid GMBH (Ludwigshafen, Germany). Commercial nisin (Nisaplin®) was purchased from Danisco Brasil Ltda. According to the manufacturer, the formulation contains NaCl (77%), denatured milk solids as fillers, and 2.5% pure nisin. Nisin was dissolved in 0.1M HCl and diluted with phosphate buffer pH 7. Bacto-agar and bacto-peptona were obtained from Becton Dickinson BD® (New Jersey, USA) and HiMedia Laboratories® (Mumbai, India), respectively. 1,1,3,3-Tetramethoxypropane 2′,7′-dichlorofluorescein diacetate (DCF-DA) were supplied by Sigma-Aldrich (St. Louis, MO, USA). Phosphoric acid and 2-thiobarbituric acid were purchased from Tedia Co (Fairfield, OH, USA) and Sprectrum Chemical Co (Gardena, CA, USA).

2.2 Liposome preparation and characterization

Liposomes were produced by film-hydration method, according to Malheiros *et al* (2010) and its full characterization is described in Boelter and Brandelli, 2016. Briefly, 76 mg of Phospholipon®90G was dissolved in 15ml of chloroform in a round round-bottom flask and the organic solvent was removed using a rotary evaporator until a thin film was formed on the walls. After 24h in a dissecator, 5ml of a warm 1mg mL^{-1} nisin solution was added to disperse the resulting dried lipid film. These mixture were homogenized at 60°C and sonicated in an ultrasonic cell disrupter (Unique, Brazil) by five cycles of 1 min, at intervals of 1 min during which the samples were kept in ice. Then, the solution was filtered through 0.22 µm membranes. The drug-unloaded liposomes are here denominated as control liposomes and the volumes used in all tests were the same of nisin-loaded liposomes.

The unloaded liposomes had 124.5 ± 6.2 nm, polydispersity index (PDI) 0.196 ± 0.08, zeta potential 2.96 ± 13.3 mV. Liposomes loaded with nisin had 162.3 ± 26.8 nm, PDI 0.264 ± 0.03 and zeta potential -5.95 ± 1.98 mV.

2.3 Strains, culture and synchronization of C. elegans

The *C. elegans* strains N2 (wild type), CF1553 (muls84), GA800 (wuls151), were provided by the *Caenorhabditis* Genetics Center (University of Minnesota, Twin Cities, MN, USA), maintained and handled at 20ºC on *Escherichia coli* OP50 in NGM (nematode growth medium) plates. Synchronous L1 population was obtained by isolating embryos from gravid hermaphrodites using bleaching solution (1% NaOCl; 0.25M NaOH), followed by floatation on a sucrose gradient to segregate eggs from dissolved worms and bacterial debris, accordingly to standard procedures described by Brenner (1974). Eggs were washed with M9 buffer (0.02 M KH_2PO_4, 0.04 M Na_2HPO_4, 0.08 M NaCl, and 0.001 M $MgSO_4$) and allowed to hatch in unseeded NGM overnight.

2.4 Exposure to liposomes with nisin, control liposomes and free nisin

Synchronized L1 worms were immersed in 85 mM NaCl solution and exposed for 30 min, to liposomes containing nisin, control liposomes and free nisin. The control samples contained only the worms and NaCl solution. The concentrations were expressed as mg mL^{-1} of pure nisin. After exposure, worms were washed 3 times with saline solution (85 mM).

2.5 LD_{50} determination

To determine the LD_{50} of liposomes and free nisin, 2500 worms per dose were exposed and washed, according to item 2.3, and then were placed on OP50-seeded NGM plates. The number of surviving worms on each plate was counted 24 hours after exposure. All of the tested doses were compared to the control group, which did not receive the treatment. Three replicates were performed. The curves of dose-response were drawn according to a sigmoidal model with a top constraint at 100%.

2.6 Development of worms

For the evaluation of development, the surface of 20 adult worms per treatment were measured. For this, the NGM plates were washed with distilled water and the nematodes were transferred to a centrifuge tubes, being washed successively until the

solution was clear. After this procedure, 15µl of the solution with the worms were mounted on 2% agarose pads with 15µl of levamisole (2.25% w/v) to anaestheticization. Pictures were taken and the flat surface area of nematodes were measured using the AxioVision software LE (version 4.8.2.0 for windows). Results were expressed as percentage of body area relative to control.

2.7 Measurement of reactive oxygen species (ROS) generation

After the exposure, 1500 worms were maintained in 100 µL of saline buffer and transferred to a 96-well plate; 2'7' dichlorofluoresceindiacetate (DCF-DA) was added at a final concentration of 0.05 mM and the fluorescence levels were measured, excitation: 485 nm; emission: 535 nm using a microplate reader (Spectramax Me2; Molecular DevicesLLC, Sunnyvale, CA, USA) heated at 20°C. The fluorescence from each well was measured for 90 min at 10 min intervals. Results were expressed as percentage of fluorescence intensity relative to control wells.

2.8 Fluorescence quantification

The GFP expressing strains (CF1553 [muls84] for superoxide dismutase - SOD and GA800 [wuls154] for catalase - CAT) were submitted to the acute exposure as described above. Worms (1500 per dose) were maintained in 100 µL of saline buffer and transferred to a 96-well plate, where total GFP fluorescence was measured using 485 nm excitation and 530 nm emission filters using a microplate reader (Spectramax Me2; Molecular DevicesLLC, Sunnyvale, CA, USA) heated at 20°C. The fluorescence from each well was measured for 10 min at 1 min intervals. Results were expressed as the mean percentage of fluorescence intensity relative to control wells.

2.9 Fluorescence microscopy

For each concentration, a slide was taken and 20 treated worms were mounted on 2% agarose pads and anaesthetized with 15µl of levamisole (2.25 % w/v). Fluorescence observations were performed for image acquisitions in fluorescence microscopy (Olympus,IX-51).

2.8 Lipid peroxidation

Thiobarbituric acid reactive substances (TBARS) were determined in adult worms (2500 per dose), 48h after exposure, as a marker of lipid peroxidation by the TBA (thiobarbituric acid) assay using a 1,1,3,3-tetramethoxypropane solution as malondialdehyde (MDA) standard. After 48 hours of exposure, the plates containing the worms are washed to remove the OP50. The nematodes were disrupted in turrax homogenizer at full amplitude for about 60 seconds, in order to release the lipid and protein content. Then the content was centrifuged at 10.000 rpm for 5 min. The supernatant was transferred to cryotubes where happen the reaction of TBARS, with the addition of 0.1M phosphoric acid solution, 20mM sodium dodecyl sulfate solution and 40mM 2-thiobarbituric acid solution. The reaction took place in a water bath for 1 hour and 30 minutes under agitation at 100 ° C. Additionally, the samples were transferred to 96 well plates and its absorbance is read at 532 nm (Spectramax Me2; Molecular DevicesLLC, Sunnyvale, CA, USA).The protein content of the samples was determined according to Bradford (1976).

2.10 Statistical Analysis

The results were subjected to variance analysis (ANOVA) and means were compared through the Tukey test at a level of 5 % of significance. The results obtained in items 2.4, 2.5 and 2.6 were submitted to Spearman's correlation, at a level of 5 % of significance. All tests were performed using the Prism 5.0 software (GraphPad Software Inc., La Jolla, United States).

3. RESULTS

3.1 Dose-response curves for liposomes and free nisin

Figure 1 shows the survival of worms in a sigmoid dose–response curve in log scale. The LD_{50} found to free nisin was 0.239 mg mL^{-1}.The determination of LD_{50} for liposomes loaded with nisin and control lipossomes was not possible experimentally. The survival rates of these liposomes were 73.6 and 94.3%, respectively (Figure 2).

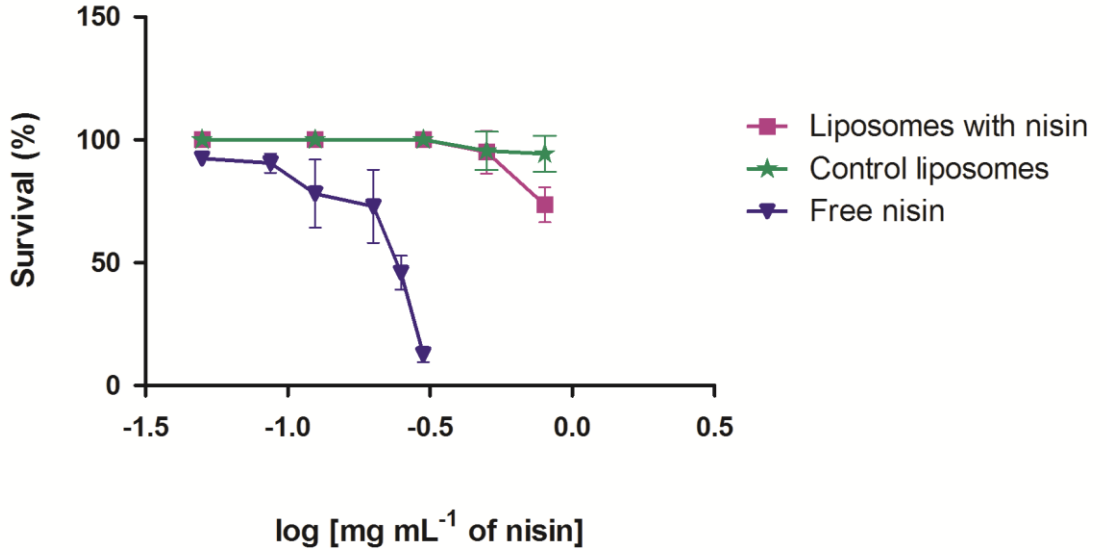

Figure 1. Dose–response curves for acute exposure of *C. elegans* to liposomes containing nisin, control liposomes and free nisin. Control liposomes do not contain nisin, but were added in the same quantities of liposomes with nisin. Data are expressed as mean ± SEM.

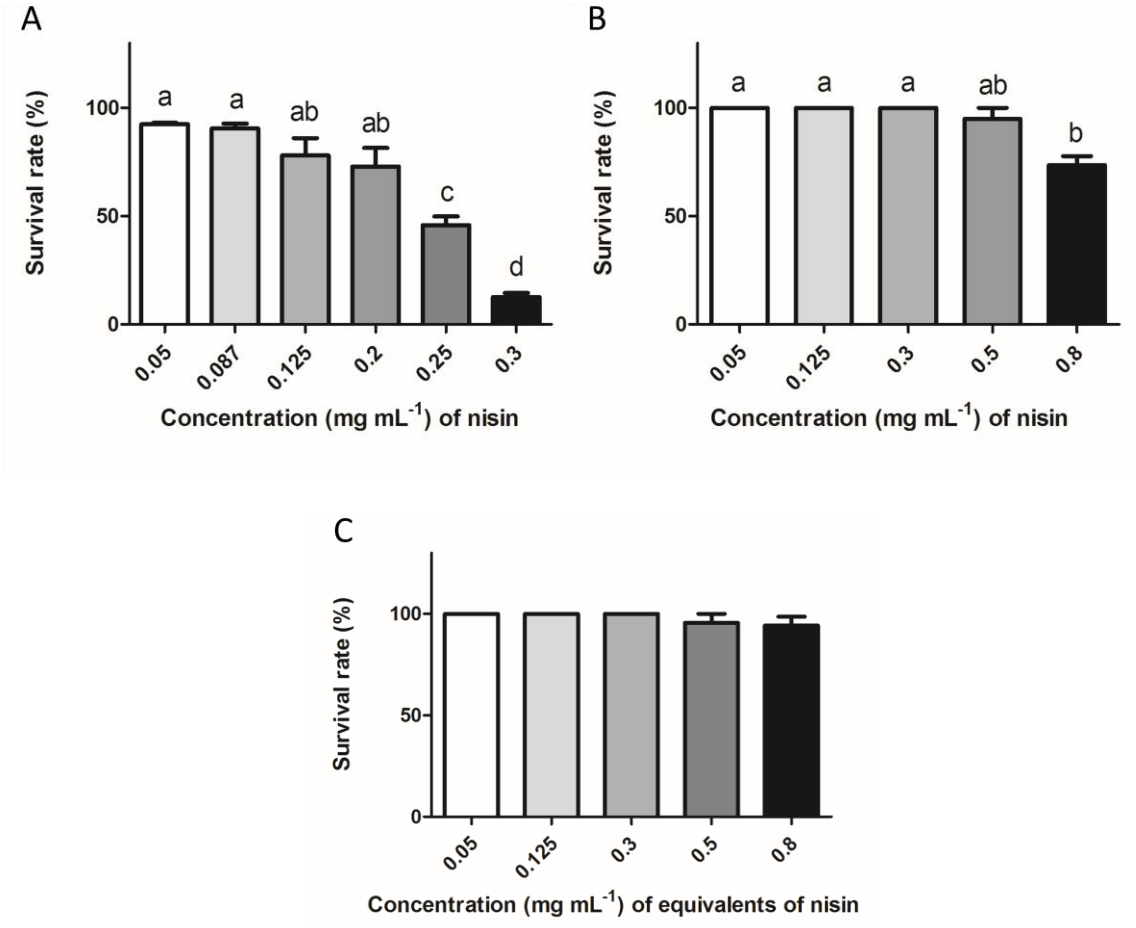

Figure 2. Survival rate of *C. elegans* after acute exposure to (A) free nisin; (B) liposomes containing nisin (1mg mL^{-1}) and (C) control liposomes. Control liposomes do not contain nisin, but were added in the same quantities (same volume) of liposomes with nisin. Data are expressed as mean ± SEM. Treatments with different letters are significantly different (P<0.05).

3.2 Development of nematodes

In the lower concentrations of liposomes (LN 0.1 mg mL^{-1} and LC 0.1 mg mL^{-1}), the body area of nematodes was significantly higher than the control. The remaining concentrations of liposomes were not significantly different from the control, as well as the lowest concentration of free nisin. However, the higher concentrations of free nisin led to a decrease of approximately 25% of body area (Figure 3), showing that the normal development of *C. elegans* was affected by nisin exposure.

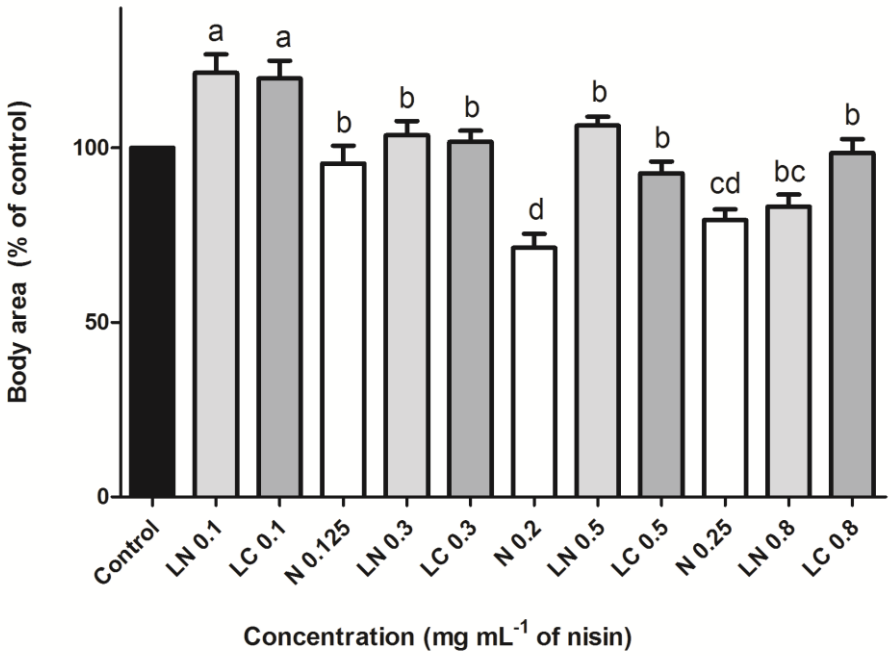

Figure 3. Body area of adult namatodes. The mean size in control group was considered as 100%. Data are expressed as mean ± SEM. Treatments with different letters are significantly different (P<0.05). LN – liposomes containing nisin; LC – control liposomes; N – free nisin. Control liposomes do not contain nisin, but were added in the same quantities (same volume) of liposomes with nisin.

3.3 ROS levels

ROS levels were determined with the dichlorofluoresceindiacetate (DCF-DA) dye, which undergoes de-esterification to dichlorofluorescein (DCFH) which is then oxidized to the DCF fluorophore by free radicals. The exposure to liposomes and to free nisin caused a significant increase in DCF-DA oxidation, reflecting in the generation of ROS (Figure 3). For all samples, the maximum concentration generated approximately four times more ROS than the control. Intermediate concentrations of control liposomes generated higher ROS levels than liposomes containing nisin. Only the lowest concentration of free nisin generated less ROS than the control.

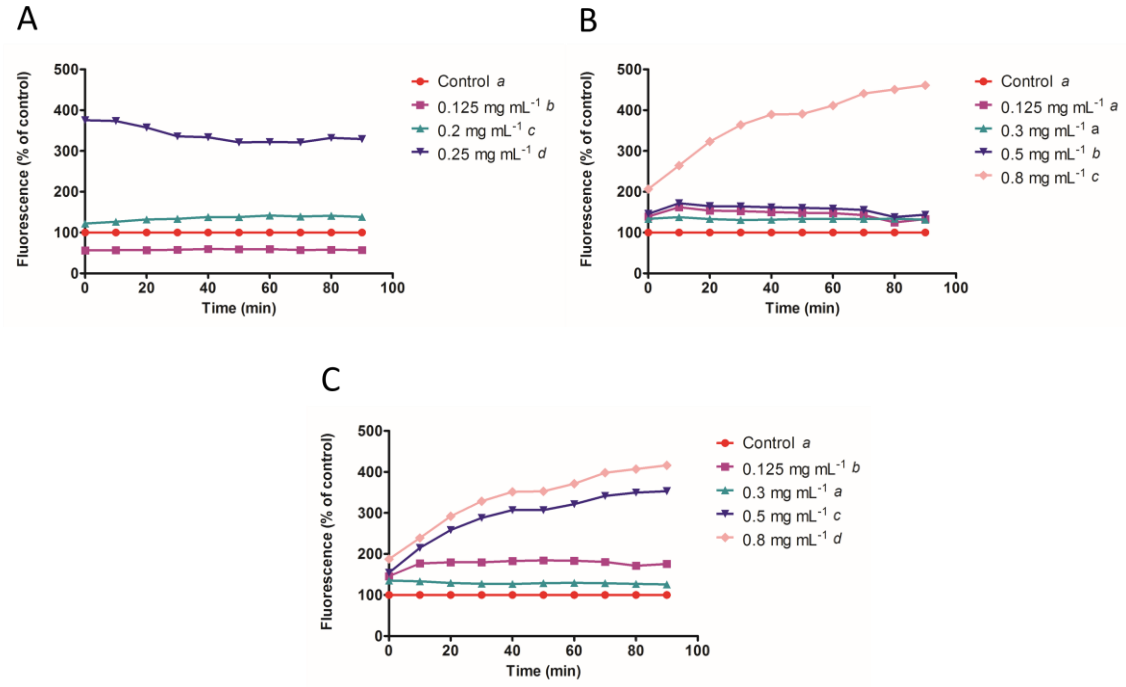

Figure 3. ROS levels measure by DCF-DA dye in worms acutely exposed to: (A) Free nisin; (B) Liposomes containing nisin and C) Control liposomes. Data are expressed as means. Treatments with different letters are significantly different (P<0.05). Control liposomes do not contain nisin, but were added in the same quantities (same volume) of liposomes with nisin.

3.4 Fluoresce quantification

A statistically significant increase in CAT levels (GA800) was observed following treatment with liposomes containing nisin and in free nisin samples (Fig. 4A), suggesting increased levels of antioxidant enzymes. However, only free nisin, at the lower concentration, induced an increase in SOD levels (Fig 4B).

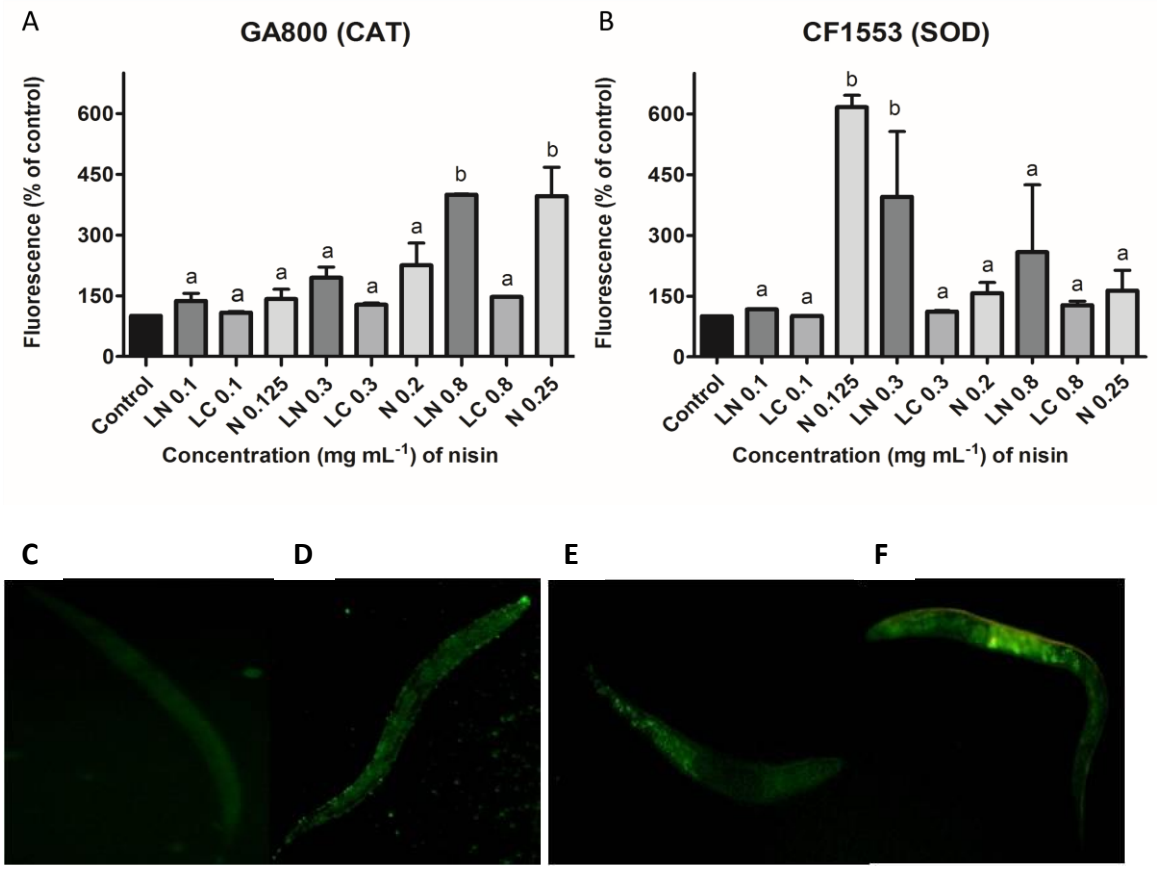

Figure 4. Quantification of fluorescence of (A) GA800 (CAT) and (B) CF1553 (SOD) strains after acute exposure to liposomes with nisin (LN), control liposomes (LC) and free nisin (N). The photographs are representative of the control animals (C and E, CAT and SOD, respectively) and of the sample with higher fluorescence (D – N 0.25 mg mL^{-1} and F – N 0.125 mg mL^{-1}). Control liposomes do not contain nisin, but were added in the same quantities (same volume) of liposomes with nisin.

3.5 Lipid peroxidation

Thiobarbituric acid reactive substances (TBARS) are formed as byproducts of the lipid peroxidation and are a signal of oxidative damage. The effect of nisin-loaded

liposomes and free nisin on generation of TBARS was evaluated. There was no significant difference between treatments compared to the control (Fig 5).

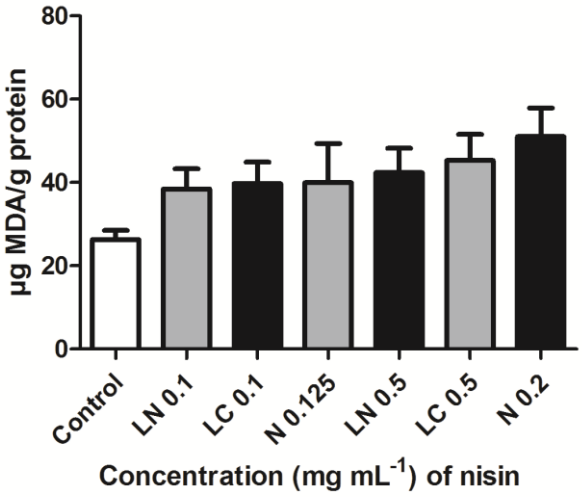

Figure 5. Quantification of thiobarbituric acid reactive substances in adult nematodes after acute exposure to liposomes containing nisin (LN), control liposomes (LC) and free nisin (N). Control liposomes do not contain nisin, but were added in the same quantities (same volume) of liposomes with nisin.

3.6 Correlation of data

The main data of this study were tested by Spearman correlation and the significant ones are listed in table 1. The test revealed a positive significant correlation between death rate (obtained from the survival experiment) and the mean quantity of reactive oxygen species in all treatments.

In liposomes containing nisin, the death rate was inversely correlated to size of worms.

Table 1. Spearman correlation test of death rate, reactive oxygen species (ROS) and size of animals exposed to free nisin, liposomes containing nisin and control liposomes.

Treatment	Correlation	r	P value
Free nisin	Death rate x ROS	0.7667	0.0214
Liposomes containing nisin	Death rate x ROS	0.7486	0.0255
	Death rate x size	- 0. 8398	0.0061
Control liposomes	Death rate x ROS	0.7886	0.0172

DISCUSSION

In recent years, liposomes have been gaining importance in medical and food areas, due to the countless possibilities of lipid combinations, substances to be encapsulated and preparation methods. However, studies indicate that MTT cell viability assay, one of the most used tests for cytotoxicity, is not compatible with the lipid nature of liposomes, leading to wrong conclusions (Angius and Floris, 2015). *In vivo* assays are very relevant since they offer information about integrated biological effects of nanoparticles. Thus, in accordance with the 3Rs policy *C. elegans* represents an alternative method for toxicological studies of liposomes. According to Charão *et al* (2015), results obtained in *C. elegans* model can be crucial to establish new approaches in nanotoxicology and to predict their effects in complex animal models. In the best of our knowledge, this is the first time that liposome toxicity is described in *C. elegans.*

Nisin is used as food preservative in more than fifty countries and in the last years has been studied not only by the food industry, but also in the medical field, for controlling common oral diseases, as caries, and even as antitumor drug (Joo *et al*, 2012; Tong, Ni, Ling, 2014). Some studies point out that nisin has no chronic or subchronic toxic effect, reproductive toxicity, genotoxicity, carcinogenic or teratogenic effects (Aranha, Gupta, Reddy, 2008; Hagiwara *et al.,* 2010). However, there are studies that report a certain degree of toxicity or adverse effects (Hagiwara *et al.*, 2010; Vaucher, Teixeira, Brandelli, 2012) in high concentrations of nisin. In the present study, no significant toxicity was observed for phosphatidylcholine liposomes loaded with nisin or control liposomes, since the highest concentrations tested killed 26.4 and 5.7% of worm population, respectively. According to Paranjpe *et al* (2014), phosphatidylcholine liposomes have been considered safe for several uses. In C elegans, Shibamura et al (2009) found no adverse effects in the life span during exposition to liposomes and Miyako et al (2015), with hybrid liposomes with carbon nanotubes observed that this nanohybrids did not affect the fertility of the worms.

We can notice that encapsulation protects against possible deleterious effects of nisin, that in its free form, in the concentration of 0.25 mg mL^{-1} killed 54% of exposed nematodes, while 100% of worms exposed to the same concentration of encapsulated nisin survived. According to Chen *et al.* (2012), the nanoencapsulation decreases the extent and the types of nonspecific toxicities and allows the increase in the amount of drug that can be effectively delivered.

The growth in *C. elegans* is determinated by a conservative genetic pathway and is considered a good parameter to evaluate toxic effects in this model (Wu *et al*, 2013). The lowest concentrations of liposomes promoted the enlargement of worms. Some authors say that subjecting *C. elegans* to mild degrees of stress can induce thermotolerance, longer lifespan, greater resistance to oxidative stress in a process known as hormesis (Cypser and Johnson, 2002; Wu *et al*, 2009 ; Zhou *et al*, 2011). We suggest that this increase of worm size may be due to hormesis. The higher concentrations of free nisin affected the development of worms, that were about 25% smaller than control samples. This same rate of size reduction was observed by Jung *et al* (2015) in *C. elegans* exposed to 50 ppm of nanosilver particles, fumed SiO_2 and multiwalled carbon nanotubes. In worms exposed to liposomes containing nisin, as the death rate increased, decreased the size of the worms. These results indicates that encapsulation protects against toxic effects of nisin.

Oxidative stress occurs when there is an imbalance between the generation of reactive oxygen species (ROS) and the antioxidant defenses of the organism. The oxidative stress state is involved in the toxicity mechanism of many compounds, since it that may cause damage to proteins, lipids and DNA (Sies, 1997). Here, we observed an increase of ROS production for liposomes and free nisin, reaching a four-fold amount compared to control samples. However, this value was reached in 0.25 mg mL^{-1} of free nisin and in 0.8 mg mL^{-1} of nanoencaptulad nisin, showing that the encapsulation reduces the toxicity effects of this substance. Spearman's correlation test showed a positive correlation between death rate and ROS levels in all treatments. Other factors may be contributing to the high death rate in free nisin. It is possible that the death in free nisin can be caused in part by osmotic stress, since Nisaplin® has a high concentration of NaCl and previous tests caused the death of 80% of worm population exposed to 0.9% saline solution (*data not shown)*. The increase of ROS levels in the higher concentrations of control liposomes suggest that the nanocapsule itself can induce oxidative stress due to the high surface area of nanoparticles, which lead to an increase of chemical reactivity of these materials, resulting in higher specific interactions with biological fluids and structures, increasing oxidation (Oberdörster *et al*, 2005; Risom *et al*, 2005; Manke *et al*, 2013). However, it is important to stress that in nanotoxicology high doses are not usual, since the nanostructures are more efficient than the drug in its free form.

Significant higher level of CAT was only detected at highest concentration of liposomes containing nisin and free nisin, where death rates are higher. However,

increase in SOD level was only detected at lowest concentration of free nisin. Increased antioxidant enzymes may be a compensatory effect to combat the increase in oxidative stress (Andrade et al, 2010; Xu et al, 2016). Control liposomes did not induced an increase of CAT and SOD levels. Halliwell and Gutteridge (1999) report that oxidative stress not always involves increased antioxidant defenses and cell death.

There was no significant increase in TBARS in all treatments, showing that there was no significant increase in lipid peroxidation in worms. This result, combined with the increased levels of CAT and SOD, demonstrate that *C. elegans* antioxidant mechanisms are combating the reactive oxygen species before free radicals can damage the membranes. According to Gems and Doonan (1998), *C elegans* has a wide range of genes and processes that contribute to protection against oxidative damage. For exemple, C elegans has more isoforms of catalases and SODs than higher animals.

The present study showed that liposomes represent a non-toxic delivery system for nisin, validating it as a good alternative to overcome the problems associated with the direct application of this antimicrobial peptide in food, keeping their efficacy and stability in a safe form.

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Manuscrito 3 - Toxicity of nanoclays with adsorbed nisin on *Caenorhabditis elegans*

Abstract: Halloysite and montmorillonite are nanoclays which have a wide range of industrial and medical applications, including food packaging. The incorporation of these clays into nanocomposites must be safe for consumers and to ensure this, toxicological screenings must be performed. The free-living nematode *Caenorhabditis elegans* is an alternative *in vivo* model to assess the toxic effects of several compounds, especially nanomaterials. In this study we evaluate the acute toxicity of three nanoclays: halloysite (HNT), unmodified montmorillonite (bentonite) and montmorillonite modified with octadecylamine (MMT-octa), in two situations: pure and with adsorbed nisin. Nisin is an important antimicrobial peptide, used as preservative in food. Lethal dose 50% (LD_{50}), growth, ROS production, quantification of CAT (catalase) and SOD (superoxide dismutase) enzymes and lipid peroxidation were the toxicological endpoints evaluated in *C elegans*. Halloysite and montmorillonite modified with octadecylamine had the lowest and highest LD_{50}, respectively. The adsoption of nisin in halloysite and bentonite provoked the decrease in surviving worms. All treatments altered the normal growth, in different degrees, being worms exposed to bentonite the most affected. ROS production increased in all treatments, especially MMT-octa, where CAT and SOD levels were not enhanced, suggesting high oxidative stress.

Keywords: acute exposure, nisin, halloysite, montmorillonite, *C. elegans*

1. Introduction

Clay minerals are one of the most promising materials of the century, since they are naturally occurring, available in thousands of tons, inexpensive, ecofriendly and have multifarious applications (Majeed et al., 2013; Maisanaba et al., 2015b), ranging from ceramics to traditional Chinese medicine, passing through agricultural applications, engineering, environmental remediation, till biomedical devices for drug and gene delivery (Murray, 2007, Ahmed et al., 2015) Currently, the use of several clays in the food industry is a reality for improving food packaging in mechanical and barrier aspects, once create an increased tortuosity of the diffusive path for a penetrant molecule, forcing

them to travel a longer way to diffuse through the matrix (Maisanaba et al. 2015a). Furthermore, they can be used as carriers of various substances, including antimicrobials in bioactive packaging (Meira et al., 2015)

Halloysite and montmorillonite are among the most frequently used solid fillers for obtaining novel polymer composites, named nanocomposites. Halloysite is a member of the kaolin group, a 1:1 two-layered aluminum hydrated phyllosilicate, presenting the shape of a predominantly hollow tube, with an outer diameter of 40–70 nm, an inner diameter of 10–20 nm and a length of 500–1500 nm, where the internal side is composed of Al_2O_3 while the external is SiO_2, which allows the selective chemical modification of these outer/inner surfaces (Rawtani and Agrawal, 2012; Abdullayev et al., 2012; Lvov and Abdullayev, 2013). Halloysite nanotubes (HNTs) are able to entrap a range of molecules with specific sizes and can serve as a viable nano-cage for active molecules, owing to the empty space inside the nanotubes. As a result, HNTs have been found to be applicable for fabrication of novel materials with controlled release, as drugs and enzymes, carriers, gene delivery vehicles, antibacterial coatings, nanostructured coatings for improved adhesion of human cells and scaffolds for tissue engineering (Fakhrullina et al., 2015). HNTs can also be incorporated into polymers to modify their functional properties (Meira et al. 2014; Unalan et al. 2014).

Montmorillonite (MMT) is a member of the smectite group, belonging to the structural family of the 2:1 phyllosilicates (Azeredo, 2013). Its layers are held together by relatively weak forces, water and other polar molecules can enter between the layers, causing the lattice to expand (Unalan et al. 2014). Montmorillonite nanoclays are attracting considerable interest due to their strong adsorptive power, high structural stability, chemical inertia, strong capacity to form stable suspensions and the possibility of having its surface modified (Baek et al., 2012). In this sense, surface modification of clays has been commonly used to achieve a greater compatibility of the clay mineral with the polymer, and therefore reach higher exfoliation degrees, and in consequence better properties (Hetzer and De Kee, 2008).

Besides the ingestion, the incorporation of the clay minerals into polymers can result in non-intentional exposure to the consumer due to the possible migration to the food product. Nanoclays has been traditionally considered safe to human (Ibarguren et al., 2014); however, several authors have already pointed out toxic effects in different experimental models (Sharma et al., 2010; Lordan et al., 2011; Baek et al., 2012; Maisanaba et al., 2013, 2014a,b; Houtman et al., 2014). The use of simpler organisms to

evaluate the potential toxicity of nanomaterials is gaining attention and in this line and following the three Rs policy (to reduce, replace, and refine the animals used for this purpose), *Caenorhabditis elegans*, one of the best characterized animals at the genetic, physiological, molecular and developmental levels, has been successfully used in toxicological evaluations of different substances (Zhang et al., 2013; Charão et al, 2015).

The *C. elegans* free-living nematode is being considered an important tool in molecular biology because its fully sequenced genome is closely homologous to the human genome, besides its relatively short lifespan (about three weeks), its small transparent body and easy and inexpensive maintenance (Fakhrullina et al., 2015). Nematodes are sensitive to many different kinds of stresses and can change their growth rate, reproductive speed, brood size, life cycle, and other properties (Dhawan et al. 1999; Anderson et al. 2001). In this context, oxidative stress is an important feature to be monitored, once reactive oxygen species (ROS) are capable of causing damage that increases gradually with aging in many organisms, originating alterations in a variety of molecules, such as lipids, proteins and DNA (Salgueiro et al, 2014). *C. elegans* possess antioxidant defense system analogous to those inherent to mammalians, such as the enzymes superoxide dismutase and catalase (Wollenhaupt et al, 2014).

Taking into account all this background, the present work aims to assess the toxicity of three nanoclays: halloysite, unmodified montmorillonite and montmorillonite modified with octadecylamine, in two situations: pure and with adsorbed nisin. Nisin is an important antimicrobial peptide, used as preservative in food. Many devices are being developed to reach a better release of this compound in food matrices and toxicological screenings are needed.

2. Material and methods

2.1 Materials

Three commercial nanoclays from Sigma-Aldrich (St. Louis, USA) were used: hydrophilic bentonite (Nanomer® PGV – unmodified montmorillonite (MMT), montmorillonite surface modified with 25-30 wt% octadecylamine (Nanomer® I.30E (MMT-octa) and a tubular clay, halloysite (HNT). Commercial nisin (Nisaplin®) was provided by Danisco Brasil Ltda (Cotia, Brazil) and contains 2.5% of pure nisin. Stock

solution of nisin was prepared by dissolving Nisaplin® in 10 mM of sodium phosphate monobasic monohydrate (pH 5.0). This suspension was then centrifuged (5000 *g* for 10 min) to remove insoluble whey proteins from the preparation and filtered through 0.22 µm membrane.

Bacto-agar and bacto-peptona were obtained from Becton Dickinson BD® (New Jersey, USA) and HiMedia Laboratories® (Mumbai, India), respectively. 1,1,3,3-Tetramethoxypropane and 2′,7′-dichlorofluorescein diacetate (DCF-DA) were supplied by Sigma-Aldrich (St. Louis, MO, USA). Phosphoric acid and 2-thiobarbituric acid were purchased from Tedia Co (Fairfield, OH, USA) and Sprectrum Chemical Co (Gardena, CA, USA), respectively.

2.2 Nisin adsorption on nanoclays

Adsorption was carried out by adding 10 mL nisin solution (1.0 mg mL^{-1} of pure nisin) to 100 mg of nanoclay. Each bacteriocin-nanoclay system was maintained during 1 h at 25°C and 80 rpm. After that, samples were centrifuged (5000 *g* for 5 min at 25°C), the supernatant was thrown away and the pellets obtained (nanoclays adsorbed with bacteriocins) were washed twice and dispersed in distilled water.

2.3 Strains, culture and synchronization of C. elegans

The *C. elegans* strains N2 (wild type), CF1553 (muls84) and GA800 (wuls151), were obtained from the *Caenorhabditis* Genetics Center (University of Minnesota, Twin Cities, MN, USA), maintained and handled at 20ºC in NGM (nematode growth medium) plates seeded with *Escherichia coli* strain OP50 as food source. Synchronized cultures of gravid hermaphrodites were obtained by lysis in an alkaline hypochlorite solution (1% NaOCl; 0.25M NaOH), followed by rinse with M9 buffer (3 g KH2PO4, 6 g Na2HPO4, 5 g NaCl, 1 ml 1 M MgSO4, H2O to 1 L sterilized by autoclaving) and floatation on a sucrose gradient to segregate eggs from dissolved worms and bacterial debris, accordingly to standard procedures described by Brenner (1974). Eggs were hatched and allowed to grow to L1 larvae in NGM plates without food overnight.

2.4 Exposure to nanclays, nanoclays with adsorbed nisin and free nisin

Synchronized L1 worms (2500 for LD_{50} determination, TBARS and development assays and 1500 for measurement of reactive oxygen species and fluorescence quantification of CAT and SOD) immersed in 85 mM NaCl solution were exposed for 30 min to nanoclays (with and without adsorbed nisin) and free nisin, in crescent concentrations. Halloysite was tested from 0.1 to 8.0 mg mL^{-1}, bentonite was tested from 0.1 to 2.0 mg mL^{-1}, MMT with octadecylamine was tested form 0.1 to 0.75 mg mL^{-1}, while free nisin was tested from 0.05 to 0.3 mg mL^{-1}. After exposure, worms were washed three times with saline solution (85 mM).

2.5 LD_{50} determination

To determine the LD_{50} of nanoclays and free nisin, the worms were exposed and washed, according to item 2.4, and then were placed on OP50-seeded NGM plates. The number of surviving worms on each plate was counted 24 hours after exposure. All of the tested doses were compared to the control group, which did not receive the treatment. Three replicates were performed. The curves of dose-response were drawn according to a sigmoidal model with a top constraint at 100%.

2.6 Development of worms

For the evaluation of development, the surface of 20 adult worms per treatment were measured. For this, the NGM plates were washed with distilled water and the nematodes were transferred to a centrifuge tubes, being washed successively until the solution was clear. After this procedure, 15µl of the solution with the worms were mounted on 2% agarose pads with 15µl of levamisole 2.25% to anaestheticization. Pictures were taken and the flat surface area of nematodes were measured using the AxioVision software LE (version 4.8.2.0 for windows). Results were expressed as percentage of body area relative to control group.

2.7 Measurement of reactive oxygen species (ROS)

After the exposure, worms were maintained in 100 µL of saline buffer and transferred to a 96-well plate; 2'7' dichlorofluorescein diacetate (DCF-DA) was added at a final concentration of 0.05 mM and the fluorescence levels were measured (excitation: 485 nm; emission: 535 nm) using a microplate reader (Spectramax Me2; Molecular DevicesLLC, Sunnyvale, CA, USA) heated at 20°C. The fluorescence from each well was measured for 90 min at 10 min intervals. Results were expressed as percentage of fluorescence intensity relative to control wells.

2.8 Fluorescence quantification

The green fluorescent protein (GFP) expressing strains (CF1553 [muls84]-superoxide dismutase - SOD e and GA800 [wuls154] – catalase - CAT) were submitted to the acute exposure as described above. Worms were maintained in 100 µL of saline buffer and transferred to a 96-well plate, where total GFP fluorescence was measured using 485 nm excitation and 530 nm emission filters using a microplate reader (Spectramax Me2; Molecular DevicesLLC, Sunnyvale, CA, USA) heated at 20°C. The fluorescence from each well was measured for 10 min at 1 min intervals. Results were expressed as the mean percentage of fluorescence intensity relative to control wells.

2.9 Fluorescence microscopy

For each concentration, a slide was taken and 20 treated worms were mounted on 2% agarose pads and anaesthetized with 15µl of levamisole 2.25 %. Fluorescence observations were performed for image acquisitions on a Olympus IX-71 microscope equipped with epifluorescence illumination.

2.10 Lipid peroxidation

Thiobarbituric acid reactive substances (TBARS) were determined in adult worms, 48h after exposure, as a marker of lipid peroxidation by the TBA (thiobarbituric acid) assay using a 1,1,3,3-tetramethoxypropane solution as malondialdehyde (MDA) standard. After 48 hours of exposure, the plates containing the worms are washed to

remove the OP50. The nematodes were disrupted in turrax homogenizer at full amplitude for about 60 seconds, in order to release the lipid and protein content. Then the content was centrifuged at 10.000 rpm for 5 min. The supernatant was transferred to cryotubes where happen the reaction of TBARS, with the addition of 0.1M phosphoric acid solution, 20mM sodium dodecyl sulfate solution and 40mM 2-thiobarbituric acid solution. The reaction took place in a water bath for 1 hour and 30 minutes under agitation at 100 ° C. Additionally, the samples were transferred to 96 well plates and its absorbance was read at 532 nm (Spectramax Me2; Molecular DevicesLLC, Sunnyvale, CA, USA).The protein content of the samples was determined according to Bradford (1976).

2.11 Statistical Analysis

The results were subjected to variance analysis (ANOVA) and means were compared through the Tukey test at a level of 5 % of significance. The results obtained in items 2.5, 2.6 and 2.7 were submitted to Spearman's correlation, at a level of 5 % of significance. All tests were performed using the Prism 5.0 software (GraphPad Software Inc., La Jolla, United States).

3. Results

3.1 LD_{50} of compounds

Figure 1 shows the percentage of survival versus the tested doses of nanoclays and free nisin. The highest LD_{50} obtained was observed in pure halloysite, while free nisin presented the lowest LD_{50} (table 1). The most toxic nanoclay was montmorillonite modified with octadecylamine. All of the tested doses were compared to the control group, which did not receive the treatment.

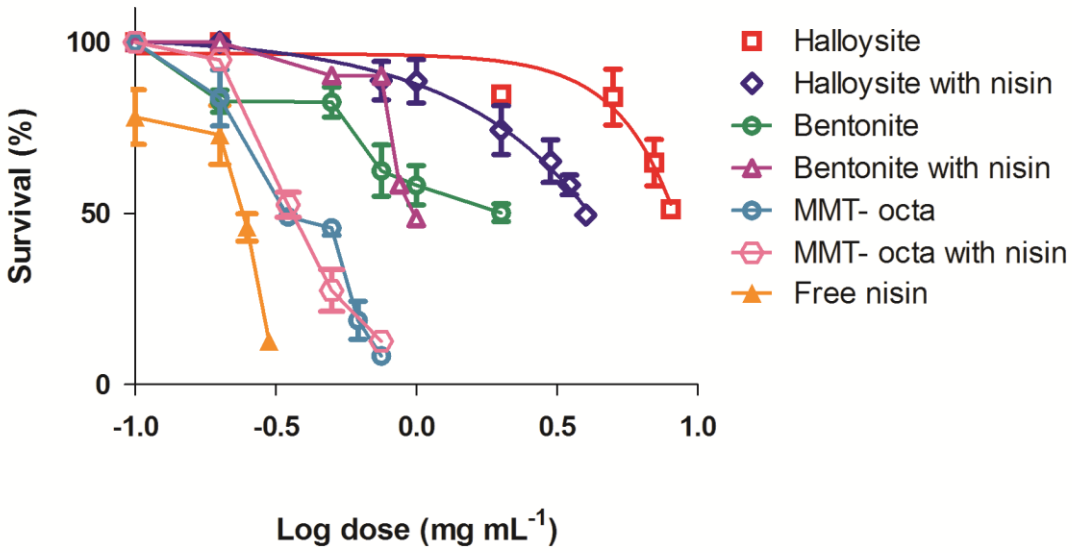


Figure 1. Dose–response curves for acute exposure of *C. elegans* to halloysite, halloysite with nisin, bentonite (MMT), bentonite with nisin (MMT-nisin), montmorillonite modified with octadecylamine (MMT-octa) and montmorillonite modified with octadecylamine with nisin (MMT-octa-nisin) and free nisin. Data are expressed as mean ± SEM.

Table 1. The LD_{50} obtained after acute exposure of *C. elegans* to different nanoclays (pure or with adsorbed nisin).

Compound	DL $_{50}$ (mg mL^{-1})
Halloysite	8.380
Halloysite with nisin	4.078
Bentonite	1.994
Bentonite with nisin	0.980
MMT-octa	0.3505
MMT-octa with nisin	0.3805
Free nisin	0.239

(MMT-octa – montmorillonite modified with octadecylamine)

3.2 Development of worms

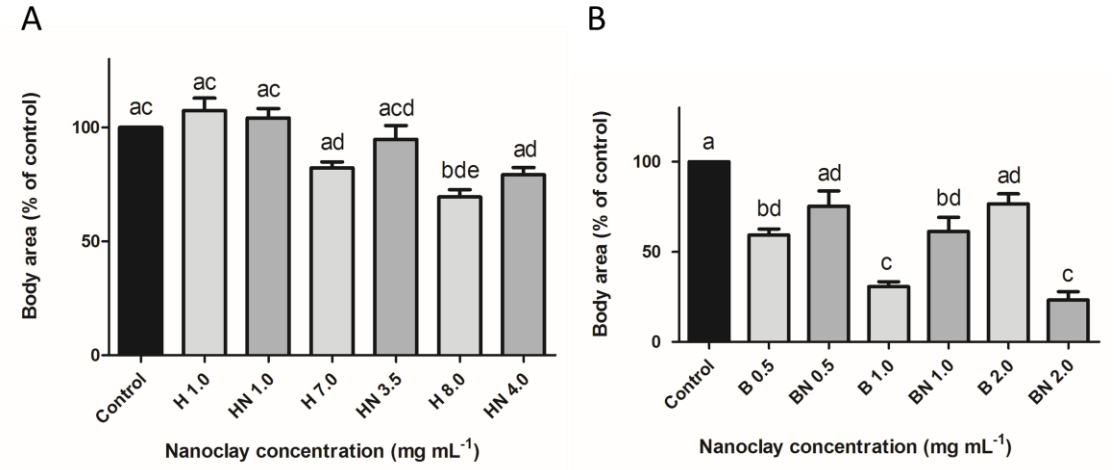
The normal development of *C. elegans* was affected by the nanoclays and free nisin exposure, as it significantly reduced the body area of the worms when compared with control group ($p<0.05$) (Figure 2).

Halloysite only affected the normal development of worms on the higher concentration tested (8.0 mg mL^{-1} of pure nanoclay) nanoclay, in which the size was about 25% smaller than the control. For worms exposed to halloysite with adsorbed nisin the difference was not statistically significant.

Bentonite was the nanoclay that most impacted the development of *C. elegans*. This nanoclay caused a crescent reduction of size, except in concentration 2.0 mg mL^{-1} of pure bentonite. The reduction in pure bentonite ranged from 40% in 0.05 mg mL^{-1} to 70% 1.0 mg mL^{-1}. However, in the 2.0 mg mL^{-1} concentration the worms were 25% smaller. Worms exposed to bentonite with nisin were 25, 40 and 75% smaller than the control, in concentrations 0.05, 1.0 and 2.0 mg mL^{-1}, respectively.

Worms exposed to MMT-octa with or without nisin were 55% smaller than the control. The higher concentrations were not statistically different from the control.

Free nisin only induced a decrease of approximately 25% of body area in the higher concentrations (0. 2 and 0.25 mg mL^{-1}).



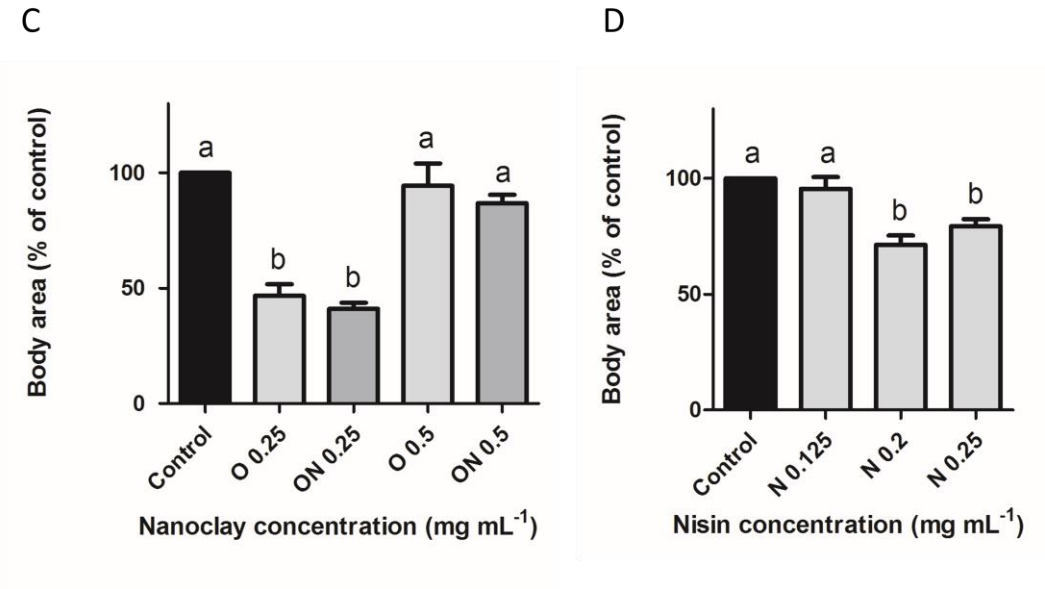


Figure 2. Body area of namatodes. The mean size in control group was considered as 100%. Data are expressed as mean ± SEM. Treatments with different letters are significantly different (P<0.05). H – halloysite; HN halloysite adsorbed with nisin; B – bentonite; BN – bentonite adsorbed with nisin; O – montmorillonite modified with octadecylamine; ON – montmorillonite modified with octadecylamine with adsorbed nisin; N – free nisin.

3.3 ROS levels

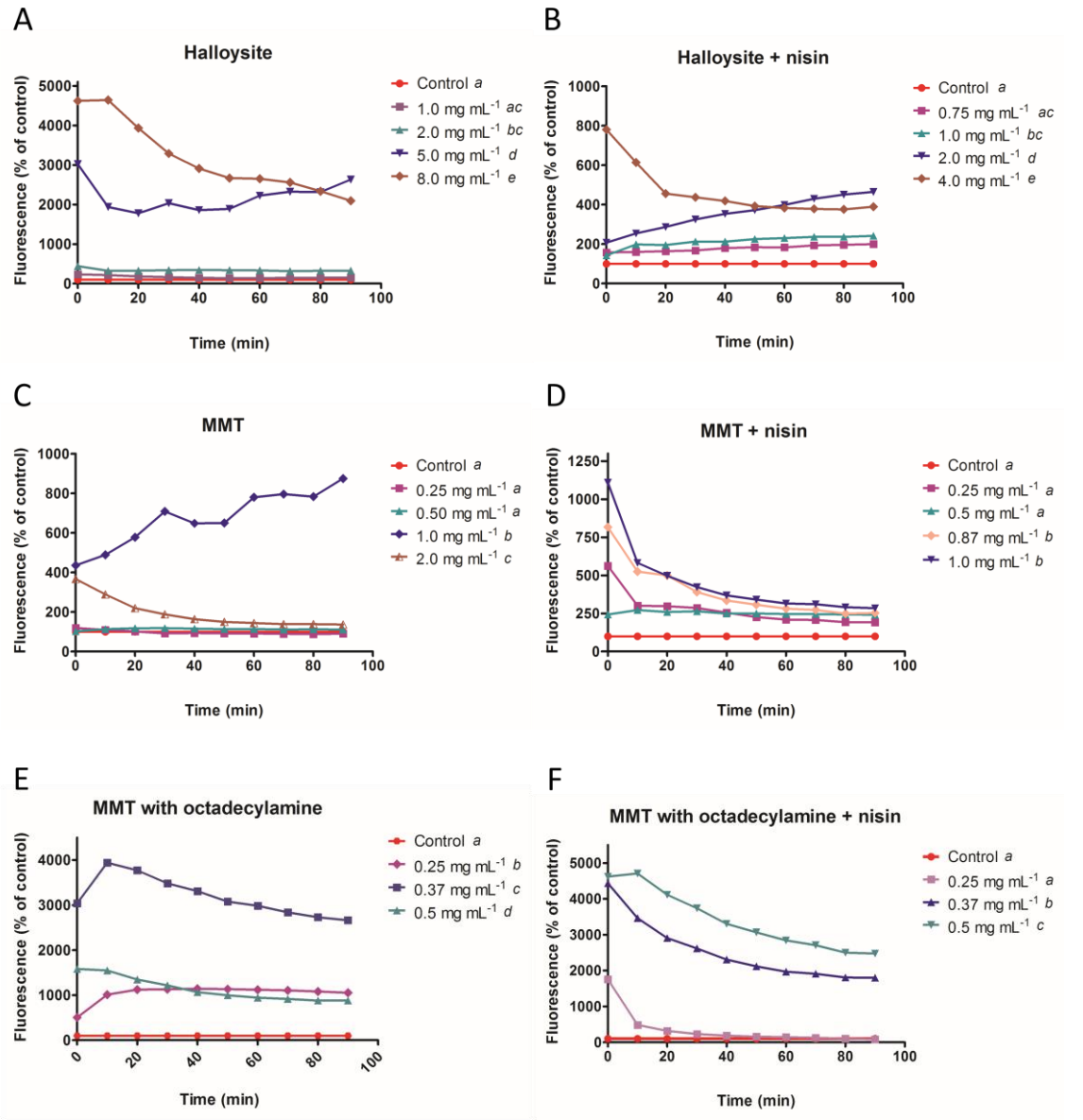
ROS levels were determined with the dichlorofluorescein diacetate (DCF-DA), which undergoes de-esterification to dichlorofluorescein (DCFH) which is then oxidized to the DCF fluorophore by free radicals. The exposure to nanoclays and to free nisin caused a significant increase in DCF-DA oxidation, reflecting in the generation of ROS (Figure2).

The highest concentration of halloysite (8 mg mL^{-1}) began fiftyfold higher than the control sample and lowered over time to half this amount. The second highest concentration (5 mg mL^{-1}) reached approximately the same level of ROS, about 25 times higher than the control. The highest concentration tested of halloysite with nisin (4 mg mL^{-1}) started at 800% and also decreased by half, about fourfold the control, which was the same value reached by the second higher concentration (2 mg mL^{-1}). However, for both types of halloysite, the lowest concentrations produced, at most, twice amount of ROS than the control sample.

For bentonite (MMT), the concentrations 1.0 and 2.0 mg mL^{-1} started generating about fourfold the amount of ROS compared to control but ended almost 9 times and 1.3 times higher than the control, respectively. The concentration 0.75 mg mL^{-1} presented an unexpected behavior, beginning with 200% of ROS and growing till 350%. The lowest concentrations weren't significantly different from control. The highest concentration of bentonite with nisin had a peak elevenfold higher than the control, decreasing to threefold. The other concentrations ended about at 250% of ROS.

MMT-octa with and without nisin, in the concentration 0.37 mg mL^{-1} (close to its LD_{50}) had peaks of 4000% of ROS, decreasing ten times this value till the end of experimental time.

Only the lowest concentration of free nisin generated less ROS than the control. The highest concentration reached fourfold the amount of control.



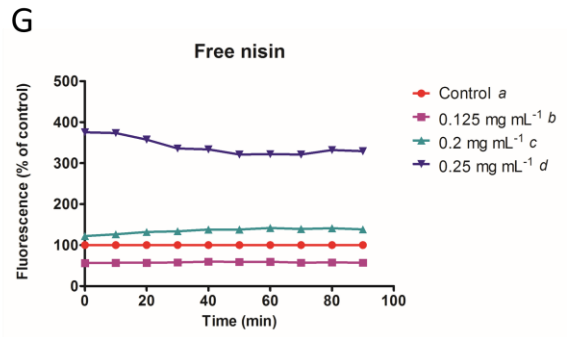


Figure 2. ROS levels measured by DCF-DA dye: (A) Halloysite; (B) Halloysite with adsorbed nisin; (C) Bentonite (MMT); (D) Bentonite (MMT) with adsorbed nisin; (E) Montmorillonite modified with octadecylamine; (F) Montmorillonite with octadecylamine with adsorbed nisin and (G) Free nisin. Data are expressed as mean ± SEM. Treatments with different letters are significantly different (P<0.05).

3.3 Fluoresce quantification

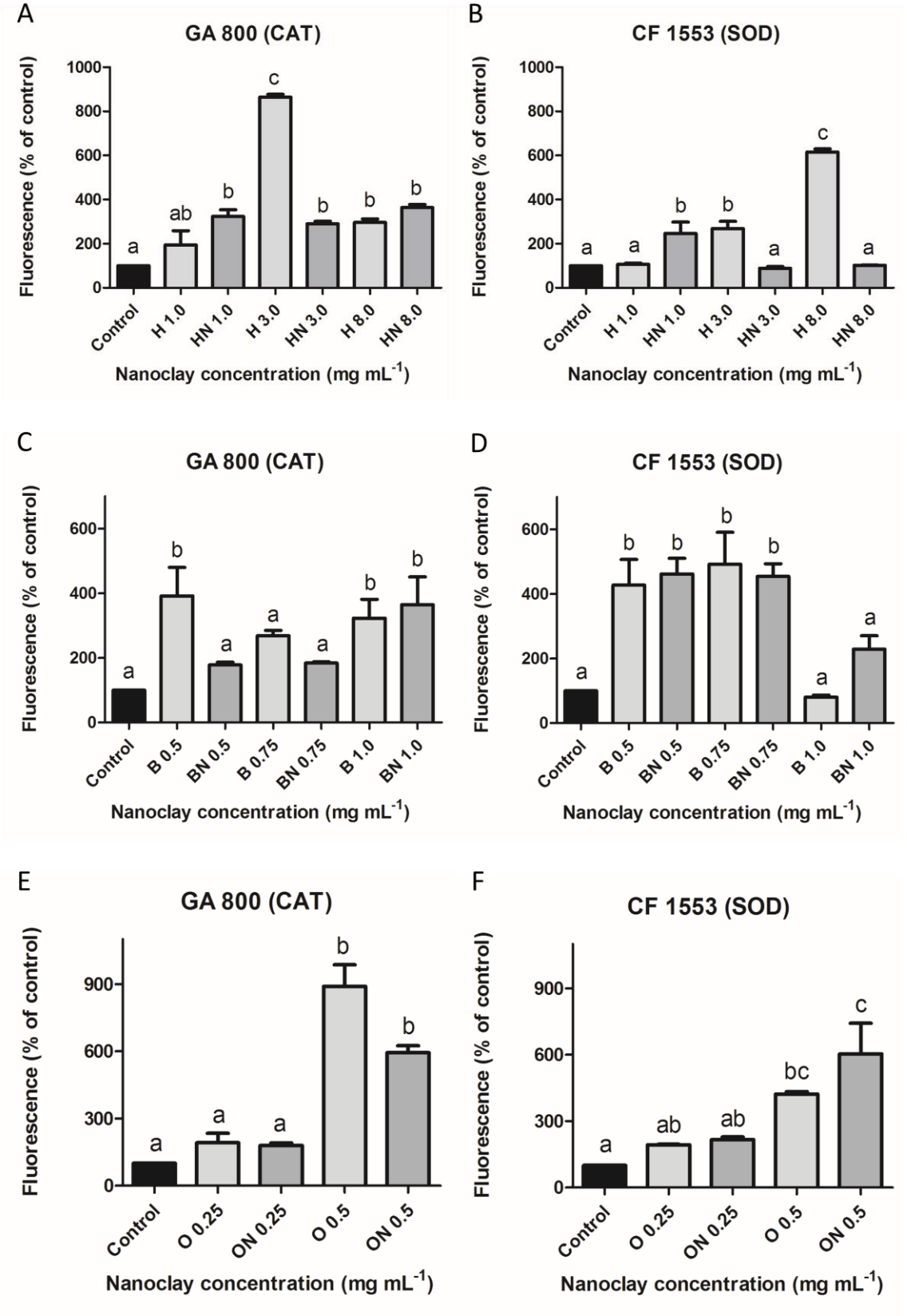
The levels of CAT and SOD enzymes were observed through fluorescence of GA800 and CF1553 strains, respectively. Each treatment showed a different pattern of quantity of fluorescence, as can be seen in figure 3 and figure 4.

The amounts of CAT and SOD in worms exposed to halloysite increased from the concentration 3.0 mg mL^{-1} on. There was a large peak of fluorescence in CAT level, ninefold higher than the control, in the concentration 3.0 mg mL^{-1}, that decreased in the concentration 8.0 mg mL^{-1}, reaching 300% of fluorescence. For SOD the peak (600%) was in concentration 8.0 mg mL^{-1}. In worms exposed to halloysite with nisin, CAT levels increased, but remained three times higher than the control in all concentrations. However, the SOD increased only in the concentration 1 mg mL^{-1}.

In animals exposed to bentonite, CAT was higher than the control (about 4.5x) only in concentrations 0.5 and 1.0 mg mL^{-1}, while SOD was 5-6 times higher in concentrations 0.5 and 0.75 mg mL^{-1}. In bentonite with nisin, CAT was only higher in concentration 1.0 mg mL^{-1} and SOD level was the same presented in bentonite alone.

The quantity of CAT and SOD was significantly higher in the concentration 0.5 mg mL^{-1} in the nematodes exposed to MMT-octa, with or without nisin. The CAT level was tenfold and fourfold higher in nanoclay without and with nisin, respectively. SOD was fourfold higher without nisin and sevenfold higher with nisin.

Worms exposed to free nisin showed a crescent amount of CAT, that reached 400% of fluorescence. However, SOD was only present in significant amounts in the lower concentration (0.125 mg mL^{-1}).



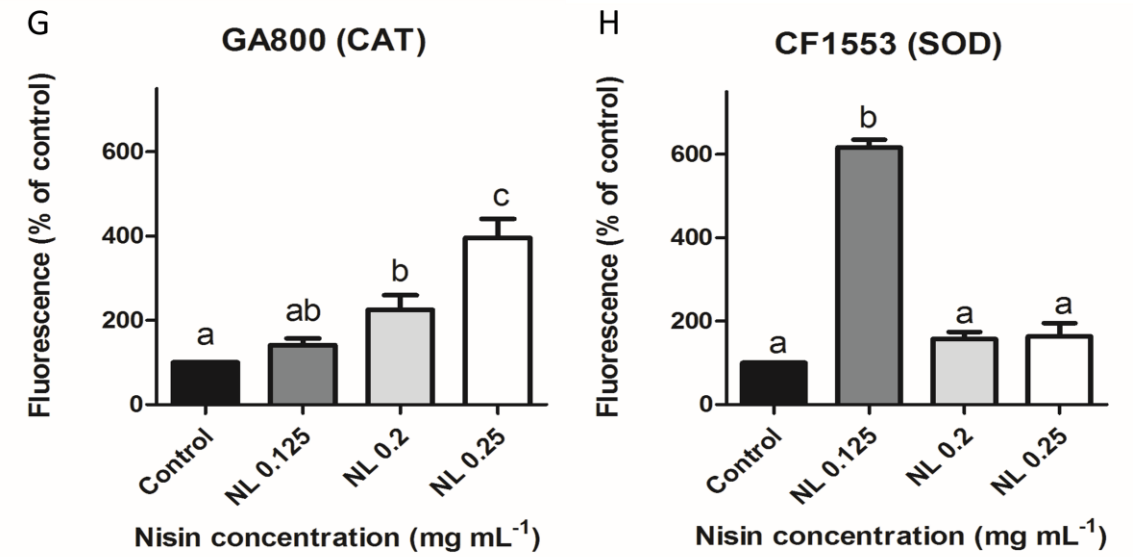


Figure 3. Quantification of fluorescence of (A) GA800 (CAT) and (B) CF1553 (SOD) strains of *C. elegans* after acute exposure to halloysite (H) and halloysite adsorbed with nisin (HN); (C) GA800 (CAT) and (D) CF1553 (SOD) strains after exposure to bentonite (MMT) and bentonite adsorbed with nisin; (E) GA800 (CAT) and (F) CF1553 (SOD) strains after exposure to montmorillonite modified with octadecylamine and montmorillonite modified with octadecylamine adsorbed with nisin; (G) GA800 (CAT) and (H) CF1553 (SOD) strains after exposure to free nisin. Data are expressed as mean ± SEM. Treatments with different letters are significantly different (P<0.05).

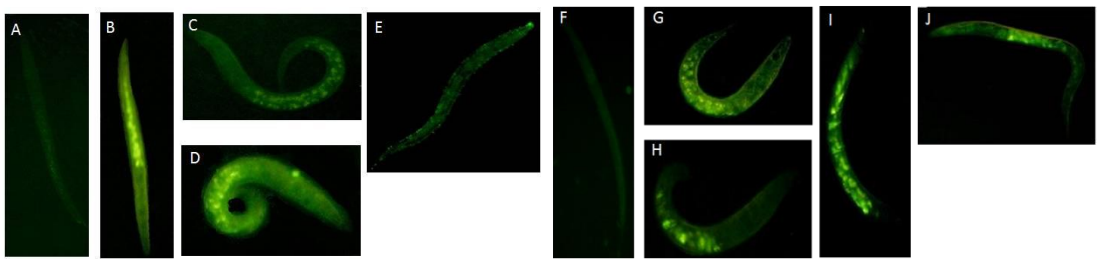


Figure 4. Representative epifluorescence microscopy images of CAT::GFP (A – E) and SOD::GFP (F – J) strains of *C. elegans* after acute exposure to treatments: (A) control – CAT; (B) halloysite 3.0 mg mL^{-1}; (C) bentonite 0.5 mg mL^{-1}; (D) montmorillonite modified with octadecylamine 0.5 mg mL^{-1}; (E) free nisin 0.25 mg mL^{-1}; (F) control – SOD; (G) halloysite 8.0 mg mL^{-1}; (H) bentonite 0.75 mg mL^{-1}; (I) montmorillonite modified with octadecylamine with nisin 0.5 mg mL^{-1} and (J) free nisin 0.125 mg mL^{-1}.

3.4 Lipid Peroxidation

Thiobarbituric acid reactive substances (TBARS) are formed during the lipid peroxidation as sign of oxidative damage. There was no significant increase in TBARS in worms exposed to haloisite, bentonite and free nisin. However, worms exposed to 0.5 mg mL^{-1} of MMT-octa, with or without adsorbed nisin, produced higher amounts of TBARS than the control animals.

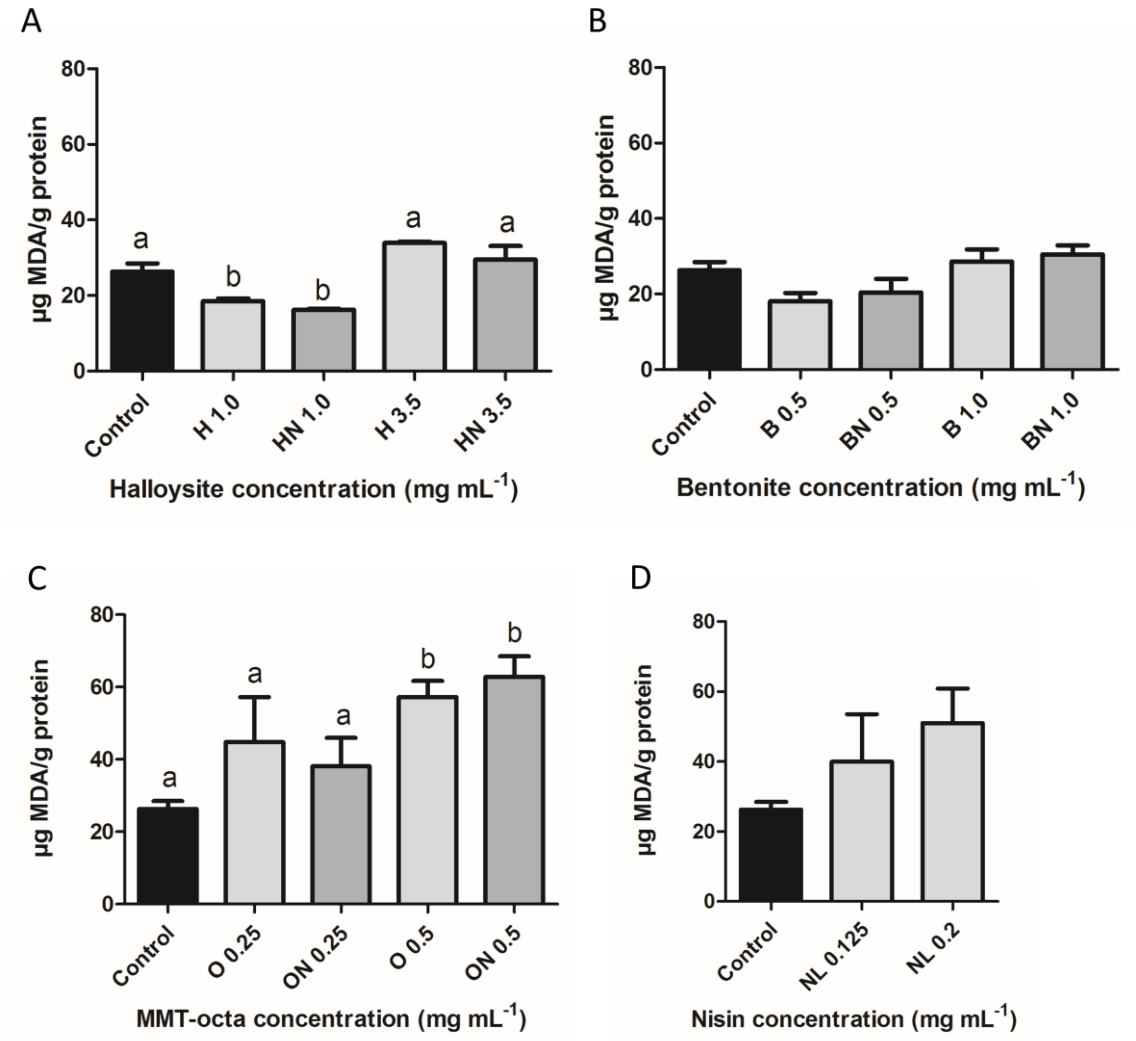


Figure 5. Quantification of thiobarbituric acid reactive substances in adult nematodes after acute exposure to (A) halloysite – H – and halloysite with nisin – HN; (B) bentonite – B and bentonite with nisin – BN; (C) montmorillonite modified with octadecylamine – O and montmorillonite modified with octadecylamine with nisin – ON and (D) free nisin – NL. Data are expressed as mean ± SEM. Treatments with different letters are significantly different (P<0.05).

3.5 Correlation between data

The main data of this study were tested by Spearman correlation and the significant ones are listed in table 2. The test revealed a positive strong significant correlation between death rate (obtained from the survival experiment) and the mean quantity of reactive oxygen species in all treatments. An important correlation was found between the ROS and the size of worms exposed to halloysite and montmorillonite modified with octadecylamine with adsorbed nisin.

Treatment	Correlation	r	P value
Halloysite	Death rate x ROS	0.9832	< 0.0001
	Death rate x size	-0.9154	0.0013
Halloysite + N	Death rate x ROS	0.9833	< 0.0001
Bentonite	Death rate x ROS	0.7500	0.0255
Bentonite + N	Death rate x ROS	0.8117	0.0108
MMT-octa	Death rate x ROS	0.7448	0.0255
MMT- octa + N	Death rate x ROS	0.9000	0.002
	Size x ROS	0.7000	0.0358
Nisin	Death rate x ROS	0.7667	0.0214

(Halloysite + N – halloysite with nisin; Bentonite + N – bentonite with nisin; MMT-octa – montmorillonite modified with octadecylamine; MMT-octa + N – montmorillonite modified with octadecylamine with nisin)

4. Discussion

Halloysite nanotubes and montmorillonite are nanoclays of special interest for application in food packaging. Thus, toxicological assessments must be performed to ensure safety for consumers. Here we used the *C. elegans* model to perform an acute toxicity evaluation.

Vergara et al (2010) investigated the toxicity of HNTs in human breast cancer and human epithelial adenocarcinoma cells and only observed significant cell death at high concentration of HNTs (1 mg mL^{-1}). In a study using HCT116 (colorectal carcinoma) and HepG2 (hepatocellular carcinoma) cells, which represent the earliest entry point and the first accumulating organ, respectively, for nanoparticles in circulation after oral delivery, results indicated that HNTs are generally safe at practical concentrations of excipients for oral dosage forms (Ahmed et al, 2013). Fakhrullina et al (2015), studing *C. elegans* exposed to halloysite, observed that this nanoclay was localized exclusively in the alimentary system and did not induce severe toxic effects on nematodes. Proteomic analysis also corroborates to the assumption of low toxicity of halloysite (Lai et al, 2013).

There are many studies with different types of montmorillonite, since layered clays are of particular interest due to the successful incorporation into composite materials and the advantageous properties that reinforced materials exhibit, which is essential when high barrier properties are needed, like in food packaging (Rhim and Ng, 2007). But for a better incorporation of the clays to the polymer matrix their compatibility should be improved by surface modification. The octadecylamine modifier is added to facilitate the interaction with polypropylene, polyethylene and ethylene vinyl acetate (EVA), to form composites, which has its mechanical and barrier properties improved. It has been suggested that the modifiers included in the montmorillonite modulates the toxicity of the resulting clay (Sharma et al, 2010), although unmodified MTT, including bentonite, has been reported to cause cytotoxicity in many cell lines (Maisanaba et al, 2015b).

Among the compounds tested in this study, halloysite nanotubes presented the highest LD_{50} (35 times higher than free nisin, the lowest one) and therefore was the less toxic substance tested. Bentonite (unmodified montmorillonite) had an intermediate toxicity and montmorillonite modified with octadecylamine was the most toxic nanoclay. In halloysite and bentonite, the adsorption of nisin provoked the fall of the LD_{50} for half, but in modified montmorillonite nisin doesn't significantly change the survival rate or worms. Nisin itself showed the smaller $LD_{50,}$ 0.239 mg mL^{-1}. The apparent high toxicity

of nisin can be partially explained by the high osmotic stress caused by the content of NaCl in Nisaplin® (77% of NaCl). This results are in line with other studies *in vitro, in silico* and *in vivo* (Takahashi *et al.*, 1983; Shirai *et al*, 1984; Aranha *et al.* 2008; Hagiwara *et al.;* Vaucher *et al.*, 2012).

Although all treatments have affected the normal development of the worms, this occurred in different degrees. Worms exposed to halloysite only had their size reduced significantly in the concentration 8.0 mg mL^{-1}. Bioinformatics analysis suggested that halloysite stimulates processes related to cell growth and proliferation (Lai et al, 2013). Fakhrullina et al (2015) also find a development deficit in *C. elegans* treated with 1mg mL^{-1} of halloysite. They suggested that the uptake of halloysite nanotubes can cause irritation in contact with the intestinal cuticle of the worms affecting the ingestion and, as a consequence, the body length. Bentonite caused the biggest drop in the size of worms, which in 1.0 mg mL^{-1} reached only 30% of normal body area. However, in the 2.0 mg mL^{-1}concentration the worms were 25% smaller than the control. We suggest that it happened due to the decrease of population. With fewer individuals per plate there are more available bacteria as food source, allowing the growth of surviving animals. The same happened in worms exposed to MMT-octa. In the concentration 0.25 mg mL^{-1}, these worms had their size reduced in 55%, but in higher concentrations presented the average size. We suppose that the nanoclay can hinder the movement of the worm, since even after successive washings, nanoparticles remain adhered to the animal body and thus affect the foraging.

The Spearman's correlation test pointed high positive relation between the death rate and reactive oxygen species produced, suggesting that oxidative stress can be the mechanism of toxicity. MMT-octa with or without adsorbed nisin reached about 70 times higher quantities of ROS and in the concentration of LD_{50} the fluorescence was 3000% (thirtyfold higher than the control sample). Halloysite in the higher concentration of LD_{50} also presented this level of ROS. Bentonite presented a different pattern, where only the concentration 1.0 mg mL^{-1} presented high levels of ROS and the other concentrations were near the control. Bentonite with adsorbed nisin started with very high levels of ROS that deceased till 400%. Free nisin also presented a fourfold higher amount of ROS compared to control in the highest concentration tested. Fakhrullina et al (2015) also attributed the toxic effecs of halloysite to the direct contact of nanotubes with cell walls and ROS generation.

The antioxidant defense, here represented by the quantification of fluorescence of strains with GPF for catalase and superoxide dismutase, also presented differences among the treatments. With halloysite treatment, the levels of CAT and SOD aren't different from control animals in low concentrations, but in higher concentrations present high peaks. The addiction of nisin to HNT change this pattern, and CAT keeps higher in all concentrations while SOD is only statistically significant in the lower concentration. In animals exposed to bentonite, with or without nisin, the SOD remains high and CAT levels fluctuate with pure nanoclay and with adsorbed nisin only the highest concentration is statistically significant. In worms exposed to MMT-octa, with or without nisin, the CAT and SOD levels were only higher than the control in the concentration 0.5 mg mL^{-1}, where the mortality is very high and lipid peroxidation is significant, indicating oxidative stress. Other treatments did not induced significant lipid peroxidation. Free nisin presented significant SOD levels in the lower concentration, while CAT was only significant in higher concentrations. In rats, Maisanaba et al (2015e) did not find alterations in lipid peroxidation and SOD activity in the liver and kidney after exposition for 90 days to modified MMT, but CAT activity, gene expression and protein abundance were increased in the kidney and Sharma et al (2014) did not observe DNA strand-breaks in the colon, liver and kidney cells of Wistar rats exposed to cloisite (montmotillonite modified with a quaternary ammonium salt).

In conclusion, from a toxicological point of view, halloysite seems to be the most appropriate nanoclay for addition in food packaging, as reinforcer or carrier, once it can be used in high doses without significant damage. Bentonite had and intermediate LD_{50} but was the compound which caused the greatest reduction in size of worms. On the other hand, MMT modified with octadecylamine showed the most toxic profile, with low LD_{50}, high ROS levels and development defict of worms exposed to this nanoclay. The nisin adsorption is supposed to have caused the reduction of LD_{50} in halloysite and bentonite treatments, but not in MMT-octa. These results showed that *C. elegans* can be a good alternative in vivo model for toxicological screening of nanoparticles, with rapid and accurate results, contributing for the characterization of nanomaterials.

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4 CONSIDERAÇÕES FINAIS

O peptídeo antimicrobiano nisina foi encapsulado em lipossomas de fosfatidilcolina, que apresentaram boas características físico-químicas, com alta eficiência de encapsulação e estabilidade durante o armazenamento. A nanoencapsulação de substâncias adicionadas a alimentos previne interações indesejadas com a matriz alimentar, além de ser um sistema de liberação controlada. A adição desses lipossomas a filmes biocompatíveis de matriz protéica de gelatina ou caseína mostrou grande potencial como embalagens antimicrobianas. Os filmes resultantes demonstraram atividade frente a patógenos alimentares (*Listeria monocytogenes, Bacillus cereus* e *Clostridium perfringens*) em ágar leite, simulante de alimentos sólidos, revelando grande potencial para evitar ou minimizar a contaminação pós-processamento. Estudos com alimentos reais são necessários para comprovar a eficácia dos filmes.

Embora muitos estudos demonstrem que o uso de nanoargilas melhora as propriedades mecânicas e de barreira de nanocompósitos, e a haloisita seja interessante para a liberação controlada da nisina, na quantidade utilizada no presente trabalho, essa melhora não foi observada.

Novos alimentos, ou mesmo novos ingredientes, como os lipossomas e as nanoargilas, precisam ser considerados seguros antes da industrialização, tentando ao máximo garantir a inocuidade para os consumidores e o ambiente. A nanotecnologia vem se desenvolvendo com rapidez e com isso são necessários métodos de avaliação toxicológica que atendam essa demanda, gerando resultados rápidos e confiáveis. O modelo *Caenorhabditis elegans* mostrou-se de cultivo fácil, rápido e econômico comparado a modelos tradicionais. Tal modelo invertebrado está dentro da política dos 3R (“reduce, refinement and replacement”), que representa um esforço da comunidade científica mundial para reavaliar o uso de mamíferos em experimentos científicos. Outra vantagem do modelo é que sua fisiologia, desenvolvimento e genoma já estão completamente elucidados e há uma grande ortologia com o genoma humano.

Embora a nisina tenha o status *GRAS* (Generally Recognized As Safe) concedido pelo FDA, na literatura há estudos demonstrando certa toxicidade dessa substância, assim como foi encontrada nesse estudo. A nisina livre apresentou a maior dose letal 50% (DL_{50}) entre as substâncias testadas. Os lipossomas produzidos mostraram-se pouco tóxicos, de maneira que não foi possível determinar suas (DL_{50}) com a técnica empregada. Dessa forma, podemos dizer que além das vantagens já citadas sobre

lipossomas, esses diminuem o impacto tóxico da nisina livre e permitem que uma quantidade maior de droga possa ser entregue.

Os lipossomas não são o único sistema de liberação controlada de bacteriocinas como a nisina. Estudos anteriores mostraram que é possível adsorver a nisina em nanoargilas, as mesmas que foram testadas nos filmes nanocompósitos como reforçadores (haloisita, bentonita e montmorilonita modificada com octadecilamina). A nisina parece aumentar a toxicidade da haloisita e da bentonita, mas não altera a mortalidade de *C. elegans* expostos à montorilonita modificada com octadecilamina. A bentonita apresentou uma toxicidade moderada e alterou significativamente o desenvolvimento dos vermes, que tiveram seu tamanho diminuído em 70%. A haloisita pura apresentou a maior DL_{50}, quase 24 vezes maior do que a montmorilonita modificada com octadecilamina, a menor DL_{50} entre as argilas. Animais expostos à MMT-octa apresentaram um grande estresse oxidativo. A toxicidade da MMT-octa pode ser explicada pelo modificador (a octadecilamina), que é o elemento responsável por tornar a argila mais compatível com outros polímeros, melhorando as propriedades mecânicas e de barreira do nanocompósito formado. Os modificadores são conhecidos por modular a toxicidade das montmorilonitas.

Testes de correlação mostraram forte relação positiva entre a taxa de mortalidade dos vermes e a geração de espécies reativas de oxigênio em todos os tratamentos, sejam lipossomas ou nanoargilas, mostrando que o estresse oxidativo pode explicar parcialmente a toxicidade desses compostos. Entretanto, para confirmar tal hipótese, novos estudos precisam ser realizados.

Do ponto de vista toxicológico e prático, a haloisita mostrou ser a argila mais adequada para a incorporação em alimentos, uma vez que possui características adequadas do ponto de vista mecânico, é funcional como carreador e potencialmente seguro para o consumo.

5 CONCLUSÕES

De acordo com os resultados obtidos no presente estudo, é possível concluir que:

- A bacteriocina nisina foi encapsulada eficientemente em nanovesículas lipídicas preparadas com fosfatidilcolina comercial (Nisaplin®) e a partir de lecitina de soja parcialmente purificada, apresentando estabilidade e alta eficiência de encapsulação.
- Os nanocompósitos de polímeros biodegradáveis (caseína e gelatina), lipossomas e a nanoargila haloisita foram ativos, capazes de liberar nisina.
- As propriedades dos nanocompósitos como embalagens foi satisfatória.
- Os nanocompósitos apresentaram atividade antimicrobiana frente a *Listeria monocytogenes* ATCC 7644, *Bacillus cereus* ATCC 14579 e *Clostridium perfringens* ATCC 3624.
- Os lipossomas apresentaram baixa toxicidade comparados à nisina livre.
- A nanoargilas haloisita e montmorilonita modificada com octadecilamina apresentaram a menor e a maior toxicidade, respectivamente.
- A incorporação de nisina nanoencapsulada e haloisita em nanocompósitos de gelatina e caseína representa uma tecnologia promissora e segura como uma barreira adicional ao controle de *L. monocytogenes* e possivelmente outros patógenos em alimentos.
- A geração de espécies reativas de oxigênio parece participar parcialmente o mecanismo de toxicidade de lipossomas e nanoargilas. No entanto, estudos adicionais precisam ser realizados para confirmar essa hipótese.

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