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Desenvolvimento de nanocompósitos contendo peptídeos antimicrobianos para uso em alimentos

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Orientador: Dr. Adriano Brandelli

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“A vida é para quem é corajoso o suficiente para se arriscar e humilde o bastante para aprender”. (Clarice Lispector)

RESUMO

Nanocompósitos antimicrobianos foram desenvolvidos a partir de bacteriocinas (nisina e pediocina), nanoargilas (montmorilonita e haloisita) e polímeros sintético (polipropileno) e biodegradável (amido de milho). A caracterização foi feita quanto a propriedades antimicrobianas, por difusão em ágar; estruturais, por meio de microscopia eletrônica de varredura e difração de raios-X; propriedades mecânicas, cujos parâmetros foram resistência à tração, deformação da ruptura e módulo de Young; propriedades térmicas, por análise termogravimétrica e, conforme o caso, calorimetria diferencial de varredura. Independentemente das diferentes técnicas e formulações empregadas para o preparo dos filmes, os resultados obtidos para essas propriedades demostraram que a incorporação de nisina e pediocina alterou a matriz polimérica original, com perda parcial de homogeneidade e cristalinidade e modificação do desempenho dos materiais. A adição de argilas foi importante para melhorar ou manter as propriedades funcionais dos filmes resultantes, enquanto que a capacidade de inibição de bactérias Gram-positivas *in vitro* (*Listeria monocytogenes, Clostridium perfringens e Staphylococcus aureus*) foi alcançada em todas as formulações, porém influenciada pela presença do nanoreforço. Nos filmes de polipropileno/montmorilonita adicionados de nisina, propriedades de barreira foram também avaliadas e os resultados mostraram que a presença do antimicrobiano não influenciou a permeabilidade ao vapor d'água, mas aumentou a permeabilidade ao oxigênio. Além disso, a migração de nisina a partir dos nanocompósitos ativos de polipropileno ocorreu durante 48 h em soluções simulantes de alimentos. Com relação aos filmes de amido, a análise complementar de espectroscopia de infravermelho evidenciou poucas interações químicas entres os aditivos nos nanocompósitos obtidos por extrusão, mas grandes alterações de bandas devido a ligações de hidrogênio estabelecidas entre os componentes durante o preparo dos filmes pela técnica de *casting*. A análise de cor refletiu a aparência dos filmes de amido, com destaque ao parâmetro *b*, o qual expressou em valores significativamente mais elevados o aspecto escuro adquirido pelos nanocompósitos devido à presença de haloisita e bacteriocinas. Algumas formulações tiveram a incorporação das bacteriocinas previamente adsorvidas em haloisita, já que paralelamente este estudo mostrou que nisina e pediocina foram capazes de adsorver em três diferentes tipos de nanoargilas. A técnica de adsorção dos peptídeos mostrou-se interessante por melhorar a cor e as propriedades estruturais, mecânicas e térmicas, apesar de afetar negativamente a transparência e atividade antimicrobiana. Finalmente, a real aplicação dos nanocompósitos em alimento foi realizada empregando-se os filmes de amido contendo nisina elaborados por extrusão para controle de *L. monocytogenes* em queijo Minas Frescal. Estes resultados apontaram o potencial de utilização dos nanocompósitos visto que o patógeno foi eficientemente inibido após 4 dias de estocagem do alimento modelo a 4ºC. Portanto, os nanocompósitos antimicrobianos desenvolvidos a partir de polipropileno e amido de milho oferecem inovação tecnológica com perspectiva de aplicação como embalagem de alimentos.

ABSTRACT

Antimicrobial nanocomposites were developed using bacteriocins (nisin and pediocin), nanoclays (montmorillonite and halloysite) and synthetic (polypropylene) and biodegradable (corn starch) polymers. The characterization was carried out in relation to antimicrobial properties, by agar diffusion assay; structural properties, by scanning eletron microscopic and X-ray diffraction; mechanical properties, which parameters was tensile strength, deformation at break and Young's modulus; thermal properties, by thermogravimetric analysis, and where applicable, scanning differential colorimetric. Regardless of different thecniques and formulations used for preparation of films, the results obtained for these properties showed that incorporation of nisin and pediocin altered the polymer matrix, with partial loss of homogeneity and cristallinity and performance modification of materials. The addition of clays was important to improve or maintain the functional properties of resultant films, whereas the inhibiton of Gram-positive bacteria *in vitro* (*Listeria monocytogenes* and *Clostridium perfringens*) was achieved in all formulations, but influenced by the presence of the nanoreinforcement. In polypropylene/montmorillonite films added with nisin, barrier properties were also evaluated and the results showed that the antimicrobial did not influence the water vapor permeability, but increased the oxygen permeability. Moreover, the nisin migration from active polypropylene nanocomposites occurred during 48 h in food simulants. In relation to starch films, the complementary analysis of infrared spectroscopy evidenced minimal chemical interactions between additives and polymer in nanocomposites obtained by extrusion, however major changes of bands due to hydrogen bonds established between components during preparation of the films by the casting technique. The color analysis reflected the appearance of starch films, especially for *b* parameter which expressed in significantly higher values the darker aspect of nanocomposites because of the presence of halloysite and bacteriocins. Some formulations was incorporated with bacteriocins previously adsorbed on halloysite, since this study showed parallel that nisin and pediocin were able to adsorb in three different types of nanoclays. The adsorption thecnique was interesting because improved color and structural, mechanical and termal properties, although it negativelly affected the transparency and antimicrobial activity. Finally, the real application of nanocomposites in food was conducted using starch films with nisin developed by extrusion to control *L. monocytogenes* in Minas Frescal cheese. These results indicated the potential use of the nanocomposites since the pathogen was effectivelly inhibited after 4 days of storage at 4ºC. Therefore, the antimicrobial nanocomposites developed with polypropylene and starch offer technological innovation with application perspective as food packaging.

SUMÁRIO

1. INTRODUÇÃO

Alimentos mais naturais, minimamente processados e sem adição de conservantes químicos estão sendo cada vez mais procurados pelos consumidores, desafiando as indústrias alimentícias à produção de alimentos seguros e com elevada vida útil. Nesta perspectiva, novas propostas de preservação de alimentos são imprescindíveis para assegurar e contemplar a produção de alimentos com qualidade em seu aspecto mais amplo.

De forma especial, o potencial de antagonismo de alguns micro-organismos, assim como de seus metabólitos antimicrobianos tem despertado interesse, sendo a atividade antimicrobiana de peptídeos bioativos sugerida para o controle do desenvolvimento de micro-organismos patogênicos e/ou deteriorantes em alimentos. Os peptídeos antimicrobianos produzidos por bactérias, denominados bacteriocinas, constituem uma estratégia promissora para a bioconservação de alimentos. Nisina e pediocina, ambas produzidas por bactérias lácticas, apresentam destaque principalmente devido ao efeito inibitório contra *Listeria monocytogenes*, patógeno encontrado em produtos lácteos.

As bacteriocinas são consideradas agentes antimicrobianos naturais e podem ser empregadas como parte da tecnologia de barreiras. Entretanto, a atividade antimicrobiana pode ser comprometida pela suscetibilidade à degradação proteolítica e a interações indesejáveis com a matriz alimentar. Por esse motivo, o desenvolvimento de embalagens contendo agentes antimicrobianos representa uma alternativa à aplicação direta de bacteriocinas em alimentos. A eficácia da atividade biológica pode até mesmo ser melhorada pelo controle da migração do peptídeo presente no filme antimicrobiano para o alimento, permitindo a inibição do desenvolvimento inicial de micro-organismos indesejáveis na superfície, bem como mantendo atividade residual ao longo da vida útil do produto.

No setor de embalagens de alimentos, polímeros são largamente empregados e vantajosos frente a outros materiais. Em paralelo aos polímeros convencionais sintéticos, aqueles obtidos a partir de recursos renováveis estão em evidência devido à maior conscientização quanto ao impacto ambiental. Porém, suas aplicações são limitadas devido a propriedades mecânicas, térmicas e de barreira geralmente insatisfatórias. Neste sentido, a adição de nanopartículas, especialmente nanoargilas, pode ser capaz de melhorar as características tanto destes materiais biodegradáveis como também dos polímeros convencionais, além de poder facilitar a liberação controlada de antimicrobianos.

Embalagens compostas por materiais híbridos, nas quais pelo menos um de seus componentes apresenta dimensões nanométricas, são denominadas nanocompósitos. Logo, o desenvolvimento de filmes nanocompósitos antimicrobianos é particularmente desejável devido à integridade estrutural e a propriedades vantajosas conferidas pelas nanopartículas dispersas na matriz polimérica, e ao efeito antimicrobiano exercido pelo agente bioativo natural associado. Portanto, a possibilidade de incorporação de bacteriocinas em nanocompósitos representa um grande potencial de inovação tecnológica no tocante a embalagens ativas para alimentos.

Neste contexto, o objetivo deste trabalho foi desenvolver filmes antimicrobianos para uso em alimentos a partir de nanoargilas, peptídeos antimicrobianos e polímeros sintético e biodegradável.

Os objetivos específicos do estudo foram:

- Desenvolver nanocompósitos de polipropileno e montmorilonita adicionados de nisina;
- Avaliar a adsorção dos peptídeos antimicrobianos nisina e pediocina em diferentes nanoargilas;
- Incorporar os peptídeos antimicrobianos em nanocompósitos poliméricos de amido com haloisita;
- Avaliar as propriedades estruturais, mecânicas, de barreira e térmicas dos filmes;
- Avaliar a atividade antimicrobiana *in vitro* dos nanocompósitos;
- Avaliar o efeito antimicrobiano dos filmes no desenvolvimento de *Listeria monocytogenes* em queijo minas frescal.

2. REVISÃO BIBLIOGRÁFICA

2.1. Peptídeos Antimicrobianos

Peptídeos com propriedades antimicrobianas são usados como primeira barreira química contra o ataque microbiano, sendo sintetizados em resposta a infecções bacterianas. São produzidos por quase todas as espécies de vida, desde micro-organismos, plantas e animais, até humanos (ESPITIA et al., 2012).

Os peptídeos antimicrobianos podem ser divididos em dois grandes grupos conforme sua biossíntese. Os peptídeos sintetizados não ribossomicamente são produzidos por micro-organismos por meio de uma enzima ou de um complexo enzimático, como gramicidinas, polimixinas, glicolipídeos, peptídeos cíclicos, lipopeptídeos, entre outros (HANCOCK, 1997). O outro grupo compreende os peptídeos produzidos por bactérias e liberados a partir da síntese proteica nos ribossomos, denominados bacteriocinas (KLAENHAMMER, 1993).

As bactérias podem representar a alternativa mais viável para obtenção de peptídeos antimicrobianos em nível comercial, os quais são produzidos por diferentes classes bacterianas, incluindo enterobacteriaceae, bactérias ácido lácticas, corineriformes, pertencentes ao gênero *Bacillus*, entre outras (BRANDELLI, 2012). Na indústria de alimentos, as bacteriocinas têm sido amplamente investigadas devido ao potencial uso como conservantes naturais (PAPAGIANNI, 2003; ESPITIA et al., 2012).

Bacteriocinas são definidas como peptídeos biologicamente ativos contra membros da mesma espécie ou espécies muito relacionadas à linhagem produtora (TAGG et al., 1976). Mais recentemente esta definição foi ampliada para incluir compostos similares que atuam contra cepas filogeneticamente distanciadas da cepa produtora. As chamadas substâncias tipo bacteriocinas (bacteriocin-like substance, BLS) englobam os compostos antimicrobianos de natureza proteica que ainda não estão completamente definidos ou não cumprem com todas as características das bacteriocinas, geralmente com um espectro de ação maior, atuando contra uma variedade de bactérias Gram-positivas, Gram-negativas e até alguns fungos (MESSENS & DE VUYST, 2002). O espectro de ação mais estreito e a síntese em ribossomos são as duas características principais que diferem as bacteriocinas de antibióticos clássicos, considerados metabólitos secundários e que inibem variável grupo de bactérias (CLEVELAND et al., 2001).

As bacteriocinas representam uma classe de antagonistas bacterianos heterogêneos que variam consideravelmente em seu modo de ação, espectro de atividade, massa molar, propriedades bioquímicas e origem genética (KLAENHAMMER, 1993). A maioria das bacteriocinas descritas até o momento apresentam baixa massa molar, variando de 30 a 60 aminoácidos e são estáveis ao calor. Geralmente, o modo de ação envolve despolarização da membrana da célula alvo ou inibição da síntese da parede celular de bactérias Gram-positivas, podendo ser efetivas contra Gram-negativas quando a membrana externa for desestabilizada, por exemplo, utilizando EDTA como agente quelante (ABEE et al., 1995). A bactéria produtora não é afetada pela ação da bacteriocina por possuir um mecanismo de imunidade específica (COTTER et al., 2005).

2.2. Classificação das Bacteriocinas

O sistema de classificação de Klaenhammer (1993) divide as bacteriocinas em quatro classes, conforme a diversidade de estruturas químicas e características.

A classe I corresponde a peptídeos pequenos (< 5 kDa) caracterizados por conter os aminoácidos não usuais dehidroalanina (Dha), dehidrobutirina (Dhb), lantionina ou β-metillantionina em sua molécula, devido à modificação pós-traducional da serina e tronina em suas formas dehidro. O aminoácido dehidro reage com a cisteína para formar anéis de tioéter de lantionina. As bacteriocinas quando contêm estes anéis são chamadas de lantibióticos. Com base nas suas características estruturais, sua carga e seu 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 subtilina, pep5 e 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. Outros exemplos são actagardina, cinamicina e mutacina A.

À classe II pertencem diversas bacteriocinas constituídas de pequenas moléculas de peptídeos termoestáveis, geralmente formadas de peptídeos não modificados menores de 10 kDa, as quais são subdivididas em três sub-classes: sub-classe IIa – correspondente a

peptídeos com uma seqüência de aminoácidos N-terminal comum (-Tir-Gli-Asn-Gli-Val-Xaa-Cis) e inclui as bacteriocinas pediocina PA-1, sakacina A e P, leucocina A, bavaricina MN e curvacina A, as quais têm atividade contra *Listeria monocytogenes.* Sub-classe IIb – contém 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). Sub-classe IIc – correspondente a peptídeos ativados por tiol, dependentes de baixas concentrações de cisteína para sua ação, como a lactococcina B. Além disso, dentro desta classe também podem ser encontradas bacteriocinas sem cisteína, já que esta classe inclui todas as bacteriocinas da classe II que não se enquadram nas sub-classes IIa e IIb. Outros exemplos são: cereína 7/8, enterocinas, acidocina B, divergicina A.

A classe III contém grandes proteínas antimicrobianas termolábeis, cuja massa molar é superior a 30 kDa, tais como a helveticinas J e V, produzidas pelos *Lactobacillus helveticus*, lactacinas A e B, e enterolisina, produzida pelo *Enterococcus faecium* (PAPAGIANNI, 2003).

A classe IV engloba proteínas complexas que requerem moléculas de carboidratos ou lipídios indispensáveis para a atividade. A composição e função dessas porções não-protéicas são desconhecidas. Nesta classe encontram-se leuconocina S, lactocina 27 e pediocina SJ-1. Porém, Cleveland et al. (2001) acreditam que este tipo de bacteriocina é um artefato devido às propriedades catiônicas e hidrofóbicas das bacteriocinas, o que resulta em complexação com outras macromoléculas em extrato bruto.

Uma nova proposta de classificação na qual as bacteriocinas são divididas em lantibióticos (classe I) e as que não contêm lantionina (classe II) foi sugerida por Cotter et al. (2005). Além disso, as bacteriocinas da classe III de Klaenhammer (1993) foram reclassificadas como “bacteriolisinas”, excluindo-se a classe IV.

Ainda não há consenso em como dividir as classes I e II em subclasses. Assim, Nes et al. (2007), mantiveram a classe IIa correspondente a bacteriocinas tipo-pediocina e classificaram a classe IIb como bacteriocinas duplo peptídeo, as que contêm um peptídeo-sinal típico na classe IIc e definiram as bacteriocinas circulares (modificadas pós-traducionalmente) como classe IId. No entanto, 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 et al. (2010). Nessa classificação, as bacteriocinas

conhecidas até o momento são divididas em 12 grupos, de acordo com as sequências de consenso dos aminoácidos que fazem parte de sua composição.

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 et al., 2002).

2.3. Nisina

A nisina foi descoberta no final dos anos 1920 e no início dos anos 1930, quando foi descrita como uma substância tóxica presente no leite que afetava adversamente a atuação das culturas iniciadoras dos queijos. Foi parcialmente purificada e analisada em 1947 por Mattick & Hirsch (*apud* Cotter et al., 2005), sendo que o desenvolvimento de uma preparação comercial contendo nisina, chamada Nisaplin, ocorreu em 1957 (Rogers & Whittier, 1928; Whitehead, 1933 *apud* DELVES et al., 1996). Essa bacteriocina foi considerada segura para uso em alimentos pelo Joint FAO/WHO Expert Committee on Food Additives (JECFA) em 1969. Na Europa, foi adicionada na lista de aditivos alimentares em 1983, designada como E234. Nos Estados Unidos, o FDA aprovou o uso da nisina em 1988. Atualmente é aprovada como preservativo de alimentos em mais de 40 países (COTTER et al., 2005), incluindo o Brasil (INS 234), onde é permitida desde 1996 para uso em queijos pasteurizados no limite máximo de 12,5 mg/kg (BRASIL, 1996).

Por se tratar de um peptídeo inócuo e sensível a proteases digestivas, por não produzir alterações nas propriedades organolépticas nos alimentos, ter status grau alimentício e histórico de uso seguro, a nisina é considerada um efetivo biopreservativo natural e a única bacteriocina permitida para uso em alimentos. É empregada em uma ampla gama de produtos, incluindo leite e derivados, tomates, ovo líquido, vegetais enlatados, sopas enlatadas, produtos cárneos, maionese e alimentos infantis (DE VUYST & VANDAMME; 1994; DELVES, 1996; DE MARTINIS, 2003).

A bacteriocina nisina é um peptídeo pequeno (3353 Da), catiônico, hidrofóbico, composto por 34 resíduos de aminoácidos. É produzida por linhagens de *Lactococcus lactis* subsp. *lactis* e sua biossíntese ocorre durante a fase exponencial de crescimento, cessando completamente quando as células entram na fase estacionária (PONGTHARANGKUL & DEMIRCI, 2004).

A nisina pertence à classe dos peptídeos lantibióticos por conter os aminoácidos *meso*-lantionina e 3-metil-lantionina, provavelmente responsáveis por importantes propriedades funcionais, como tolerância a acidez, termoestabilidade a baixo pH e modo de ação bactericida especifico (DE VUYST & VANDAMME, 1994). Além disso, a nisina ocorre em duas formas afins, A e Z, de estruturas e espectros de ação similares, mas diferem em um resíduo de aminoácido na posição 27, sendo histidina na nisina A e asparagina na nisina Z. Porém essa modificação estrutural confere à nisina Z características de alta solubilidade e difusão quando comparada à nisina A, o que deveria ser considerado quando da aplicação em alimentos. Entretanto, somente nisina A é comercializada, sendo disponível na forma de pó, o qual não é completamente solúvel (DE VOS et al., 1993).

Bactérias Gram-positivas dos gêneros *Lactococcus, Streptococcus, Staphylococcus, Micrococcus, Pediococcus, Lactobacillus, Listeria* e *Mycobacterium* são efetivamente inibidas pela nisina. O efeito deste antimicrobiano em células vegetativas é exercido sobre a membrana citoplasmática devido à formação de poros que alteram a força próton motriz e o pH de equilíbrio causando perda de íons e hidrólise de ATP, o que resulta em morte celular. Outro mecanismo de ação reconhecido e relatado por diferentes autores é a capacidade de a nisina ligar-se ao lipídeo II, o principal transportador das subunidades de peptideoglicano do citoplasma para a parede celular, impedindo assim a síntese da parede celular ou, ainda, usando o lipídeo II como uma molécula "âncora" para facilitar sua inserção na membrana celular, o que levaria à formação de poros e, consequentemente, à morte da célula (COTTER et al. 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, muitas bactérias Gram-positivas demonstraram ser resistentes à nisina devido à habilidade em sintetizar uma enzima, nisinase, que inativa a bacteriocina (ABBE et al., 1995; KAUR et al., 2011).

Esporos de *Bacillus* e *Clostridium* spp. são também suscetíveis a nisina, por isso, ela é amplamente utilizada na indústria de laticínios como preventivo de estufamento tardio em queijos. A ação contra esporos é causada pela ligação aos grupos sulfidril dos resíduos proteicos. Os esporos se tornam mais sensíveis à nisina quanto maior for o dano provocado pelo calor, o que é um importante fator do uso da nisina como preservativo de alimentos em alimentos processados termicamente (DELVES et al., 1996; MONTVILLE & CHEN, 1998).

2.4. Bacteriocinas do Tipo-Pediocina

Um grupo de peptídeos antimicrobianos bacterianos importante e bem estudado são os peptídeos antimicrobianos do tipo pediocina, muitas vezes denominados bacteriocinas do tipo-pediocina ou bacteriocinas da classe IIa, produzidos por uma variedade de bactérias ácido lácticas. Os primeiros peptídeos a serem identificados e caracterizados deste grupo foram pediocina PA-1 (do qual o termo bacteriocinas do tipo pediocina foi derivado), leucocina A, sakacina P, curvacina A e mesentericina Y105 (FIMLAND et al., 2005).

As bacteriocinas do tipo-pediocina são geralmente pequenas (<5 kDa) e não-modificadas, apresentam 36 a 48 resíduos de aminoácidos, cuja similaridade de sequência está entre 40% a 60%. Todas as pediocinas identificadas até o momento contêm duas cisteínas unidas por uma ponte dissulfeto na porção N-terminal, conhecida como "*pediocin box*": -Y-G-N-G-V-X_1-C-X_2-K/N-X_3-X_4-C-, com X_{1-4} representando resíduos polares carregados ou não-carregados (RODRÍGUEZ et al., 2002; PAPAGIANNI & ANASTASIADOU, 2009).

No geral, as bacteriocinas da classe IIa tem um espectro de atividade bastante estreito, reconhecidas por sua atividade antilisterial. Elas também são ativas contra algumas outras bactérias Gram-positivas, como *Clostridium* spp. e *Enterococcus* spp (PAPAGIANNI & ANASTASIADOU, 2009).

A representante mais estudada da "família das pediocinas" é a pediocina PA-1, também chamada de pediocina AcH. A maior parte do conhecimento atual sobre ela foi gerado a partir de 1992, ano em que a determinação de sua sequência de aminoácidos, a aplicação de protocolos mais avançados de purificação e a identificação do operon facilitaram grandemente as pesquisas sobre o assunto (RODRÍGUEZ et al., 2002). É a única bacteriocina da classe IIa sintetizada não apenas por diferentes espécies, como também por diferentes gêneros de bactérias lácticas. Inicialmente foi detectada em *Pediococcus acidilactici* PA 1.0 e H (GONZALEZ & KUNKA, 1987; BHUNIA et al., 1987 *apud* NASCIMENTO et al., 2008). Desde então, outras linhagens e espécies de *Pediococcus* foram descritas como produtoras de pediocina AcH, embora, em muitos casos a bacteriocina recebia diferentes nomes (por exemplo, pediocinas PA-1, AcH, JD, Bac e 347) antes de sua identificação e percepção de que todas eram a mesma molécula (RODRÍGUEZ et al., 2002). Ennahar et al. (1996) isolaram em queijo Munster uma linhagem de *Lactobacillus plantarum* também produtora desta bacteriocina.

Há potencial uso da pediocina PA-1 como preservativo de alimentos, considerando que já é comercialmente explorada como um ingrediente alimentar fermentado por *Pediococcus acidilactici*, ALTA^{TM} 2345, com o objetivo de estender a vida útil de uma variedade de alimentos, particularmente, a inibição do desenvolvimento de *Listeria monocytogenes* em produtos cárneos prontos para consumo. O uso dessa bacteriocina em carnes, queijos e saladas é protegido por patentes americanas e européias (ENNAHAR et al., 2000; RODRÍGUEZ et al., 2002).

A pediocina PA-1 é um peptídeo que contém 44 aminoácidos, catiônico com ponto isoelétrico em pH básico, sem modificações pós-traducionais. O peso molecular pode ser 4628 ou 4624 Da, na ausência ou presença de pontes dissulfeto, respectivamente (RODRÍGUEZ et al., 2002). Possui solubilidade em soluções aquosas, atividade em ampla faixa de pH e não é afetada por calor ou congelamento (NES et al., 1996). Demonstra ser mais efetiva que a nisina contra patógenos alimentares como *Listeria monocytogenes* e *Staphylococcus aureus* (CINTAS et al., 1998).

O modo de ação bactericida da pediocina PA-1 em células sensíveis inicia-se quando a bacteriocina liga-se a receptores na superfície da célula, provavelmente devido a sua carga positiva fazendo com que interações com as membranas bacterianas contendo fosfolipídios carregadas negativamente e/ou com as paredes celulares bacterianas com caráter ácido. Posteriormente, as moléculas inserem-se na membrana pela porção C-terminal, havendo permeabilização devido ao caráter anfifílico/hidrofóbico de parte da sequência da pediocina. Alterações na conformação ocorrem, desestabilizando-a e ocorrendo a formação de poros, com a conseqüente dissipação da força próton motriz. Ocorre uma aceleração do consumo de ATP e o processo finalmente leva à célula a morte, que pode ocorrer com ou sem lise celular, provavelmente dependente da ativação concomitante de autolisinas celulares (RODRÍGUEZ et al., 2002; FIMLAND et al., 2005; PAPAGIANNI & ANASTASIADOU, 2009).

2.5. Aplicações de Bacteriocinas em Sistemas Alimentares

Na produção de alimentos é importante que ações adequadas sejam realizadas para garantir a segurança e a estabilidade durante a vida-de-prateleira. Atualmente, os consumidores requerem alimentos de alta qualidade (nutricional e organoléptica), sem conservantes químicos, seguros e com elevada vida útil. Observa-se também uma crescente

demanda dos consumidores por alimentos convenientes, que possam ser adquiridos prontos para o consumo ou de rápido preparo, refrigerados ou congelados (ROSS et al., 2002; DE MARTINS et al., 2003; SOUZA et al., 2005).

Para atender a estas exigências, as indústrias alimentícias estão produzindo alimentos mais frescos, submetidos ao processamento mínimo, com menor quantidade ou isentos de conservadores químicos e que dependem, em grande parte, de sua manutenção em baixas temperaturas ou atmosferas modificadas para garantir a segurança microbiológica (ENHHAR et al., 1996; GÁLVEZ et al., 2007). Dessa forma, evidencia-se uma cadeia produtiva mais complexa e longa, com a exigência de introdução de novas e complementares tecnologias de preservação de alimentos, considerando o risco de contaminação, em paralelo à emergência de patógenos psicrotróficos, como *Listeria monocytogenes* (GARCÍA et al., 2011).

Para fornecer suficiente proteção contra a contaminação microbiana, as bacteriocinas devem ser aplicadas aos alimentos como uma parte de um sistema com múltiplos obstáculos ao desenvolvimento de micro-organismos patogênicos e deteriorantes. A tecnologia de métodos combinados tem demonstrado efeitos sinérgicos ou adicionais de bacteriocinas quando em conjunto com outros compostos ou tratamentos físicos, permitindo a redução da adição de antimicrobianos sintéticos e da 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 (DEEGAN et al., 2006, GÁLVEZ et al., 2007).

A bioconservação de alimentos consiste no emprego de uma microbiota natural ou controlada, bem como de metabólitos antimicrobianos de micro-organismos para controle de agentes patogênicos e deteriorantes. Os peptídeos antimicrobianos de bactérias ácido lácticas (BAL) têm atraído considerável interesse para a bioconservação de alimentos devido ao seu *status* GRAS – *Generally Regarded as Safe*. Com isso, podem ser introduzidos por alimentos fermentados sem prévia purificação ou concentração (COTTER et al., 2005). As duas únicas bacteriocinas disponíveis comercialmente, nisina e pediocina PA-1, são produzidas por BAL (DEEGAN et al., 2006). Deste modo, as bacteriocinas podem ser incorporadas na matriz alimentar por meio de culturas iniciadoras produtoras de bacteriocinas (culturas de BAL para produção *in situ*), pela adição direta de aditivos antimicrobianos purificados (como a nisina, comercializada como Nisaplin®) ou pela adição de ingredientes contendo bacteriocina (como observado para a pediocina PA-1 através do produto ALTA^{TM} 2345) (SCHILLINGER et al., 1996; COTTER et al., 2005).

A eficácia das bacteriocinas em alimentos depende de vários fatores, como aqueles relacionados ao próprio alimento: 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 (gordura ou proteínas), baixa solubilidade e até mesmo distribuição das moléculas de bacteriocina na matriz alimentar, 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).

Frente a estas limitações, há possibilidade de aplicar as bacteriocinas ex situ na forma de preparações imobilizadas, em que a bacteriocina parcialmente purificada ou o meio de cultivo concentrado é ligado a um suporte. Este suporte age como um reservatório e um difusor das moléculas da bacteriocina no alimento, exige menor quantidade de bacteriocina (comparando-se à aplicação no volume total do alimento) e pode também proteger o peptídeo da inativação por enzimas ou por interação com componentes do alimento, por exemplo. Entre as formas de imobilização, citam-se: adsorção às células produtoras e a superfícies, como sílica; encapsulação em lipossomas; incorporação em géis de revestimento e filmes de diferentes materiais, como alginato de cálcio, gelatina, celulose, proteínas de soja, amido de milho, colágeno, celofane, coberturas de silício, polietileno, nylon e outros filmes plásticos poliméricos (GALVÉZ et al., 2007).

De modo especial, sistemas de embalagem de alimentos e carreadores de peptídeos oferecem oportunidades inovadoras para explorar o potencial de peptídeos antimicrobianos visando à segurança dos alimentos. Embora as embalagens de alimentos tradicionais ofereçam suporte mecânico e proteção contra influências externas e deveria ter mínima interação com o alimento, embalagens antimicrobianas/bioativas 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).

2.6. Embalagens de alimentos contendo peptídeos antimicrobianos

Os agentes antimicrobianos podem ser diretamente incorporados em polímeros, podem ser adsorvidos ou revestir a superfície do polímero, ou podem ser imobilizados aos polímeros por ligações iônicas ou covalentes (APPENDINI & HOTCHKISS, 2002). De qualquer forma, o modo de atividade dos agentes antimicrobianos é um importante fator. As bacteriocinas são ideais para aplicação em embalagens antimicrobianas porque elas interagem com a superfície externa do micro-organismo (parede celular e membrana plasmática) e não necessitam ser internalizadas para exibirem o efeito antimicrobiano (MILLS et al., 2011). Neste sentido, Quintavalla & Vicini (2002) estabelecem que o controle da contaminação microbiana nos alimentos pode ocorrer mediante três mecanismos: redução da taxa de crescimento dos micro-organismos, prolongamento da fase *lag* e inativação por contato.

Peptídeos incorporados diretamente nos filmes poliméricos devem ser capazes de difundir para a superfície da embalagem ao longo do tempo para serem efetivos. A nisina, incorporada a uma concentração de 1000 UI/cm^2 em filmes de caseinato plastificados com sorbitol, foi capaz de reduzir a contagem de *Listeria innocua* em queijo inoculado na superfície em 1,1 log (CAO-HOANG et al., 2010). A bactéria foi inoculada também em profundidade no queijo e o efeito antimicrobiano foi dependente da distância entre a superfície de contato dos filmes contendo nisina e a matriz do queijo, sendo que a inativação foi de 1,1, 0,9 e 0,25 log UFC/g para distâncias da superfície de 1, 2 e 3 mm, respectivamente (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 de *Salmonella* Enteritidis em clara de ovo líquida (JIN & ZHANG, 2008). Em contrapartida, no estudo de Liu et al. (2009), a nisina foi incorporada diretamente em PLA por extrusão, ou seja, após a fusão do PLA em 160ºC juntamente com ácido lático, lactídeo ou glicerol, a temperatura foi diminuída para 120ºC (para evitar a inativação do antimicrobiano) e o Nisaplin foi adicionado, dando sequência à extrusão. Os extrusados resultantes foram capazes de suprimir o crescimento de *L. monocytogenes*, demonstrando atividade antimicrobiana significativa (LIU et al. 2009).

O revestimento da superfície com o peptídeo antimicrobiano é uma alternativa quando o polímero requer condições de processamento extremas durante o preparo do material da embalagem, tais como alta temperatura ou pressão, o que pode resultar em inativação do agente antimicrobiano (APPENDINI & HOTCHKISS, 2002). Em alguns casos, o revestimento é feito pelo contato do filme ou imersão em solução contendo o peptídeo. Neste sentido, Scannell et al. (2000) usaram alternativamente lacticina 3147 e nisina adsorvidas na superfície de sacos plásticos de polietileno/poliamida através do contato direto do material polimérico com a solução da bacteriocina. Os filmes com nisina demostraram atividade inibitória contra *Listeria innocua* e *Staphylococcus aureus*, mantendo a atividade por 3 meses a temperatura ambiente e sob refrigeração. Quando aplicados em queijos Cheddar, intencionalmente inoculados na superfície com *Listeria innocua*, reduziram em 2 ciclos logaritmos os níveis da bactéria quando estocados a 4ºC durante um período de 12 semanas. Entretanto, a lacticina 3147 não adsorveu ao plástico usado neste estudo, o que pode estar relacionado a natureza dos dois componentes, requerendo adsorção de ambos os componentes para atividade ou interferência de outras proteínas na preparação da lacticina 3147 (SCANNELL et al., 2000). Ainda neste estudo, embalagens bioativas à base de celulose impregnadas com nisina (7650 UA/cm^2) reduziram os níveis de *Listeria* em pouco mais que 2 logs e *Staphylococcus aureus* em 1,5 log quando intercaladas em pedaços de presunto e queijo por um período de 24 dias a 4ºC (SCANNELL et al., 2000).

Filmes de polietileno revestidos com nisina (preparados a partir de uma solução estoque de nisina a uma concentração de 6400 UA/mL) mostraram-se efetivos para inibição de *Micrococcus luteus* em caldo e da microbiota bacteriana em leite, resultando na redução de 0,9 log em leite cru e 1,3 log em leite pasteurizado estocado a 4ºC por sete dias (MAURIELLO et al., 2005).

A liberação controlada da nisina foi alcançada em filmes com camadas hidrofóbicas e hidrofílicas compostas por etilcelulose/hidroxiproprilmetilcelulose/etilcelulose (EC/HPMC/EC) (GUIGA et al., 2010). A nisina presente nos filmes compostos por duas camadas (EC/HPMC) totalmente dessorveu dentro de 0,5 h, enquanto os filmes de três camadas (EC/HPMC/EC) aumentaram o tempo de liberação da nisina para 20 h e mostraram atividade antimicrobiana significativa. A liberação controlada do agente antimicrobiano pode ser altamente vantajosa, assegurando que um nível constante atinja a superfície do alimento. Isso pode também eliminar o risco de inativação do conservante pelos componentes dos

alimentos ou diluição abaixo da concentração ativa devido à migração em grande quantidade para a matriz alimentar (APPENDINI & HOTCHKISS, 2002).

Tripas de celulose revestidas internamente com pediocina produzida por *Pediococcus acidilactici* K em meio a base de leite desnatado, demonstraram adequada retenção da bacteriocina quando da aplicação de 9,30 mg/cm^2 resultando em zonas de inibição de *Listeria monocytoges* em ágar semi-sólido. Além disso, sacos plásticos revestidos com a pediocina inibiram significativamente o crescimento de *L. monocytogenes* inoculada artificialmente sobre a superfície de peito de peru, presunto e carne bovina fresca após 12 semanas a 4ºC quando comparados a controles não inoculados embalados com os mesmos plásticos antimicrobianos (MING et al., 1997). Enquanto que filmes de acetato de celulose contendo pediocina comercial ALTA® 2351, dispostos entre fatias de presunto, reduziram o número de *Listeria* em 2 logs após 15 dias de estocagem a 12ºC (SANTIAGO-SILVA et al., 2009).

A eficácia das bacteriocinas em embalagens depende de vários parâmetros como pH, temperatura, concentrações de sais e gordura. Estes parâmetros apresentam importante papel na solubilidade, bioatividade, estabilidade, taxa de desorção do filme e difusão nas matrizes alimentares (GUIGA et al., 2010).

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 com soluções simulantes de alimentos (BRASIL, 2010). Migração total se refere à quantidade de componentes transferida dos materiais em contato com alimentos ou seus simulantes, sendo que migração específica se refere à quantidade de um componente não polimérico particular transferido do material para o alimento ou simulante.

2.7. Nanocompósitos

O uso de cargas em nanoescala nas matrizes poliméricas tem sido explorado para atender aos requerimentos de desempenho, bem como considerações de custo e processamento da maioria das aplicações atuais de polímeros. As partículas em tamanho

nanométrico oferecem maior área superficial, propiciando vantagens no comportamento de polímeros quando comparadas a cargas macro e microscópicas (ESTEVES et al., 2004; AZEREDO et al., 2009).

A classe de materiais formada por híbridos em que pelo menos um dos componentes tem dimensões nanométricas, geralmente entre 1 e 100 nm, é denominada nanocompósitos. Tal como acontece nos compósitos tradicionais, um dos componentes serve de matriz, na qual as partículas do segundo material se encontram dispersas (ESTEVES et al., 2004).

As cargas utilizadas como precursores na obtenção de nanocompósitos apresentam diferentes dimensões na escala nanométrica, citando-se: as lamelares, que apresentam uma dimensão (argilas, grafite); fibrilares e tubulares, as quais apresentam duas dimensões nanométricas (nanofibras e nanotubos de carbono, nanowhiskers de celulose); isodimensionais, com as três dimensões nanométricas (nanopartículas esféricas de sílica e de carbonato de cálcio, nanopartículas metálicas, negro-de-fumo) (MAI & YU, 2006). Dentre estes potenciais precursores de nanocompósitos, as nanoargilas têm sido frequentemente utilizadas devido a sua disponibilidade, baixo custo, processamento relativamente simples e melhorias significativas nas propriedades funcionais dos polímeros (AZEREDO, 2009).

Os silicatos em camada ou filossilicatos, especialmente a argila montmorilonita (MMT), têm sido amplamente investigados. Isso se deve à habilidade da MMT em dispersar em folhas individuais e a possibilidade em modificar sua superfície química através de reações de troca de íons com cátions orgânicos ou inorgânicos (GIANNELIS, 1996; PAIVA et al., 2008). A MMT possui partículas de tamanhos que podem variar de 2 μm a tamanhos bastante pequenos como 0,1 μm 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, com fórmula geral $M_x(Al_{4-x}Mg_x)Si_8O_{20}(OH)_4$ (PAIVA et al., 2006). A MMT é um material naturalmente hidrofílico, o que torna a sua esfoliação difícil em uma matriz polimérica hidrofóbica. Por esta razão, 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).

Recentemente os nanotubos de haloisita (HNT) têm atraído interesse como nanopartículas de polímeros. A haloisita é um membro importante do grupo das caulinitas com composição $Al_2Si_2O_5(OH)_4.nH_2O$. Apresenta uma estrutura tubular oca como morfologia

dominante, semelhante a de nanotubos de carbono, e suas dimensões típicas são em escala nanométrica. A superfície da HNT é composta de siloxano e tem poucos grupos hidroxil, o que indica que HNT possui um potencial para formação de ponte de hidrogênio, favorecendo uma boa dispersão na matriz polimérica. Além disso, a maior rigidez dos nanotubos de HNT em comparação com plaquetas de MMT e a alta razão comprimento-diâmetro dos tubos podem promover excelente nanoreforço em nanocompósitos poliméricos (CARLI et al., 2011).

Há três processos principais para preparação de nanocompósitos poliméricos com argilas:

- esfoliação-adsorção (método de "casting"): argila é esfoliada utilizando um solvente no qual o polímero é solúvel. Após a adsorção às camadas delaminadas da argila, o solvente é evaporado e a estrutura do nanocompósito esfoliado é formada;

- intercalação por polimerização *in-situ*: a argila é inchada dentro do monômero líquido (ou em uma solução do monômero) de modo que a polimerização possa ocorrer permitindo que o polímero consiga produzir a intercalação das camadas da argila;

- intercalação no estado fundido (via extrusão, "melt processing"): a argila é misturada com a matriz de polímero no estado fundido. O polímero pode migrar para o espaço interlamelar e formar tanto nanocompósitos intercalados como esfoliados. Nesta técnica, nenhum solvente é necessário (ALEXANDRE et al., 2000).

O polipropileno é um dos polímeros mais utilizados atualmente devido ao seu baixo custo, fácil processabilidade, reciclabilidade, boas propriedades mecânicas, resistência química e ao calor, com grande aplicação no setor alimentício (PRACHUM et al., 2011; SIRACUSA et al., 2012). No tocante a nanocompósitos, a combinação deste polímero com montmorilonita é comumente usada objetivando melhorar suas propriedades. Zehetmeyer et al. (2012) obtiveram aumento significativo na resistência ao impacto, rigidez e propriedades de barreira ao oxigênio em filmes de polipropileno (PP) incorporados com montmorilonita (MMT) modificada organicamente com um sal de amônio quaternário. Quando aplicados para embalagem de suco de laranja, os filmes foram capazes de manter as características físico-químicas e microbiológicas do produto por 10 dias. As análises físico-químicas avaliadas foram densidade, sólidos solúveis totais, açúcares redutores e não-redutores, ácido cítrico, sólidos insolúveis e ácido ascórbico. Quanto às análises microbiológicas, foram realizadas contagem de micro-organismos aeróbios mesófilos, bolores e leveduras e coliformes totais e termotolerantes (ZEHETMEYER et al., 2012).

Como a maioria dos materiais de embalagem tradicionais é formada a partir de materiais não biodegradáveis, os quais consomem combustíveis fósseis para sua produção e contribuem para a poluição ambiental, filmes biodegradáveis estão despertando interesse. Porém, estes filmes exibem baixas propriedades mecânicas e de barreira e, assim, a incorporação de nanoargilas em biopolímeros é uma alternativa promissora para a melhoria destas propriedades e para a expansão do uso de materiais ambientalmente "amigáveis" em detrimento aos plásticos convencionais. Além disso, a biodegradabilidade do material de embalagem pode ser elevada pela introdução de partículas inorgâncias (SOZER & KOKINI, 2009). O amido é um dos polímeros mais utilizados para compor materiais biodegradáveis pelo seu baixo custo, rápida taxa de biodegradação e disponibilidade. Park et al. (2003) observaram um aumento significativo da tensão de ruptura, da capacidade de alongamento e permeabilidade ao vapor d'àgua em compósitos de amido-nanoargilas com 5% de montmorilonita. Da mesma forma, Slavutsky et al. (2012) evidenciaram que a solubilidade e a permeabilidade ao vapor de água diminuíram com o aumento do teor de montmorilonita em filmes de amido de milho e ambas as propriedades foram afetadas pelo método de dispersão.

As argilas constituem barreiras não somente à água, mas também a gases, já que os forçam a seguir uma via tortuosa ao redor das camadas de argila, levando uma longa passagem para difundir através do filme. Outros benefícios relatados sobre a melhoria do desempenho de uma diversidade de polímeros como resultado do uso de nanopartículas de argila incluem o aumento da transição vítrea e das temperaturas de degradação térmica (AZEREDO, 2009). Nanotubos de haloisita contribuíram para aumentar a estabilidade térmica de compósitos com amido, além de reforçar a viscosidade da pasta, a força de tração e a barreira ao vapor d´água dos filmes, de acordo com Xie et al. (2011).

A utilização de nanomateriais em embalagens possibilita, ainda, 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 e KOKINI, 2009). O uso de antimicrobianos como compostos bioativos em embalagens de alimentos está ganhando interesse na indústria e por parte de pesquisadores em vista do potencial para promover a qualidade e a segurança.

A incorporação de antimicrobianos em embalagens pode ajudar no controle do crescimento superficial (local mais suscetível à contaminação) de micro-organismos patogênicos e deteriorantes nos alimentos e, consequentemente, evitar desperdícios e doenças

transmitidas por alimentos (ASSIS et al., 2012). Os materiais em nanoescala apresentam uma alta razão superfície-volume quando comparado aos homólogos em microescala, o que permite a ligação a mais cópias de moléculas bioativas, podendo até mesmo reduzir a quantidade incorporada de antimicrobianos normalmente utilizados (AZEREDO, 2009).

3. RESULTADOS E DISCUSSÃO

Os resultados obtidos neste trabalho estão apresentados na forma de artigos. Além dos artigos científicos, elaborou-se uma revisão bibliográfica a respeito de nanocompósitos de polímeros biodegradáveis como embalagens para alimentos, a qual fará parte de um capítulo no livro **"**Green Polymer Composites Technology: Properties and Applications" a ser publicado pela Editora CRC Press.

Os resultados são apresentados como quatro artigos técnico-científicos que contemplam os estudos realizados ao longo deste doutorado. O primeiro artigo está publicado no periódico Food and Bioprocess Technology e refere-se ao desenvolvimento e caracterização de nanocompósitos de polipropileno e montmorilonita adicionados de nisina. O segundo artigo foi submetido ao periódico Food Chemistry e trata da adsorção de nisina e pediocina em nanoargilas. O terceiro artigo está formatado para a revista Carbohydrate Polymers e está relacionado à elaboração de filmes de amido pelo método de *casting* com a incorporação de nisina e pediocina, bem como a avaliação do efeito da adição de nanotubos de haloisita (adsorvidos ou não de bacteriocinas) sobre a matriz polimérica. O quarto artigo está formatado para o periódico Food and Bioprocess Technology e trata-se da caracterização de filmes antimicrobianos de nisina em matriz polimérica de amido com nanopartículas de haloisita preparados pelo método de extrusão e sua aplicação em queijo Minas Frescal para controle de *Listeria monocytogenes*.

Posteriormente, elaborou-se uma discussão geral a respeito dos resultados apresentados nesta tese.

ARTIGO 1 – Polypropylene/montmorillonite nanocomposites containing nisin as antimicrobial food packaging

Abstract

Antimicrobial nanocomposites prepared with polypropylene, montmorillonite and nisin were developed as food packaging material. Nisin was incorporated at 1, 2.5 and 5% (w/w) and the characterization included antimicrobial, mechanical, thermal, barrier and structural properties. Composite films inhibited the Gram-positive bacteria *Listeria monocytogenes, Staphylococcus aureus* and *Clostridium perfringens* when tested on skimmed milk agar plates. Antimicrobial activity was released in food simulants after contact with the nanocomposites, increasing until 48 h in solutions containing the surfactant Tween 20 or acetic acid. The addition of nisin caused no significant modification in deformation at break values as compared with control films. However, results of tensile strength and Young modulus differed significantly among samples. The higher value for Young modulus was observed for films with 5% nisin. Water vapor barrier properties were not significantly different among control and antimicrobial films, whereas oxygen permeability was higher for nanocomposites containing nisin. The nanocomposites tested had no significant differences in the melting temperature (165 to 167°C), and the crystallization temperature ranged from 121 to 129°C, with lower values for films containing 5% nisin. Scanning electron microscopy showed that nanocomposites containing 1 and 2.5% nisin present similar homogeneity to that of control films. Some film properties were affected after nisin incorporation in PP/MMT matrix but active antimicrobial films were obtained, showing suitable behavior as a food packaging material.

Keywords: polypropylene; montmorillonite; nanocomposite; antimicrobial

Introduction

The actual tendency of consumers for better quality, minimally processed, healthier, safe and convenient food products, including the demand for additive-free foods or the use of natural compounds for food preservation, increased attention for antimicrobial active packaging concept. This technology could play a role in extending shelf life of food and reduce the risks of pathogen contamination (Appendini and Hotchkiss 2002; Quintavalla and Vicini 2002). Meanwhile, the choice of film and antimicrobial substance is often made on a case-by-case

basis considering at least target foods and bacteria and storage conditions (La Storia *et al*. 2013).

Nisin is an interesting alternative to synthetic additives in packaging materials. This substance produced by *Lactococcus lactis* ssp. *lactis* is considered a natural preservative and the most widely used bacteriocin for food applications (Cotter *et al*. 2005; Snyder and Worobo 2014). Nisin is a non-toxic heat stable antimicrobial peptide, sensitive to digestive proteases and does not contribute to off-flavors. This bacteriocin has a wide spectrum activity against Gram-positive bacterial strains like those belonging to *Listeria* and *Staphylococcus* genera, and shows an effective growth inhibition of *Bacilli* and *Clostridia* spores (Arauz *et al*. 2009). A better efficiency in food protection can be achieved by incorporating nisin into packaging films rather than direct addition in the food matrix (Marcos *et al.* 2013), since some loss of nisin activity can occur due to proteolytic degradation or cross-reaction with food components such as lipids or proteins (Gálvez *et al*. 2007).

Nisin has a dual mode of action against target pathogens. It can bind to lipid II, the main transporter of peptidoglycan subunits from the cytoplasm to the cell wall, and at sufficiently high concentrations, it inhibits cell wall synthesis leading to cell death (Snyder and Worobo 2014). Furthermore, nisin can use lipid II as a docking molecule to initiate a process of membrane insertion and pore formation that leads to rapid cell death (Cotter *et al*. 2005). Then, nisin is interesting for incorporation into antimicrobial packaging because it interacts with the microbial surface and does not have to be internalized to exhibit the antimicrobial effect. In addition, the antimicrobial substance incorporated into packaging materials can control microbial contamination by reducing the growth rate and maximum growth population, extending the lag-phase of the target microorganism, or by inactivating microorganisms by contact (Quintavalla and Vicini 2002).

Concerning innovative packaging systems, nanocomposites comprise a new class of materials in which the dispersed phase is nanostructured (Azeredo 2013). Nanoclays, especially hydrated alumina-silicate clays called montmorillonites (MMT), are nanostructures commercially available for food packaging. MMTs are considered environmentally friendly, naturally abundant and inexpensive (Hatzigrigoriou and Papaspyrides 2011). In particular, polymer-layered silicate nanocomposites have attracted great interest, because they often exhibit remarkable improvement in material properties when compared with the pristine polymer and they have the potential to introduce novel properties and features to the food packaging industry (Hatzigrigoriou and Papaspyrides 2011; Azeredo 2013).

Polypropylene (PP) is one of the most widely used polymers in large-scale production due to low cost, recyclability and easy processing (Castel *et al*. 2011). Regarding nanocomposites for food packaging, better mechanical and oxygen barrier properties is obtained in PP films incorporated with an organically modified MMT. When these PP/MMT nanocomposites were applied for orange juice packaging, the physicochemical and microbiology quality after 10 days of storage was maintained (Zehetmeyer *et al*. 2012).

In relation to studies about nisin in PP polymer matrix, a nisin-incorporated whey protein isolate coating on PP film has been previously reported (Lee *et al*. 2008). However, to the best of our knowledge, the incorporation of bacteriocins for the development of polymer-clay nanocomposites as food packaging has not been reported yet. Besides, antimicrobial agents are often adsorbed onto polymer surfaces or immobilized to polymers by ion or covalent linkages (Appendini and Hotchkiss 2002) and the direct incorporation of nisin into the polymeric matrix is not common (Scaffaro *et al*. 2011). This last approach was chosen in this study to develop antimicrobial nanocomposites of PP and MMT plus nisin, without using organic solvent, which has positive environmental and economic implications. Therefore, the aim of this investigation was to develop and characterize PP/MMT nanocomposite films containing nisin.

Materials and Methods

Materials

A commercial grade of polypropylene (Braskem S.A., Triunfo, Brazil) with a melt flow index (MFI) 3.5 g 10^{-1} min^{-1} (230°C/2.16 kg) and density 0.905 g cm^{-3} (23°C) was used. The nanoclay montmorillonite (MMT) Cloisite 15A, modified with a quaternary ammonium salt, was purchased from Southern Clay Products (Austin, TX, USA). Commercial nisin (Nisaplin®) was provided by Danisco Brasil Ltda (Cotia, Brazil).

Preparation of nanocomposites

Nanocomposites were prepared with a clay content of 2% (w/w) by melt mixing using a co-rotation twin-screw extruder Coperion with a screw diameter of 18 mm and L/D 44 (model ZSK18, Stuttgart, Germany), prior to addition of the antimicrobial. The temperature profile (feed to die) was 165-190°C, with a speed of 350 rpm and a constant feed rate of 5 kg h^{-1}. The nanocomposites were granulated in a granulator (Sagec SG-35, Diadema, Brazil) as described

by Zehetmeyer *et al*. (2012). For nisin incorporation, the granular materials were finely ground under a nitrogen atmosphere and the antimicrobial was mixed as a powder at three different concentrations: 1, 2.5 and 5% (w/w), based on previous works incorporating nisin in polymeric films (Bastarrachea et al 2010; Scaffaro *et al* 2011). The active antimicrobial films were obtained by compression molding at 180°C in a hot press (Carver Inc., Model 3710-ASTM, Wabash, IN, USA). The material was kept between the plates at atmospheric pressure for 3 min until melting and then it was pressed under 2.5 MPa for 2 min. An additional sample without nisin was also prepared and used as control.

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. Then, 10 ml BHI soft agar (7.5 g l^{-1}) inoculated with indicator strain *Listeria monocytogenes* ATCC 7644 (10^7 CFU ml 1^{-1}) was poured onto plates. Petri dishes were stored at 4°C during 12 h to initiate nisin desorption and after incubated at 37°C for 24 h. The antimicrobial activity is evidenced by clear zones (no micro-organism growth or survival) surrounding film pieces.

Similar test was carried out on skimmed milk agar (SMA) to simulate a condition for solid food packaging. The SMA comprised 5.0 g l^{-1} peptone, 3.0 g l^{-1} yeast extract, 100 ml l^{-1} UHT skimmed milk, and 12 g l^{-1} agar. In this case, *L. monocytogenes* ATCC 7644, *Bacillus cereus* ATCC 14579, *Staphylococcus aureus* ATCC 1901 and *Clostridium perfringens* ATCC 3624 were used as indicator strains.

All bacteria were stored at -20°C in 20% (v/v) glycerol and propagated twice on BHI broth before use. All determinations were carried out in triplicate (n=3).

Nisin migration test

The migration of nisin from the packaging films was tested into three simulant solutions (Anvisa 2001): distilled water, physiological solution with 5% (v/v) Tween 20 (Merck KGaA, Darmstadt, Germany) and a 3% (v/v) acetic acid solution (Dinamica Ltda, Diadema, Brazil). An amount of 0.25 g of each film was immersed into 3 ml of simulant solutions and shaken at 150 rpm at 25°C. After 3, 24 48 and 72 h, aliquots were removed for determination of antimicrobial activity onto BHI agar plates previously inoculated with a swab submerged in the indicator strain suspension (*L. monocytogenes* ATCC 7644), which corresponded to

approximately 10^7 UFC ml^{-1}. The reciprocal value of the highest dilution that produced an inhibition zone was taken as the activity unit (AU) per ml (Motta and Brandelli 2002).

Mechanical properties

Tensile tests were carried out using films with 8 mm X 50 mm of size under crosshead speed of 25 mm min^{-1} in a universal testing machine (EMIC DL 10000, São José dos Pinhais, Brazil) equipped with a 50 N load cell, according to ASTM D-638 standard. The samples were acclimatized for 24 h at 23 ± 2°C with humidity 50% ± 5 before analysis.

Scanning electron microscopy (SEM)

The film surfaces and cross-sections were analyzed using a JEOL microscope (model JSM-5800, Tokyo, Japan) operated at a voltage of 10 kV. Samples were coated with gold layer prior to analysis in order to increase their electrical conductivity. Images were registered at 1000 X and 200 X magnification in order to study their homogeneity.

Differential scanning calorimetry (DSC)

Crystallization characteristics were determined using a DSC Thermal Analyst 2100 (TA Instruments, New Castle, DE, USA) where linear heating and cooling experiments were performed at 10°C min^{-1} under a dry nitrogen atmosphere (50 mL min^{-1}). All samples (ca 10 mg) were heated from ambient temperature to 220°C and kept for 5 min to erase the thermal history. The samples were then cooled down to -30°C and heated again until 220°C. To calculate the crystallinity of PP, a value of melting enthalpy of 190 J g^{-1} was used (Zehetmeyer *et al*. 2012).

Thermogravimetric analysis (TGA)

A thermogravimetric analyzer model QA 50 (TA Instruments, New Castle, DE, USA) was used for the thermal stability evaluation. The samples were heated from 25 to 800°C at the rate 10°C min^{-1} under nitrogen atmosphere (50 mL min^{-1}).

X-Ray diffraction (XRD)

XRD measurements were performed using a Siemens D-500 diffractometer (Siemens, Karlsruhe, Germany). Nisin powder and films were scanned in the reflection mode using an incident Cu K_α radiation ($\lambda = 1.54$ Å), at a step width of 0.05°min^{-1} from $2\theta = 1°$ to 35°C.

Oxygen and water vapor permeability

An oxygen permeation analyzer OX-TRAN 2/21 (Mocon, Minneapolis, MN, USA) was used to determine oxygen barrier properties of the films at 23°C and 0% relative humidity, in duplicate, according to ASTM F-1927. The detector used was a coulometric oxygen sensor. The test was terminated automatically after stabilization in the permeation and using synthetic air (20% O_2) as permeant gas.

Permeability to water vapor was performed with a PERMATRAN-W 3/33 (Mocon, USA) at 37.8°C and 90% relative humidity, in duplicate, according to ASTM F-1249. The detector used was an infrared sensor, and the test was terminated automatically after stabilization in permeation.

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, using the SAS software (version 9.3).

Results and Discussion

Antimicrobial activity and nisin migration

Incorporation of nisin at three different concentrations (1, 2.5 and 5%) into nanocomposites resulted in active packaging films with visible inhibition zones against the indicator strain *L. monocytogenes* (Fig. 1A), increasing as higher amounts of nisin were added. As expected, control film without nisin had no antimicrobial activity. When the test was carried out on SMA plates, as a food simulant, inhibition zones were obtained against *L. monocytogenes, S. aureus* and *C. perfringens* and only a smaller inhibition zone was observed for nanocomposite with 5% nisin against *B. cereus* (Table 1). These results indicate that nisin can be released from the nanocomposites prepared by compression molding at 180°C (temperature required for PP melting) and the materials displayed antimicrobial properties against the Gram-positive bacteria in BHI and SMA agar.

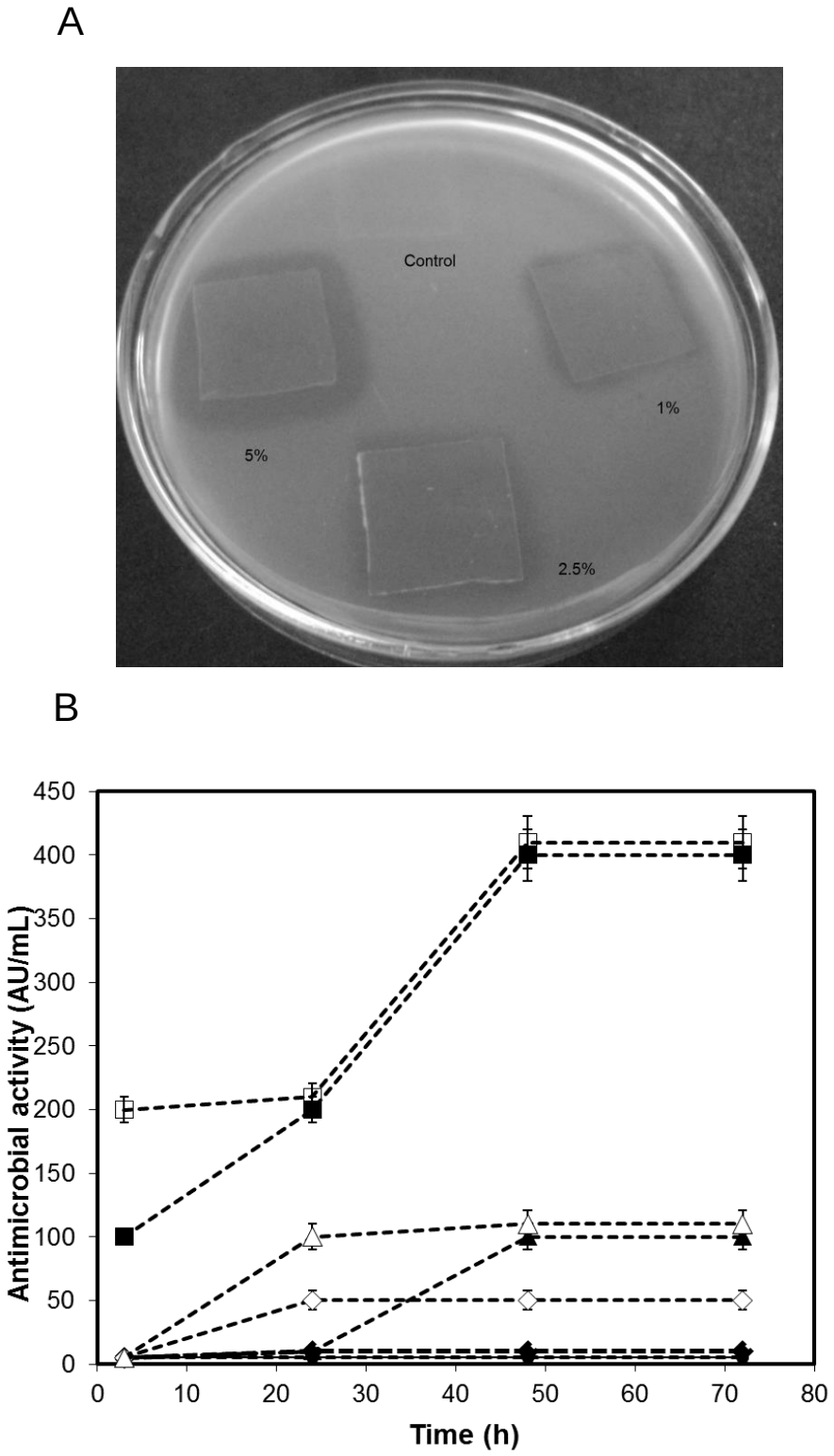


Fig. 1. Antimicrobial activity of the nanocoposites of PP/MMT incorporated with nisin. (A) Composite films containing 1, 2.5 or 5% nisin were tested agaist *Listeria monocytogenes* in BHI agar plates. A sample without nisin addition was used as control. (B) Antimicrobial activity released from PP/MMT nanocomposites containing 1% (diamonds), 2.5% (triangles)

or 5% (squares) nisin after periods of time in saline solution containing 0.5% Tween 20 (open symbols) or 3% acetic acid (full symbols). A sample without nisin addition was used as control (circles).

Table 1. Inhibition zones of nanocomposites of PP/MMT incorporated with nisin against indicator microorganisms on skimmed milk agar plates.

Indicator microorganism	Nanocomposites			
	Control	1% nisin	2.5% nisin	5% nisin
Listeria monocytogenes ATCC 7644	-	+	++	++
Staphylococcus aureus ATCC 1901	-	-	+	++
Clostridium perfringens ATCC 3624	-	+	++	++
Bacillus cereus ATCC 14579	-	-	-	+

−: no halo of inhibition

+: the inhibition area confined to the film perimeter

++: the inhibition area spreads beyond the film perimeter

Complementarily, migration of nisin from the nanocomposites into simulant solutions was evaluated. Figure 1B shows antimicrobial activity, expressed as UA/mL, detected in physiological solutions with Tween 20 and acetic acid solutions after immersion of films for up to 48 h, since no additional release occurred in a longer period.

After 3 h of contact with films containing 5% nisin, simulant solutions containing Tween 20 showed 200 UA/mL, remaining the same value after 24 h and exhibiting 400 UA/mL after 48 h. Films containing 2.5% nisin displayed 100 UA/mL after 48 h immersed in solutions containing Tween 20. However, no antimicrobial activity was detected in this same simulant solutions after contact with nanocomposites containing 1% nisin.

In acetic acid solutions, migration of the bacteriocin occurred after 24 h of contact with all the nanocomposites and the evaluation was carried out using previously neutralized aliquots for antimicrobial activity test (Fig. 1B). After 48 h, films with 2.5% nisin released 100 UA/mL in acetic acid solution, whereas other films containing nisin released the same values of antimicrobial activity in simulant solutions with Tween 20.

In distilled water, antimicrobial activity was not observed indicating that nisin was not released, or migrated at a concentration below de detection limit. These results are in agreement with Siragusa *et al*. (1999) that showed that nisin incorporated into a polyethylene

based plastic film was extracted with saline solution containing 0.5% Tween 20, but not with distilled water. Surfactants, commonly used as food emulsifier, can be capable to enhance nisin activity (Jung *et al*. 1992). Tween 80 exerts its enhancing effect by facilitating diffusion of the bacteriocins and it has been demonstrated that it prevents nisin adsorption on polypropylene disposables (Joosten and Nuñez 1995). In this sense, the mild concentration of Tween 20 used in this study induced a leaching of antimicrobial activity probably favoring the diffusion of nisin incorporated into PP/MMT nanocomposites. On the other hand, lower pH was responsible for nisin migration when acid acetic was used to simulate acid foods, since this bacteriocin is more soluble at acid than neutral pH. This effect was also verified by Mauriello *et al*. (2005) when studying low-density polyethylene (LDPE) films coated with nisin.

Concerning to the time of nisin release, the present work demonstrated that 48 h are necessary for complete migration of the antimicrobial from films. A comparative study on ethylcellulose/hydroxypropylmethylcellulose films (two layer films) and ethylcellulose/hydroxypropylmethylcellulose/ethylcellulose (three-layer films) added with nisin showed that the three-layer films delayed nisin desorption (Guiga *et al*. 2010). Nisin release from two-layer films was total within 30 min, while it was complete in approximately 20 h when three-layer films were used. Thus, nanocomposites prepared with PP, MMT and nisin are promising for further studies about controlled release of this antimicrobial from packaging. This purpose could be highly advantageous, ensuring that a constant level of antimicrobial agent reaches the food surface. Controlled release of the bacteriocin also eliminates the risk of its inactivation by food components or dilution below the active concentration due to migration into the bulk food matrix (Appendini and Hotchkiss 2002).

Mechanical properties

Tensile tests of nanocomposites were conducted to determine how mechanical properties were influenced through the incorporation of nisin. Adequate mechanical properties are very important for polymeric films designed for packaging purposes considering that the primary packaging function is to provide physical protection to the food integrity. Therefore, Young modulus, tensile strength and deformation at break were investigated as a function of the nisin incorporation in a PP/MMT matrix (Fig. 2). The addition of nisin caused no significant differences in the values of deformation at break as compared with control films ($P>0.05$). However, results of tensile strength and Young modulus differed significantly among samples

(P<0.05). Addition of antimicrobials to polymers has been reported as capable to modify mechanical performance (Liu *et al*. 2009; Bastarrachea *et al*. 2010; Scaffaro *et al*. 2011; Ramos *et al*. 2012). The Young modulus was significantly increased (P<0.05) for films with 5% nisin as compared to other films (Fig. 2). Higher tensile strength was also observed for nanocomposites containing 5% nisin, although this value was not significantly different from control and films with 1% nisin. Films with 2.5% nisin showed the lower values of tensile strength among the obtained films. One reason for this fact is a possible irregular dispersion of nisin in the film, as demonstrated by Scaffaro *et al*. (2011) in EVA films incorporated with nisin. In this sense, it is important to consider that mechanical performance involves several factors, such as crystallinity, polymer plasticization, filler matrix interface, which affect the stress-strain properties in a complex way (Persico *et al*. 2009).

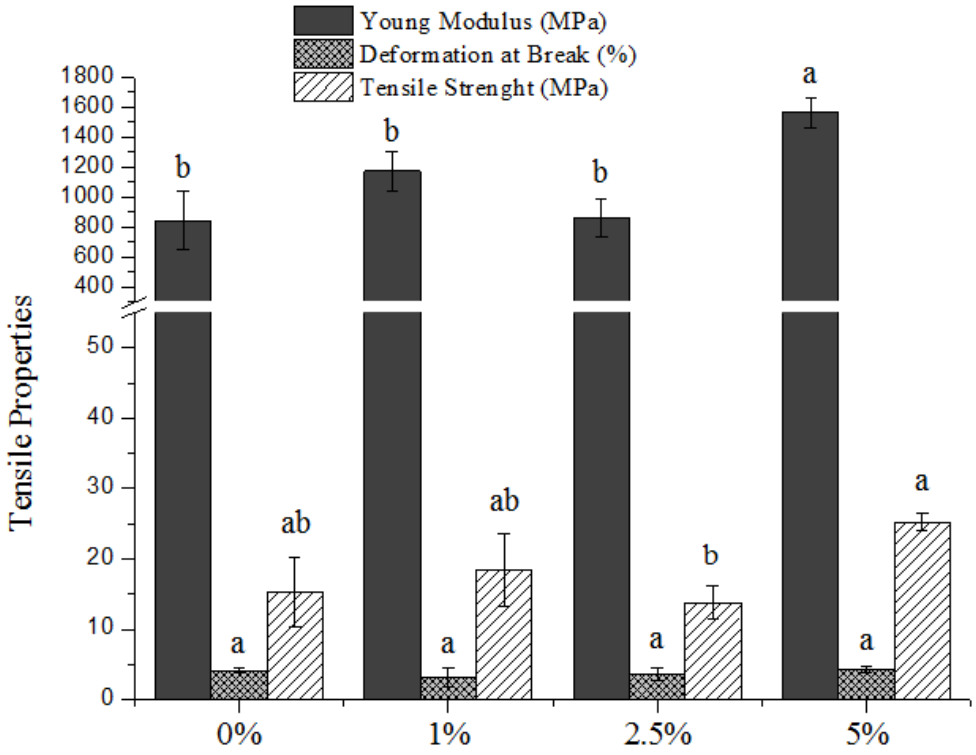


Fig. 2. Tensile properties of the control and PP/MMT nanocomposites containing different concentrations of nisin. Values are the means of three independent determinations ± S.E.M. The same letters indicate no significant difference (P<0.05).

The use of nanoclays, especially MMT, tends to improve mechanical properties when added to polymers like PP (Castel *et al*. 2011). Then, the presence of nanoparticles could explain the maintenance of tensile parameters in PP/MMT films even after nisin incorporation

(films with 1% and 2.5% of nisin). The reinforcement of the polymeric matrix by the montmorillonite is due to the high modulus, high aspect ratio of this filler, dispersion level, and its ability to reinforce in two directions (Castel *et al*. 2011).

Thermal properties

Thermogravimetric analyses (TGA) were performed to determine the influence of nisin on the thermal stability of PP/MMT nanocomposites. Figure 3 shows the TGA curves of control film and nanocomposites containing nisin. The TGA patterns of the samples were quite similar and evinced that increasing concentrations of nisin did not affect thermal stability of the polymer matrix. Table 2 displays the characteristic temperatures $T_{10\%}$ and $T_{50\%}$ corresponding respectively, to the initial decomposition temperature (10% of degradation) and to the maximum degradation rate temperature. As it can be seen, no significant differences were observed for $T_{10\%}$ and $T_{50\%}$ values in all samples.

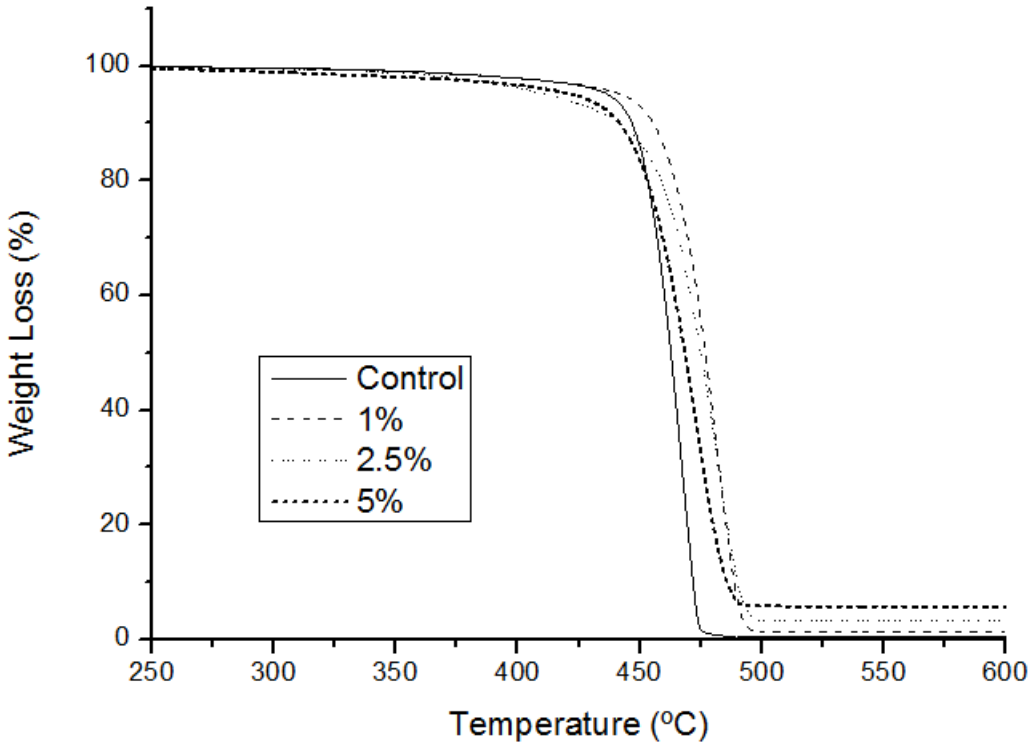


Fig. 3. Thermogravimetic analysis (TGA) of the control and PP/MMT nanocomposites containing different concentrations of nisin.

Table 2. TGA and DSC parameters obtained for nanocoposites of PP/MMT incorporated with nisin.

Sample	$T_{10\%}$ (°C)	$T_{50\%}$ (°C)	T_m (°C)	T_c (°C)	X_c (%)
Control	447 ± 9^a	463 ± 9^a	167.3 ± 1.6^a	126.5 ± 1.3^a	57 ± 0.6^b
1% nisin	456 ± 9^a	477 ± 10^a	167.6 ± 1.7^a	128.8 ± 1.3^a	77 ± 0.8^a
2.5% nisin	443 ± 8^a	475 ± 9^a	167.4 ± 1.6^a	129.1 ± 1.3^a	54 ± 0.5^c
5% nisin	442 ± 8^a	468 ± 9^a	165.9 ± 1.6^a	121.0 ± 1.2^b	47 ± 0.5^d

DSC analyses were carried out to study the effect of incorporation of nisin on the crystallization behavior of PP/MMT nanocomposites. PP is a semicrystalline polymer and its properties are strongly influenced by the crystalline phase (Castel *et al*. 2011). Nevertheless, Table 2 revealed that the nanocomposites tested had no significant difference (P>0.05) in the melting temperature (T_m ranging from 165 to 167°C). The crystallization temperature (T_c) ranged from 121 to 129°C, and the lower values were observed for films with 5% nisin, which were significantly different from others. The degree of crystallinity (X_c) presented the higher value for film containing 1% nisin (77%), whereas nanocomposite with 5% nisin showed 47%, the lowest crystallinity value as compared to the control film. The presence of some additives, such as antimicrobials, could decrease available space for crystal growth thus reducing its crystallinity and inducing variations in tensile properties (Bastarrachea *et al*. 2010; Ramos *et al*. 2012). In contrast, nisin added to PP/MMT nanocomposites caused an oscillation in the values of cristallinity, not decreasing with increasing nisin concentration.

The crystallization is a complex process and influenced by several opposing factors. An acceleration of crystallization rate (evidenced by higher temperature of crystallization, *T*c) and increase in crystallinity degree at low levels of (nano)filler addition has been observed for several composites, due to a nucleation effect. On the other hand, at higher particle contents, retardation of the crystallization rate has been observed even to those systems where nucleation was observed at low levels. A certain amount of particles inclusion is needed to hinder the chains mobility and induce retardation of crystallization process (Paul and Robeson 2008). In this study, montmorillonite and nisin play different roles in PP nanocomposites crystallinity. At low nisin levels the clay can spontaneously induce a nucleation effect onto polypropylene. At higher levels, in contrast, the nisin particles can hinder the diffusion of PP chains and lower its crystallinity.

Barrier properties

The maintenance of food organoleptic properties and increasing shelf life can be partially achieved by packages. In a special manner, nanofillers can act as physical barriers that delay the passage of oxygen across the polymer matrix. The addition of MMT into PP films can improve oxygen barrier properties (Zehetmeyer *et al*. 2012). However, in this study, the incorporation of the antimicrobial nisin into PP/MMT nanocomposites increased significantly ($P<0.05$) oxygen permeability as compared to control films, probably due to the modification of the polymer matrix structure in the presence of the additive (Table 3). Thus, the resistance of films to oxygen diffusion was reduced, as also observed by Ramos *et al*. (2012) after incorporation of carvacrol and thymol into PP films. Those authors attributed this behavior to the increase in free volume in the polymer structure caused by the chemical interaction between polymer chains and additive molecules or the decrease in the material crystallinity and the presence of certain porosity in the film surface. The oxygen barrier performance of a film is interesting because oxygen promotes many food degradation mechanisms, such as oxidations and modification of organoleptic properties (Siracusa 2012). Nevertheless, the results for water vapor permeability obtained for the nanocomposites did not differ significantly ($P>0.05$) (Table 3). The higher polarity of PP/MMT films could justify the slight variation of water vapor permeability values obtained for different samples. The water vapor barrier properties for the packaged food product, whose physical and chemical deteriorations are related to its equilibrium moisture content, are of great importance for maintaining or extending its shelf life (Siracusa 2012).

Table 3. Oxygen permeability and water vapor permeability values of the antimicrobial films and the control.

Samples	Permeability to O_2 (mm cm^3 m^{-2} day^{-1} atm^{-1})	Water vapor permeability (µm g m^{-2} day^{-1} $mmHg^{-1}$)
Control	93.7 ± 9.3^{b}	137 ± 1.7^{a}
1% nisin	413.5 ± 7.6^{a}	146 ± 4.5^{a}
2.5% nisin	439.5 ± 24.5^{a}	176 ± 18.2^{a}
5% nisin	376.4 ± 79.6^{a}	167 ± 12.9^{a}

Values are means ± standard deviation. Treatments followed by the same letter within the same column are not significantly different ($P>0.05$).

Structural properties

Figure 4 shows the XRD diffractograms of the nanocomposites. This analysis is important to evaluate the level of intercalation in clay powders and nanocomposites by the measurement of clay interlayer spacing (*d001*) from the 2θ position of the clay (*001*) diffraction peak using Braggs's law. The MMT used in this study is characterized by a diffraction peak at 2θ = 2.6° corresponding to the basal reflection (*d001*), accounting for a 3.4 nm interlayer distance (data not shown). Nevertheless, only small changes in the position of the clay diffraction peak were observed and it ranged between 2θ = 2.6 - 2.8° for the control and the nisin containing nanocomposites, suggesting that interlayer spacing of the clay tends to decrease when added to PP matrix, but was not affected by the addition of the antimicrobial agent. Similarly, Zehetmeyer *et al*. (2012) found a peak of 2θ = 2.75° for PP films with 2% of the same MMT, produced in planar sheet extruder. In addition, another remarkable peak is the one at 31.7-31.8° that belongs to the characteristic diffraction pattern of sodium chloride (NaCl), which is a component of Nisaplin®. This peak can be only observed for the nanocomposites containing nisin and the intensity is higher as the amount of Nisaplin® increased. This effect has been also reported for poly(butylene adipate-*co*-terephthalate) films with 1000 and 3000 IU/cm^2 of nisin (Bastarrachea *et al*. 2010).

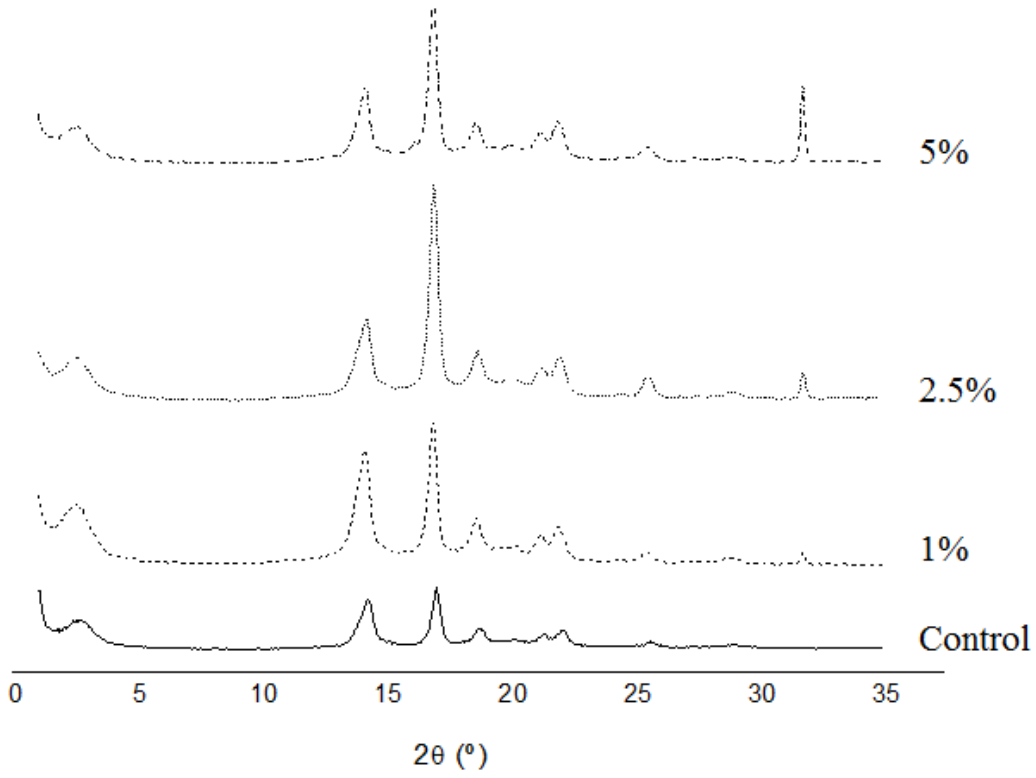
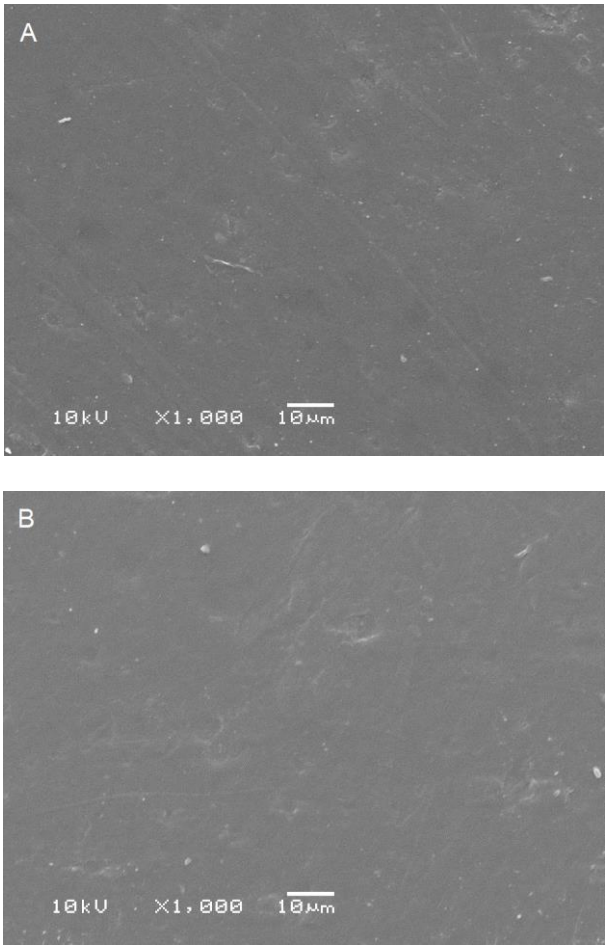


Fig. 4. X-ray diffraction (XRD) patterns of the control and PP/MMT nanocomposites containing different concentrations of nisin.

A relation between DRX and thermal properties can be established, since nanocomposites with 1% nisin showed the higher levels of intensity (Fig. 4) and the higher percentage of cristallinity (Table 2). The opposite occurred for films containing 5% nisin. According to Chivrac *et al* (2006), this behavior indicates how a foreign substance incorporated in a system is able to block the crystal growth and hence the final cristallinity.

The scanning electron micrographs of nanocomposites are shown in Figures 5 and 6. The morphology of the control and films with 1 and 2.5% nisin was quite similar, with no apparent effect of the addition of the antimicrobial to PP/MMT (Fig. 5). However, nisin agglomerates are visible on the film containing 5% nisin in Fig. 5, suggesting that during the preparation of the film some additive migrated to the film surface. A similar behavior was reported by Scaffaro et al. (2011) during the preparation of poly(ethylene-co-vinyl acetate) films with two commercial formulations of nisin.



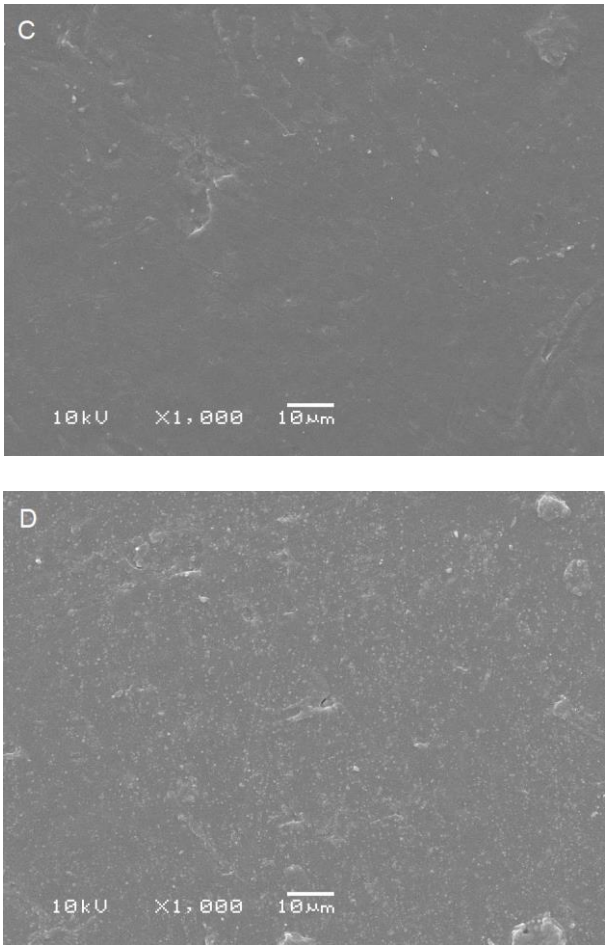


Fig. 5. SEM micrographs (1000x) of the surfaces for control films (A) and samples with 1% (B), 2.5% (C) and 5% (D) of nisin.

Figure 6 shows the cross-section images obtained from films. Small holes and pores were observed in nanocomposites containing 2.5 and 5% nisin. According to Linssen *et al*. (2003), the formation of holes in a polymer matrix is caused by separation of polymer chains. Some heat-sensitive components in the Nisaplin might have degraded and "evaporated" at higher temperature, then pores were created and particles were segregated. This result is consistent with other works (Liu *et al*. 2009; Bastarrachea *et al*. 2010) and is also correlated with the barrier and tensile properties observed in this work.

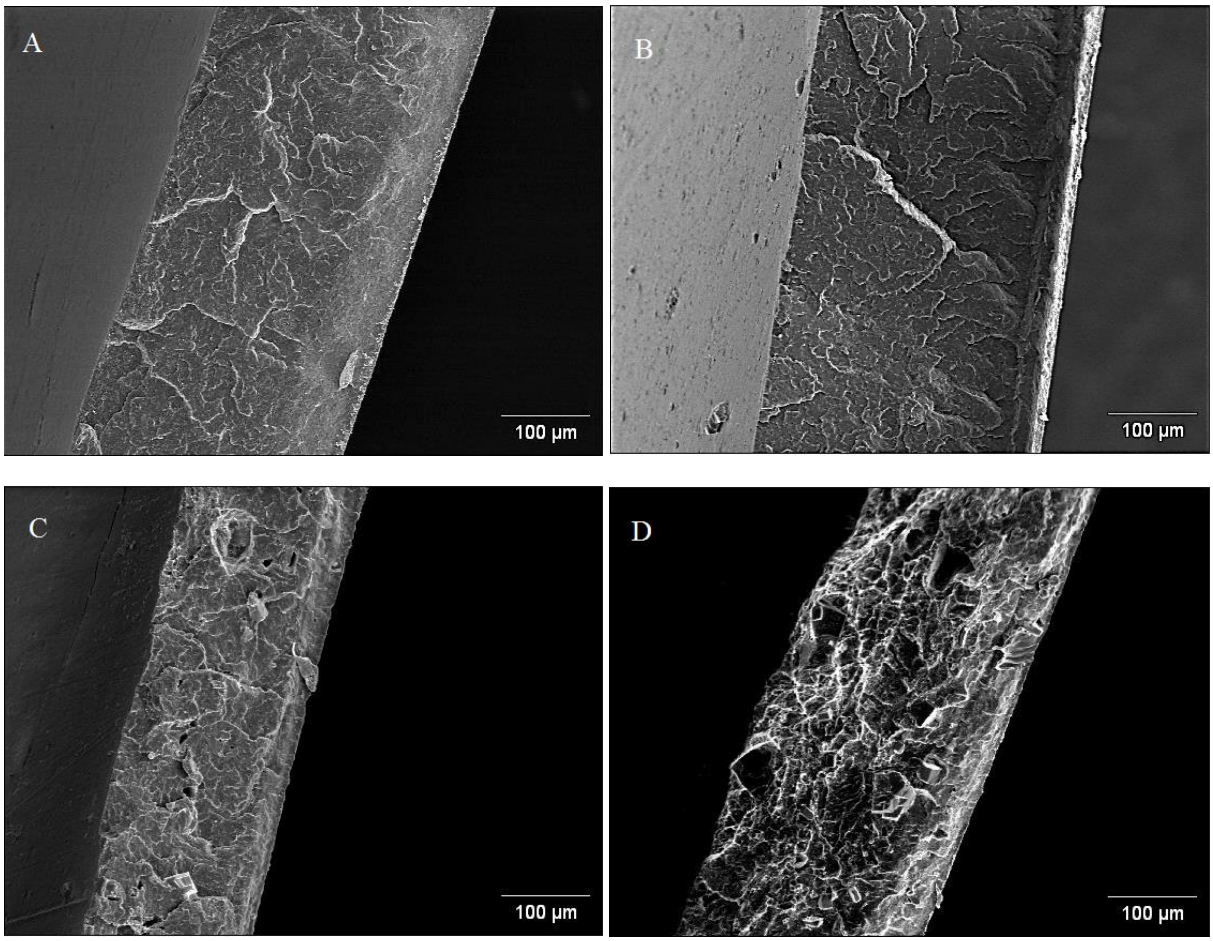


Fig. 6. SEM micrographs (200x) of the cross-sections for control films (A) and samples with 1% (B), 2.5% (C) and 5% (D) of nisin.

Conclusions

Results from the present research suggest that antimicrobial films could be prepared incorporating nisin to a previous mixture of MMT and PP by compression molding at 180°C without losing bioactivity. The resulting nanocomposites have some properties comparable to control films and revealed a great potential in antimicrobial food packaging to reduce post-process growth of food pathogens. Further studies will be conducted to optimize film preparation aiming the controlled release of nisin to be applied in real food systems.

Acknowledgments

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ARTIGO 2 - Adsorption of nisin and pediocin on nanoclays

Abstract

Three different nanoclays (bentonite, octadecylamine-modified montmorillonite and halloysite) were studied as potential carriers for the antimicrobial peptides nisin and pediocin. Adsorption occurred from peptide solutions in contact with nanoclays at room temperature. Higher adsorption of nisin and pediocin was obtained on bentonite. The antimicrobial activity of the resultant bacteriocin-nanoclay systems was analyzed using skimmed milk agar as food simulant and the largest inhibition zones were observed against Gram-positive bacteria for halloysite samples. Bacteriocins were intercalated into the interlayer space of montmorillonites as deduced from the increase of the basal spacing measured by X-ray diffraction (XRD) assay. Infrared spectroscopy suggested non-electrostatic interactions such as hydrogen bonding between siloxane groups from clays and peptide molecules. Transmission electron microscopy did not show any alteration in morphologies after adsorption of antimicrobial peptides on bentonite and halloysite. These results indicate that nanoclays, especially halloysite, are suitable nanocarriers for nisin and pediocin adsorption.

Keywords: bacteriocin; adsorption; nanoclay; halloysite; montmorillonite

1. Introduction

Antimicrobial peptides, such as bacteriocins, have become of great interest in food preservation, health care and pharmaceutical applications. These molecules differ from traditional antibiotics and are less likely to cause pathogen resistance (Ibarguren, Audisio, Torres, & Apella, 2010; Brandelli, 2012). Bacteriocins present natural occurrence, wide spectrum range of antimicrobial activity and proteinaceous nature, which implies a putative degradation in the gastrointestinal tract of man and animals (Cleveland, Montville, Nes, & Chikindas, 2001). In terms of food safety, the bacteriocins from lactic acid bacteria (LAB) have received much attention due to their generally recognized as safe (GRAS) status and potential use as natural preservatives (Cotter, Hill, & Ross, 2005; Papagianni & Anastasiadou, 2009).

The most studied LAB bacteriocin, nisin, is a polypeptide of 34 amino acids produced by *Lactococcus lactis* strain. This bacteriocin contains lanthionine and methyllanthionine residues, presents a molecular mass of 3500 Da, and display a wide

spectrum of activity against Gram-positive bacteria and on spores of Bacilli and Clostridia (Arauz, Jozala, Mazzola, & Penna, 2009). Unlike nisin, pediocin AcH (same of PA-1) presents 44 amino acids (molecular mass of 4629 Da), is produced by *Pediococcus acidilactici* and is commercially exploited as a bacteriocin-containing fermentate powder. Pediocin is part of a group of bacteriocins belonging to the class IIa, characterized as ‘‘antilisterial’’ bacteriocins (Papagianni & Anastasiadou, 2009).

These bacteriocins are commonly incorporated in food by direct addition for controlling pathogenic bacteria. However, some loss of antimicrobial activity can occur due to proteolytic degradation or potential interaction with food components (Cleveland et al., 2001; Malheiros, Daroit, & Brandelli, 2010). In this sense, nanostructures may represent an interesting alternative as bacteriocin carriers, not only for food but also for medical applications, improving their stability and efficacy (Brandelli, 2012).

Nanoclays or layered silicates typically have a stacked arrangement of silicate layers with a nanometric thickness. They have been used for remediation of environmental contaminants, delivery of drugs and various active molecules, and to enhance polymer mechanical and barrier properties in packaging films (Parolo et al., 2010; Rawtani & Agrawal, 2012; Azeredo, 2013). Furthermore, mineral clays may allow a controlled release of antimicrobials and are considered as safe food additives according to FDA (US Food and Drug Administration) and EFSA (European Food Safety Authority) (Ibarguren et al., 2014). Their basic building blocks are tetrahedral sheets in which silicon is surrounded by four oxygen atoms, and octahedral sheets in which a metal like aluminum is surrounded by eight oxygen atoms. Montmorillonite (MMT) is a member of the smectite group, belonging to the structural family of the 2:1 phyllosilicates. It is one of the most widely used natural clays with a general formula of $M_x(Al_4{-}_xMg_x)Si_8O_{20}(OH)_4$, where M is a monovalent cation and *x* is the degree of isomorphous substitution (between 0.5 and 1.3) (Pavlidou & Papaspyrides, 2008; Azeredo, 2013). Halloysite (HNT) is an important member of the kaolin group of clay minerals, with a composition of $Al_2Si_2O_5(OH)_4.H_2O$ with 1:1 layer (Rawtani & Agrawal, 2012). Studies on the use of nanoclays as carriers for bacteriocins are restricted to nisin onto raw montmorillonite (Ibarguren et al., 2014).

The aim of this study was to evaluate and compare the interaction of the bacteriocins nisin and pediocin with three different type of clay nanoparticles: montmorillonite modified with octadecylamine, unmodified montmorilonite (hydrophilic bentonite) and halloysite.

2. Materials and methods

2.1. Materials

Commercial nisin (Nisaplin®) was provided by Danisco Brasil Ltda. According to manufacturer, the formulation contains NaCl and denatured milk solids as fillers, and 2.5% 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. Different working solutions of nisin were prepared by dilution of the stock nisin solution previously filter-sterilized through 0.22 µm membranes (Milipore). Nisin solutions were stored at 4°C until their use.

Pediocin (ALTA^{TM} 2345) was provided by Kerry Ingredients & Flavours, USA. To reach the desired concentrations, pediocin was diluted with 10 mM sodium phosphate buffer (pH 7.0). Until their use, pediocin solutions were also stored at 4°C.

Three commercial nanoclays from Sigma-Aldrich were used: hydrophilic bentonite (Nanomer® PGV), montmorillonite (MMT) surface modified with 25-30 wt% octadecylamine (Nanomer® I.30E) and a unmodified tubular clay, halloysite (HNT).

2.2 Nisin adsorption on nanoclays

Adsorption assays were carried out by adding 1 mL nisin solution (0.1, 0.25, 0.5, 1.0, 1.25, 1.5, 2.0 and 2.5 mg mL^{-1}) or 1 mL pediocin solution (10, 50, 100, 150, 200 and 300 mg mL^{-1}) to 10 mg of each nanoclay. These bacteriocin-nanoclay systems were maintained during 1 h at 25ºC and 80 rpm. Preliminary experiments showed that this time was enough to reach equilibrium. After that, aliquots of supernatants were recovered by centrifugation (5000 *g* for 5 min at 25ºC) and residual antimicrobial activity was determined. The pellets obtained after centrifugation (nanoclays adsorbed with bacteriocin) were washed twice with 10 mM phosphate buffer solution pH 7.0, redispersed in the same buffer and also assessed for antimicrobial activity. Additionally, nisin and pediocin solutions at different concentrations were evaluated for initial antimicrobial activity.

2.3 Antimicrobial activity evaluation

The antimicrobial activity was detected by agar diffusion assay. An aliquot of 10 µL of bacteriocin solutions, supernatants after adsorption and adsorbed nanoclays were applied on

BHI agar plates previously inoculated with a swab submerged in indicator strain (*Listeria monocytogenes* ATCC 7644) suspension, which corresponded to a 0.5 McFarland turbidity standard solution (approximately 10^7 CFU mL^{-1}). Plates were allowed at 4ºC for 24 h to favor bacteriocin migration before incubation at 37°C for 24 h. The reciprocal value of the highest dilution that produced an inhibition zone was taken as the activity unit (AU) per mL (Motta & Brandelli, 2002). A percentage of adsorbed bacteriocin activity was calculated as follows: [(initial activity of bacteriocin solution - residual activity at supernatant after adsorption)/ initial activity of bacteriocin solution] x 100.

The bacteriocin-adsorbed nanoclays were also tested using 1% (w/v) skim milk agar previously inoculated with a swab submerged in suspensions of the Gram-positive bacteria *Bacillus cereus* ATCC 9634, *Clostridium perfringens* ATCC 3624, *Staphylococcus aureus* ATCC 25923 and *Listeria monocytogenes* ATCC 7644. The diameter of the inhibition zones was measured and expressed as mm.

2.4 Characterization of samples after adsorption

After exposure to bacteriocin solutions at the saturation level, the nanoclays were washed as described in the Section 2.2. The samples were freeze-dried and submitted to X-ray diffraction (XRD), transmission electron microscopy (TEM) and infrared spectroscopy analyses. Nisaplin, ALTA^{TM} 2345 and commercial nanoclay samples were also analyzed as controls.

XRD measurements were performed using a Siemens D-500 diffractometer. Samples were scanned in the reflection mode using an incident X-ray of Cu Kα (λ = 1.54 Å), at a step width of 0.05°s^{-1} from 2θ = 2°-45°. The dispersion of the layers in the nanocomposites, as well as the basal spacing of the clays, were estimated from the (001) diffraction.

Fourier Transform Infrared (FTIR) spectra were measured using a FTIR Varian 640 IR Thermo Scientific spectrometer in attenuated total reflectance (ATR) mode with a diamond crystal. The scans were collected between 400 and 4000 cm^{-1}.

The morphology of bentonite and halloysite samples adsorbed or not with bacteriocins were examined by TEM (JEOL JEM-1200 Ex II) which was operated at an accelerating voltage of 80 kV.

3. Results and discussion

3.1 Nisin and pediocin adsorption on nanoclays

The bacteriocins nisin and pediocin adsorbed onto the three nanoclays tested, regardless the different characteristics of these nanoparticles. However, the rate of adsorption varied substantially. The adsorption of nisin was higher onto hydrophilic bentonite (Table 1), since no residual antimicrobial activity was detected in the supernatants exposed to this nanoclay until the concentration of 2.0 mg/mL nisin solution. For MMT modified with octadecylamine, nisin solutions with concentrations greater than 1.25 mg/mL led to saturation of the clay surface, evidenced by increasing values of residual antimicrobial activity from this point (Table 1). Halloysite presented the lowest adsorption potential comparing to the other clays because lower percentages of adsorbed nisin activity were obtained when it was exposed to increasing concentrations of nisin solutions (Table 1).

Table 1. Residual antimicrobial activity and adsorbed nisin activity after nanoclays exposure to bacteriocin solutions.

Concentration of nisin solutions (mg/mL)	Initial antimicrobial activity of nisin solutions (AU/mL)[a]	Residual antimicrobial activity (AU/mL)[a]; Adsorbed nisin activity (%)[a]		
		Hydrophilic bentonite	Montmorillonite modified with octadecylamine	Halloysite
0.1	400	0; 100	0; 100	0; 100
0.25	800	0; 100	0; 100	0; 100
0.5	1600	0; 100	0; 100	0; 100
1.0	4800	0; 100	0; 100	100; 97.9
1.25	6400	0; 100	0; 100	400; 93.7
1.5	7200	0; 100	100; 98.6	800; 88.9
2.0	9600	0; 100	200; 97.9	1600; 83.3
2.5	12800	100; 99.2	400; 96.9	3200; 75.0

[a] Values are the average of three independent experiments.

Pediocin exhibited a similar behavior when adsorbed onto nanoclays (Table 2). At the concentration of 300 mg/mL pediocin, the surfaces of bentonite and MMT modified with octadecylamine became saturated. Halloysite, however, adsorbed no more pediocin above the concentration of 150 mg/mL, as shown in Table 2.

Table 2. Residual antimicrobial activity and adsorbed pediocin activity after nanoclays exposure to bacteriocin solutions.

Concentration of pediocin solutions (mg/mL)	Initial antimicrobial activity of pediocin solutions (AU/mL)[a]	Residual antimicrobial activity (AU/mL)[a]; Adsorbed pediocin activity (%)[a]		
		Hydrophilic bentonite	Montmorillonite modified with octadecylamine	Halloysite
10	100	0 ; 100	0 ; 100	0 ; 100
50	800	0 ; 100	0 ; 100	0 ; 100
100	1600	0 ; 100	0 ; 100	0 ; 100
150	6400	0 ; 100	0 ; 100	200; 96.9
200	12800	0 ; 100	0 ; 100	400; 96.9
300	25600	100 ; 99.6	100 ; 99.6	800; 96.9

[a] Values are the average of three independent experiments.

MMTs and HNTs are natural hydrophilic nanoparticles. However, modifications with organic compounds like surfactants by ion exchange reactions are usually common in order to improve the compatibility of clays with polymers for their application in nanocomposites (Azeredo, 2013). For comparison, this study also used the MMT modified with octadecylamine that presents a hydrophobic characteristic. In this sense, results evidenced that nisin and pediocin interact with both surfaces types, regarding polarity. Bower, McGuire, & Daeschel (1995) also showed that nisin could adsorb onto silica surfaces with low and high hydrophobicity. On the other hand, Karam et al. (2013) detected lower amount of nisin on hydrophobic surfaces *versus* the hydrophilic ones.

Once adsorbed on nanoclays, nisin maintained its antimicrobial activity and inhibition zones were visible when aliquots of nisin-adsorbed nanoclays (suspended in buffer after washing) were applied on BHI agar plates inoculated with *L. monocytogenes* (Fig. 1).

These results are consistent with previous investigations, which shown that nisin adsorbed on silica surfaces retains biological activity (Daeschel, McGuire, & Al-Makhlafi,1992; Bower et al., 1995; Wan et al., 1996). Higher inhibition zones were obtained for halloysite samples adsorbed with nisin (Fig. 1), which could be attributed to higher desorption of nisin when in contact with the agar medium.

In contrast, after adsorption of pediocin on nanoclays, halos were only visualized for halloysite-adsorbed samples on BHI agar plates (Table 3). Nanoclays without nisin and pediocin did not exhibit any antimicrobial activity as expected.

In this sense, Dawson, Harmon, Sotthibandhu, & Han (2005) reported that adsorption of nisin onto surfaces can alter the peptide conformation and also result in formation of nisin multi-layers and dimers, which can reduce its biological activity. Bower et al. (1995) found that nisin adsorbed in small amounts on low-hydrophobic surfaces but these samples displayed more antimicrobial activity than higher-hydrophobicity surfaces, similar to results obtained for halloysite surface in the present study.

While the bactericidal mechanisms of adsorbed nisin and pediocin have not been determined, it may require the release of these bacteriocins from the adsorbed clay surface to promote damage in the cell membrane. Nisin has been consistently shown to kill Gram-positive bacteria and the mechanism of its activity in solution involves a multi-step process that destabilizes the phospholipid bilayer of the cell and creates transient pores (Bonev et al., 2000; Cotter et al, 2005). Whereas, pediocin causes destabilization of membrane functions including loss of intacellular K^+, entrance of lactose from the medium inside the cells and cell lysis of some strains (Pappagiani & Anastasiadou, 2009).

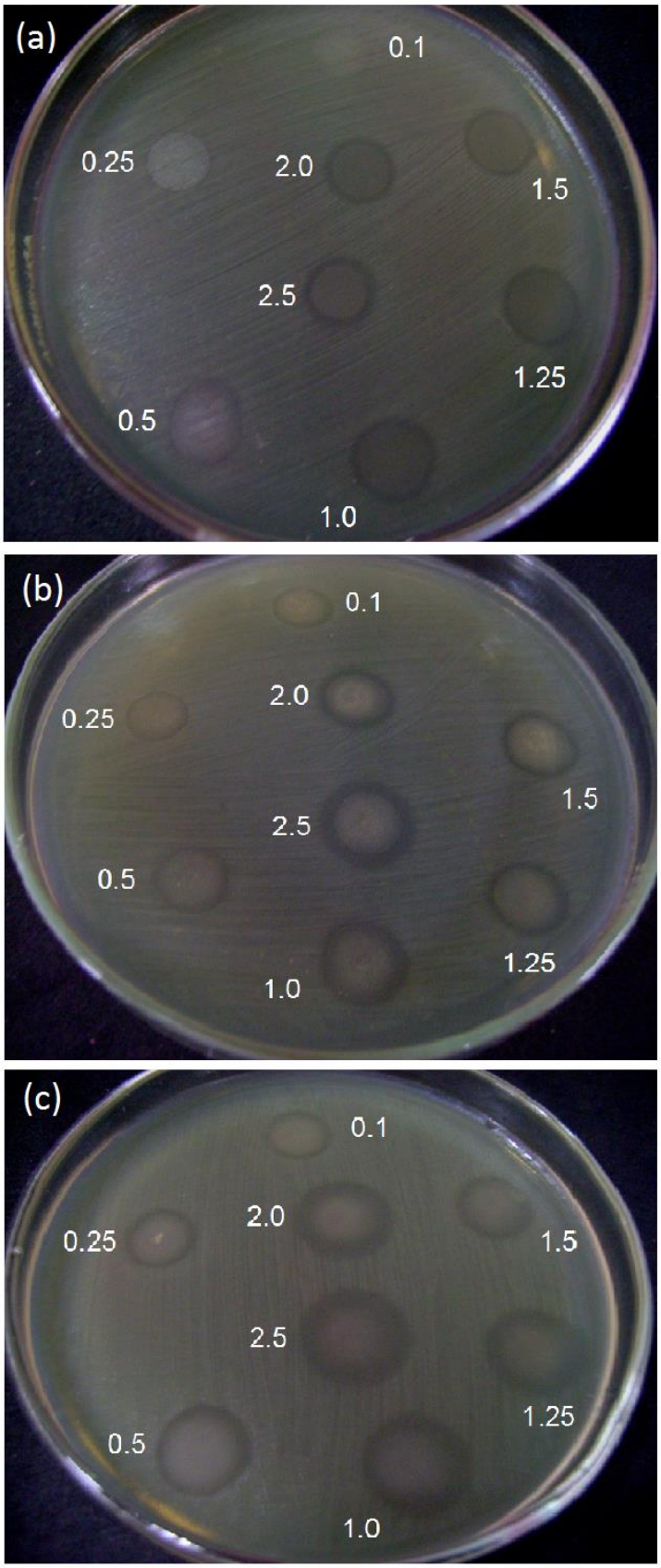


Fig 1. Antimicrobial activity of nanoclays (a) bentonite, (b) MMT modified with octadecylamine, (c) HNT after nisin adsorption.

Table 3. Zone of inhibition of pediocin adsorbed-halloysite samples.

Concentration of pediocin solutions (mg/mL)	Inhibition zone diameters (mm) [a]		
	Listeria monocytogenes on BHI agar	*Listeria monocytogenes* on skimmed milk agar	*Clostridium perfringens* on skimmed milk agar
10	8	12	12
50	10	13	13
100	10	14	13
150	11	14	15
200	14	15	16
300	14	15	16

[a] Values are the average of three independent experiments.

3.2 Characterization of bacteriocin-adsorbed nanoclays

The XRD analysis was performed for nanoclay characterization. This technique uses the scattered intensity of an X-ray beam on the sample, revealing information about the crystallographic structure, chemical composition, and physical properties of the material studied (Espitia et al., 2012). In relation to clay powders, XRD patterns display the level of intercalation by the measurement of interlayer spacing (*d001*) from the 2θ position of the clay (*001*) diffraction peak using Braggs's law. The XRD spectra for bacteriocin-adsorbed nanoclays are depicted in Figure 2.

The hydrophilic bentonite clay shows basal reflection peak *(d001)* at 2θ = 6.6° (Konwar & Karak, 2011). In this work, bentonite showed a basal reflection peak *(d001)* at 2θ = 6.5°, accounting for a 1.37 nm interlayer distance (Fig. 2a). The position of this clay diffraction peak changed to 2θ = 5.8° after nisin adsorption, increasing the interlayer spacing of the clay to 1.53 nm with the presence of the antimicrobial agent. A similar behavior was observed after pediocin adsorption with an increasing of interlayer space of the bentonite to 1.41 nm (Fig. 2a). This slight modification can suggest intercalation of nisin and pediocin molecules, or a part of them, between bentonite layers. These results are in agreement with Ibarguren et al. (2014) who reported that nisin adsorbed on raw montomorillonite probably by

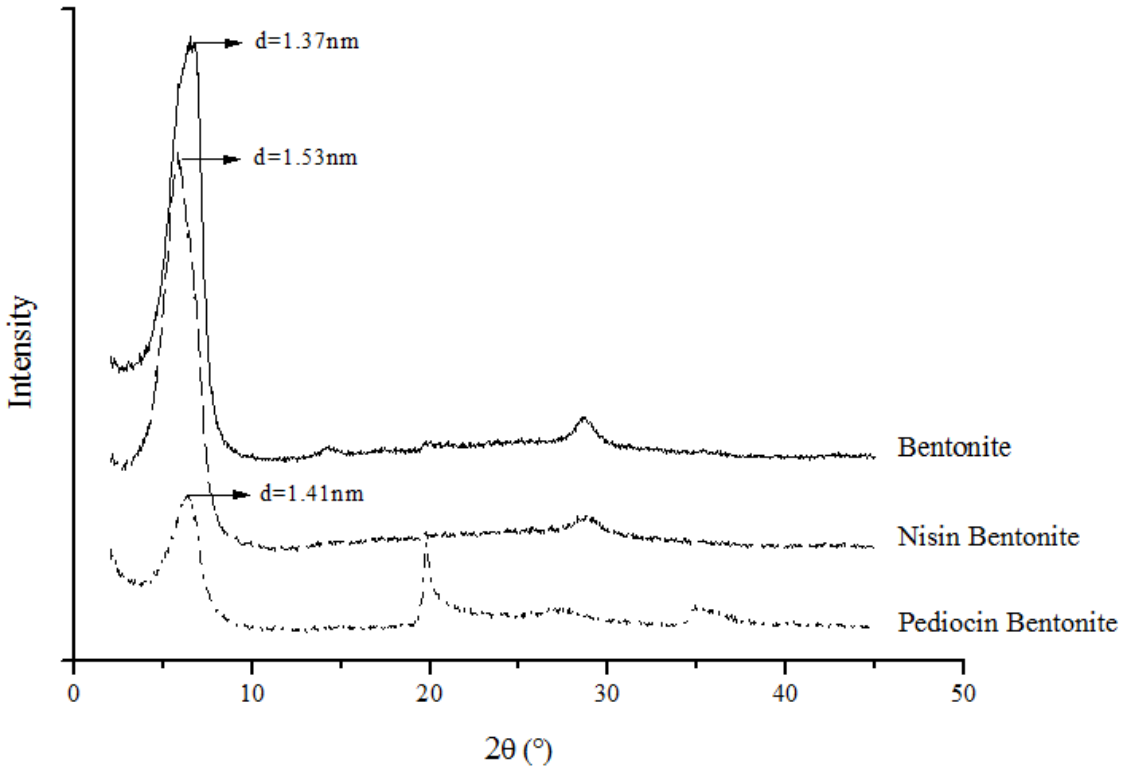
“frustrated intercalation”, suggesting that the cationic portions of the peptide molecule interacted with the clay mineral structure.

Fig. 2b shows MMT modified with octadecylamine with a basal reflection peak *(d001)* at $2\theta = 4.2°$ corresponding to 2.12 nm of interlayer spacing, which was altered to 2.26 nm for nisin-adsorbed nanoclay ($2\theta = 3.9°$). However, after pediocin adsorption, two (*d001*) diffraction lines appeared: one at 4.8º corresponding to 1.84 nm and a second line at lower angles (3.4º), indicating a modification of the interlayer space distance due to an intercalation of the pediocin molecules in the solid matrix (Fig. 2b). Parolo et al. (2010) also found intercalation of tetracycline after adsorption on montmorillonites by an extra reflection peak, coexisting two basal spacing that indicates a stacking of the nanoclay layers containing the antibiotic in the interlayer.

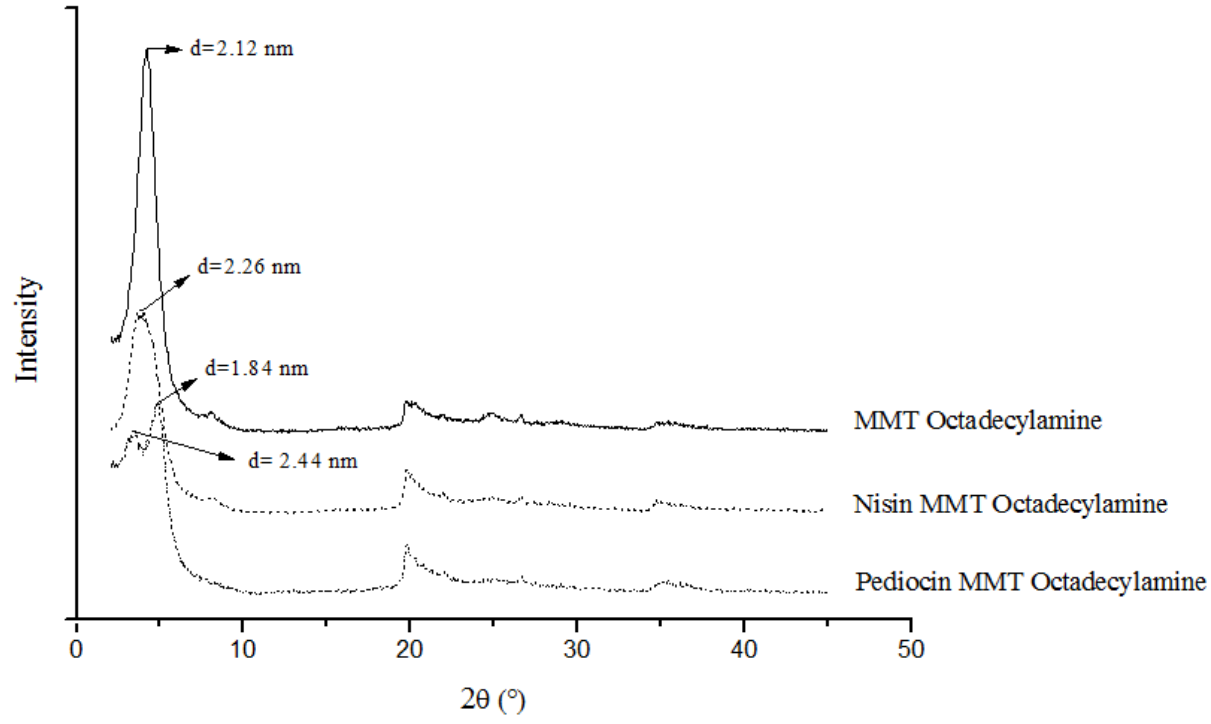
HNT is a non-swelling clay and dispersion cannot be accessed by XRD. It is possible to observe a diffraction peak at $2\theta = 11.7°$, corresponding to a basal spacing of 0.75 nm (Fig. 2c). This result confirms the tubular structure at the nanoscale of halloysite (Joussein et al., 2005). After nisin and pediocin adsorption, the diffraction peak of HNT internal channel was not shifted, confirming that the channel structure of the primary particles remained unchanged.

Nisin powder was also analyzed by XDR and a remarkable peak at 31.7-31.8° was detected (Fig. 2d). This corresponds to the characteristic diffraction pattern of sodium chloride (NaCl), a component of Nisaplin® (Bastarrachea et al., 2010). Nevertheless, this band is not present in adsorbed-nanoclays samples. In contrast, pediocin powder did not exhibit peaks when analyzed by XRD, indicating no crystalline substances are present in its formulation (data not shown).

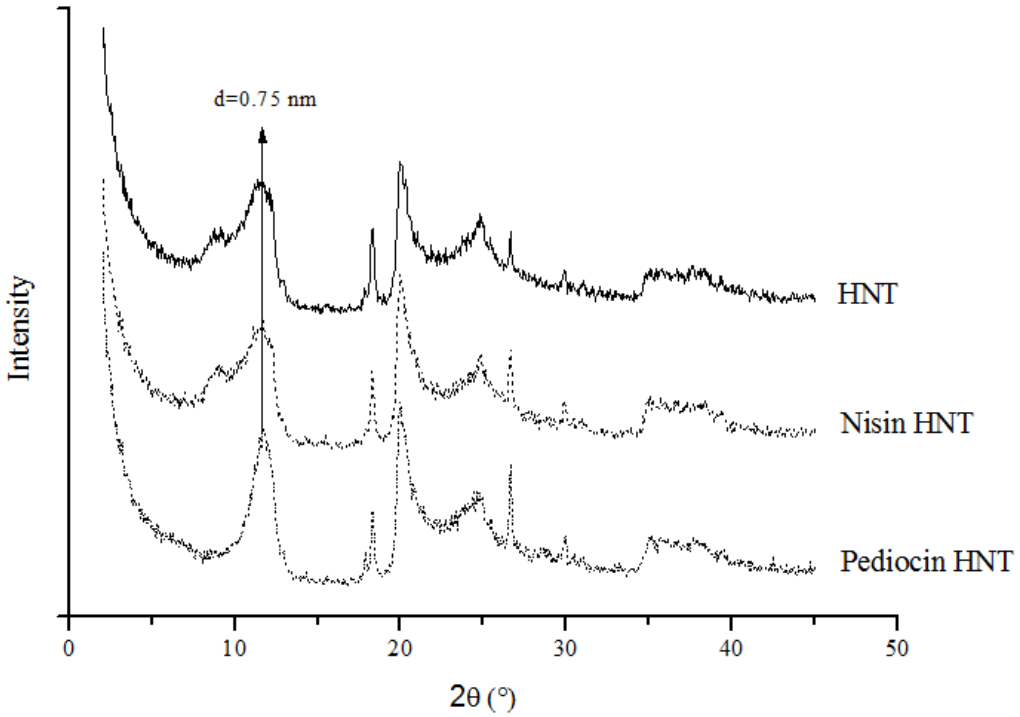
(a)


d=1.37nm
d=1.53nm
d=1.41nm
Bentonite
Nisin Bentonite
Pediocin Bentonite
Intensity
0
10
20
30
40
50
2θ (°)

(b)


d=2.12 nm
d=2.26 nm
d=1.84 nm
d= 2.44 nm
MMT Octadecylamine
Nisin MMT Octadecylamine
Pediocin MMT Octadecylamine
Intensity
0
10
20
30
40
50
2θ (°)

(c)



(d)

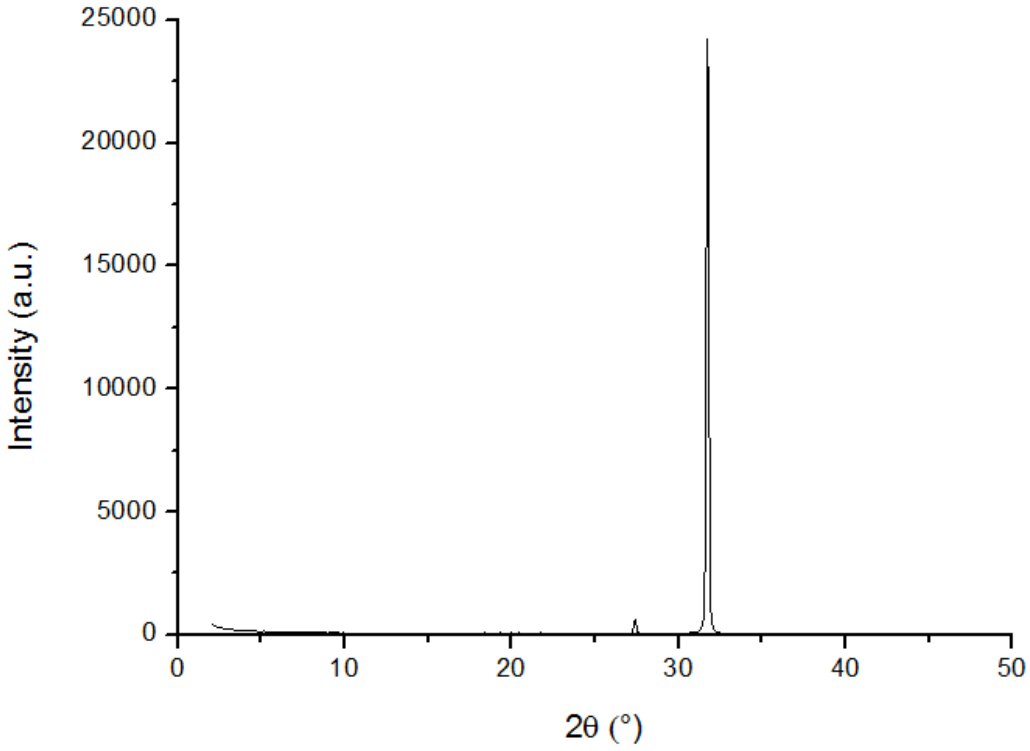


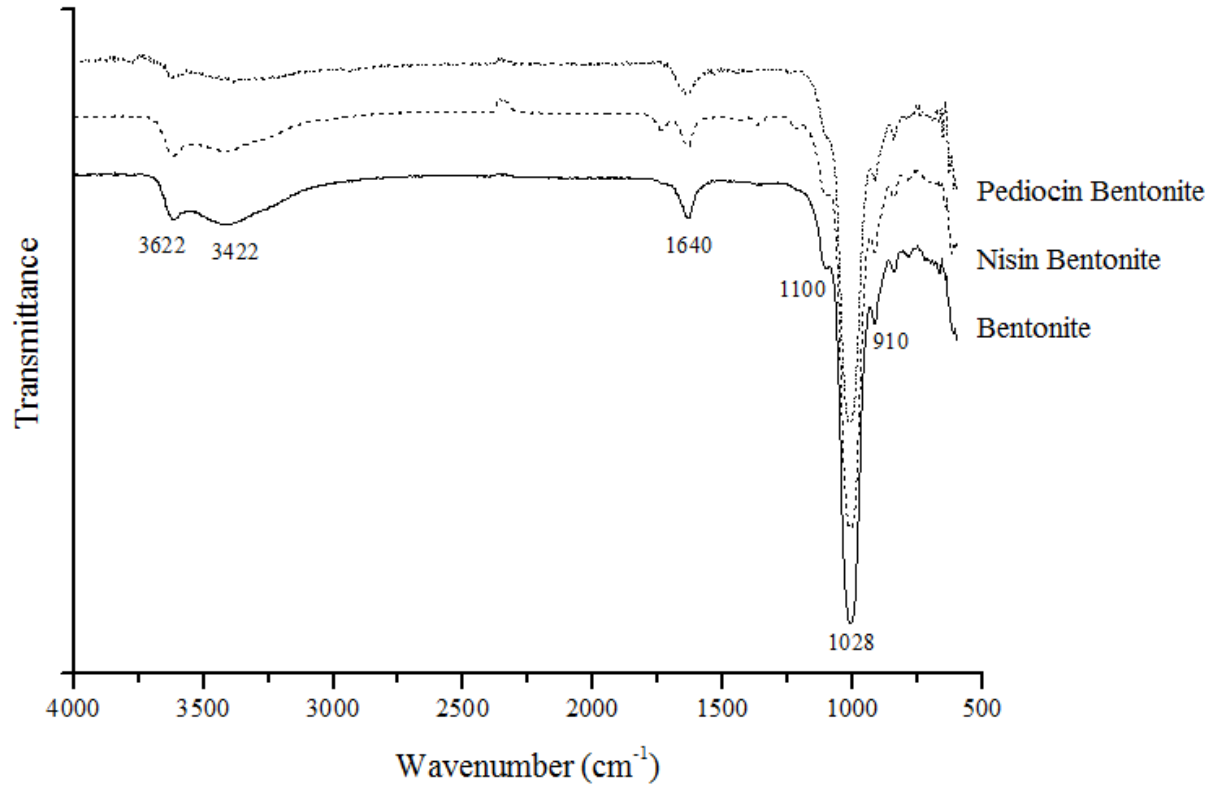
Fig 2. XRD patterns of (a) bentonite, (b) MMT modified with octadecylamine, (c) HNT, before (solid line) and after nisin or pediocin adsorption(dotted lines), (d) XDR pattern of nisin preparation.

FTIR spectroscopy is a sensitive technique largely used to reveal adsorption of surfactants or pollutants onto nanoclays. In this work, FTIR was used to explore the molecular environment of the nanoclays after interaction with nisin and pediocin (Fig. 3). The common features in the FTIR spectra of nanoclays were the presence of characteristic bands around 3622 cm^{-1} attributed to the -OH stretching vibration of structural hydroxyl groups, 1640 cm^{-1} related to the -OH deformation of water and 1028 cm^{-1} assigned to Si-O stretching vibration. The band around 910 cm^{-1} was assigned to isolated Si-O groups and it was more intense in HNT than other clays. A shoulder around 1100 cm^{-1} observed in bentonite and HNT is attributed to Si-O deformation (Du et al., 2008).

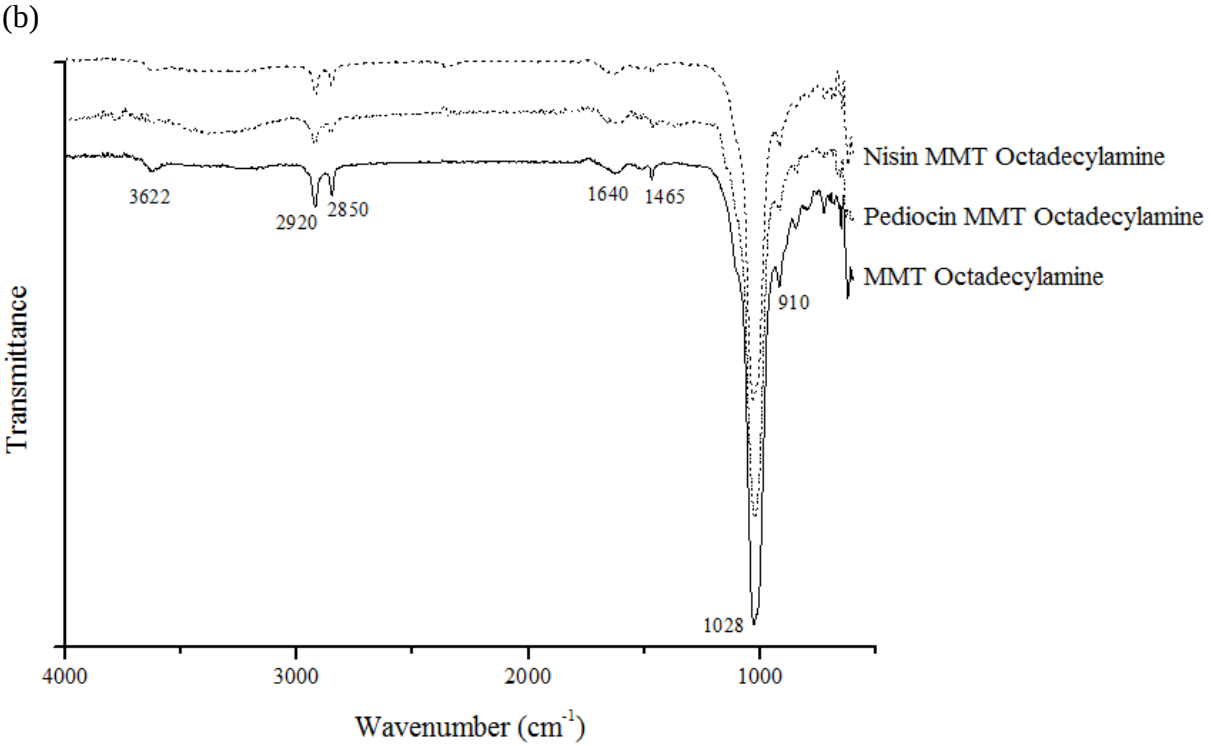
In particular, bentonite showed a band around 3420 cm^{-1} related to the -OH stretching of water, indicating Si-OH interaction with physisorbed water (Fig. 3a). This band appears broad and weaker in the samples with nisin and pediocin. Other bands due to the presence of octadecylamine are visible in Fig. 3b: 2920 cm^{-1}, related to C-H asymmetric stretching of CH_2 or CH_3; 2850 cm^{-1}, corresponding to C-H symmetric stretching of CH_2 or CH_3; and 1465 cm^{-1} that is attributed to CH_2 scissoring (Chuayjuljit, Thongraar,& Saravari, 2008). On the other hand, HNT presents a band at 3694 cm^{-1} indicating the presence of silanol groups forming hydrogen bonds (Fig. 3c).

The commercial nisin preparation, Nisaplin®, also contains NaCl, carbohydrate and moisture in the formulation. In this context, the major bands were obtained at 3420 and 1634 cm^{-1} (Fig. 3d). In the same way, pediocin formulation shows bands at 3299 and 1585 cm^{-1} (Fig. 3d). Absorption in this first area indicates stretching of the O-H and N-H bonds and the spectra in the region of 1720-1580 cm^{-1} are attributed to the amide bands (Kong & Yu, 2007). Otherwise, pediocin spectrum presented another intense band at 1024 cm^{-1} corresponding to C-N stretching.

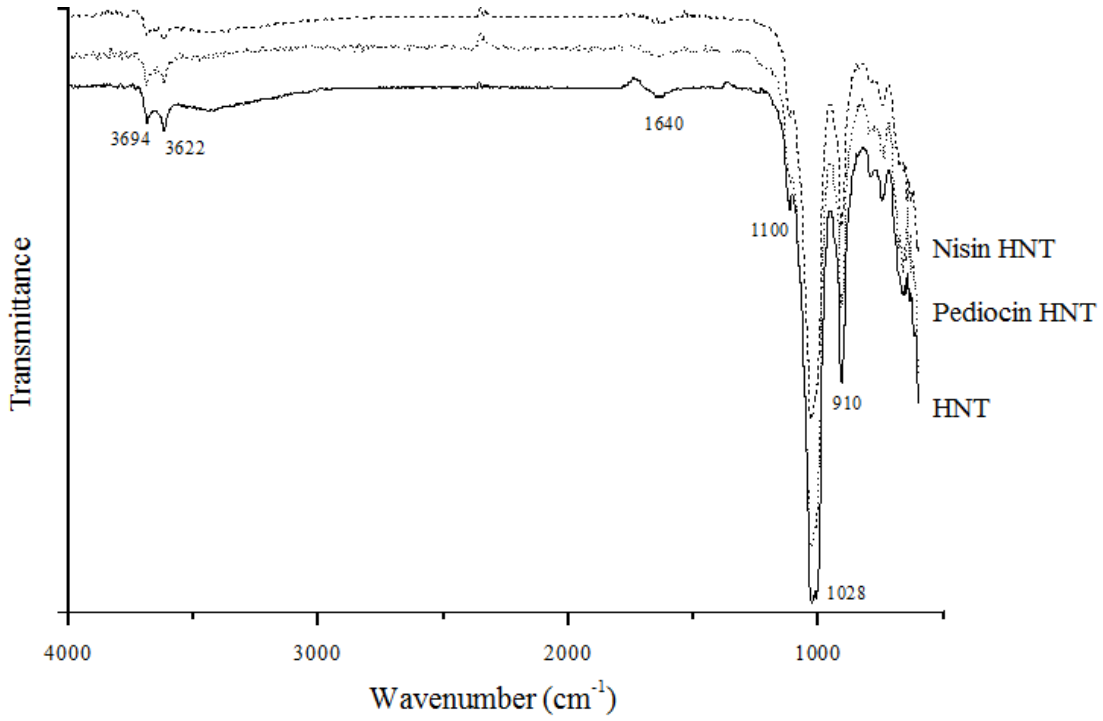
(a)


Transmittance
3622
3422
1640
1100
910
1028
Pediocin Bentonite
Nisin Bentonite
Bentonite
4000
3500
3000
2500
2000
1500
1000
500
Wavenumber (cm-1)

(b)


Transmittance
3622
2920
2850
1640
1465
910
1028
Nisin MMT Octadecylamine
Pediocin MMT Octadecylamine
MMT Octadecylamine
4000
3000
2000
1000
Wavenumber (cm-1)

(c)



(d)

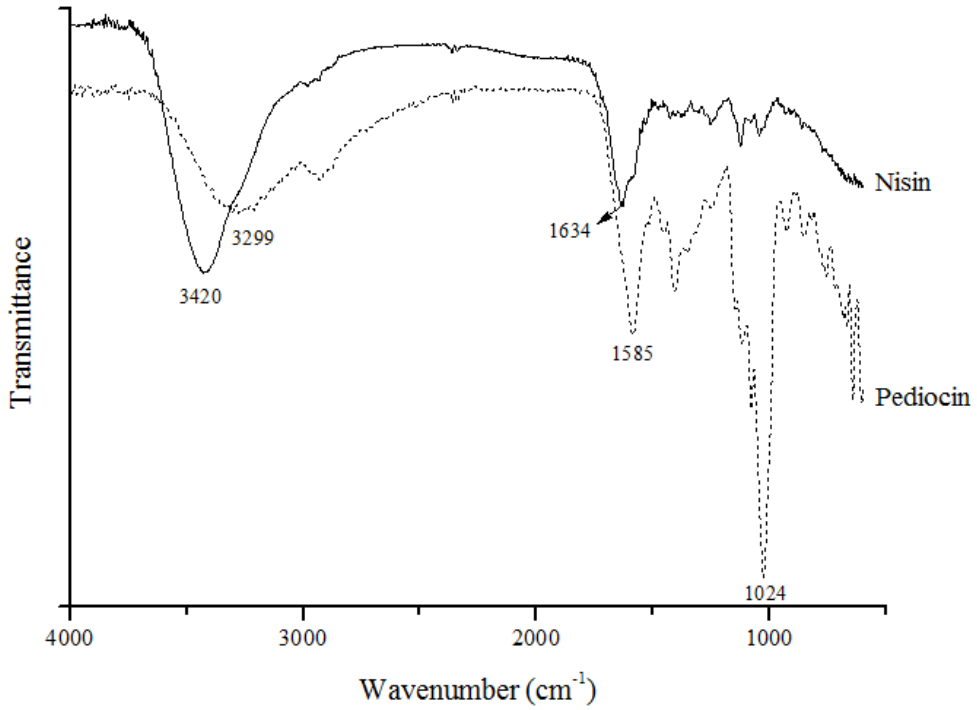


Fig 3. FT-IR spectra of (a) bentonite, (b) MMT modified with octadecylamine, (c) HNT, before (solid line) and after nisin or pediocin adsorption (dotted lines). (d) FTIR spectra of nisin and pediocin.

Non-electrostatic interactions may exist between the organic species and montmorillonites, for example hydrogen bonding and van der Waals force (Parolo et al., 2010). Also, HNT surface is composed of siloxane and has a few hydroxyl groups, which indicates that HNT also possesses potential for hydrogen bonding (Du et al., 2008). In our study, no additional bands in FTIR spectra were observed after nisin and pediocin adsorption on nanoclays. This result could be explained by the small quantity of nisin and pediocin in relation to the quantity of nanoclays used during adsorption studies. The adsorbed amount is affected by various factors such as peptide properties (size, structure stability, amino acid composition, 3-D conformation), the solid substrate surface characteristics and environmental conditions (Van der Veen, Norde, & Stuart, 2004). However, the intensity of siloxane bands (Si-O) were weaker in samples with bacteriocins, which indicate a possible interaction between the peptide with the nanoclays by the formation of hydrogen bonds. In the case of HNT spectra, samples adsorbed with nisin and pediocin showed a minor shift of the siloxane bands comparing to HNT alone, reflecting that non-eletrostatic interactions occurred after adsorption.

Electrostatic attraction may play an important role on the interaction of organic substrates with montmorillonite (Parolo et al. 2010). Nisin has a net positive charge of +5 and likely interacts with the negatively charged interface of lipid model membranes (El Jastimi & Lafleur, 1997; El Jastimi, Edwards, & Lafleur, 1999). Adsorption of nisin to hydrophilic surfaces by electrostatic interactions was previously cited (Daeschel et al., 1992; Bower et al. 1995). In the same way, pediocin presents positively charged residues, mostly located in the hydrophilic N-terminal region (Papagianni &Anastasiadou, 2009). Nisin consists of 34 amino acids including three lysine and two histidine residues. Also, pediocin presents Lys11 and His12 that are part of the cationic patch in the N-terminal β-sheet-like region of the molecule. Then, the protonation of these residual amino groups could favor electrostatic interactions between nisin and pediocin molecules and negatively-charged surfaces of the clay minerals (Papagianni & Anastasiadou, 2009; Ibarguren et al. 2014).

The morphology of MMT and HNT is illustrated in Fig. 4. Montmorillonite is a 2-to-1 layered smectite clay mineral with a platy structure with an inner octahedral layer sandwiched between two silicate tetrahedral layers. Individual platelet thicknesses are just one nanometer, but surface dimensions are generally 300 to more than 600 nm, resulting in an unusually high aspect ratio. Hundreds or thousands of these layers are stacked together with van der Waals forces to form clay particles (Pavlidou & Papaspyrides, 2008; Hashemifard,

Ismail, & Matsuura, 2011). In contrast, HNT exhibits a hollow tubular structure as the dominant morphology, resembling that of carbon nanotubes and its typical dimensions are on the nanoscale: 10-50 nm in outer membrane, 5-20 nm in inner diameter with 2-40 nm in length (Joussein et al., 2005; Rawtani & Agrawal, 2012). After nisin adsorption, no morphological differences were observed by TEM images (Fig 4 b and d). Similarly, pediocin adsorption did not alter nanoclay morphologies (data not shown).

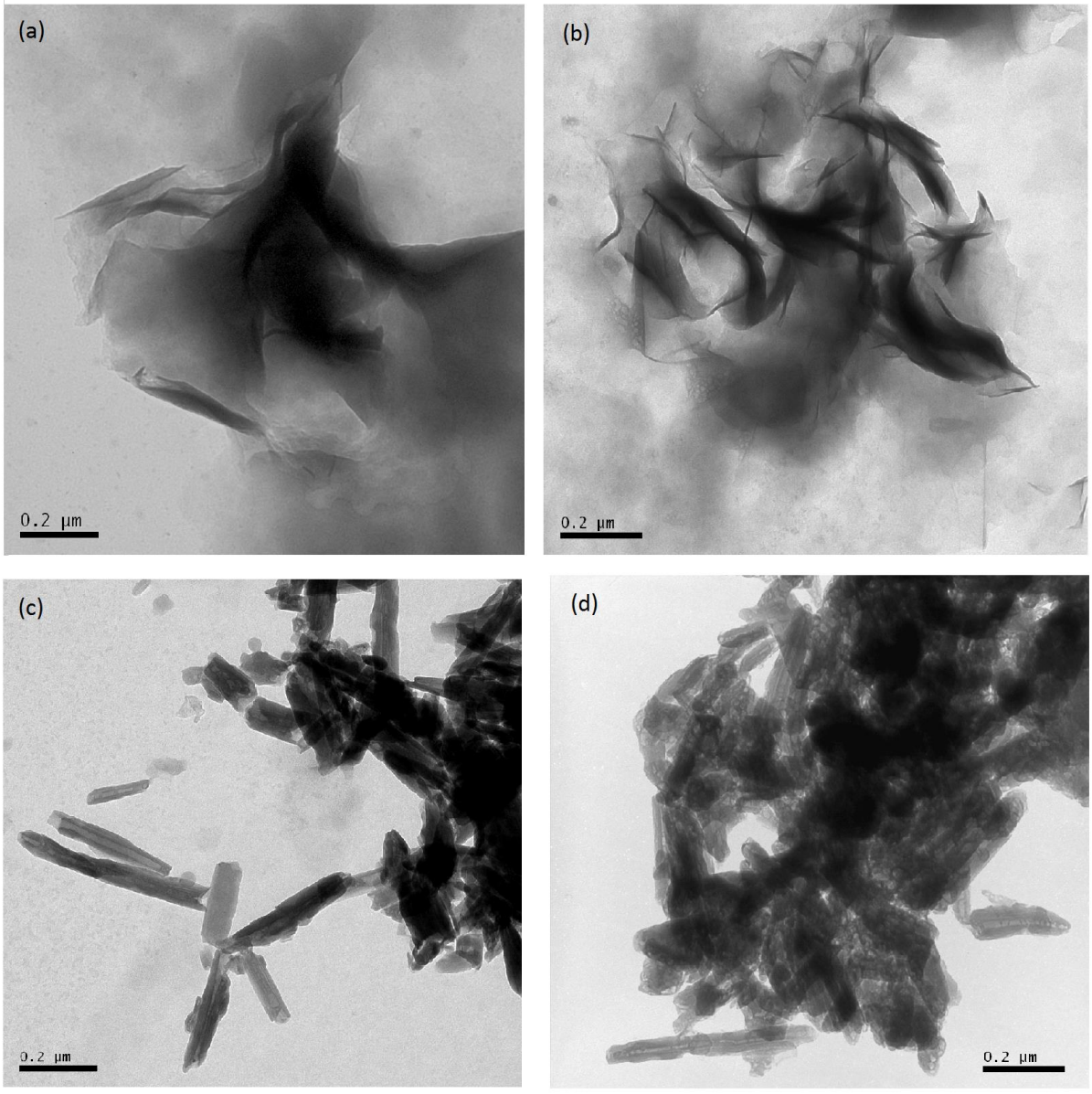


Fig 4. TEM micrographs of (a) bentonite; (b) nisin-adsorbed bentonite; (c) HNT; (d) nisin-adsorbed HNT.

4. Conclusions

Nisin and pediocin were able to adsorb on bentonite, MMT modified with octadecylamine and HNT. XRD analyses provide evidence on the intercalation of the peptide molecules on silicate layers. Non-eletrostatic interactions were inferred by FTIR analysis after bacteriocin adsorption on nanoclays. Meanwhile, the antimicrobial activity detected using BHI and skimmed milk agar plates was better when halloysite nanotubes were used as support agent. Therefore, halloysite adsorbed with nisin or pediocin proved to be a promising strategy as antimicrobial delivery systems.

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ARTIGO 3 - Biodegradable starch films prepared by casting with halloysite as nanoreinforcement and bacteriocins nisin and pediocin as antimicrobial agents

Abstract

Corn starch was used as a polymeric matrix to the development of new antimicrobial packaging prepared by casting method and using nisin or pediocin for food preservation. Halloysite clay was selected as nanofiller to promote reinforcement in film properties and a novel approach was tested using bacteriocins previously adsorbed on nanoclay before incorporation in film form solutions. Active packaging against *Listeria monocytogenes* and *Clostridium perfringens* were obtained, but the nanotubes retained antimicrobial activity comparing to films without nanofiller addition, namely as N and P samples. Results from XRD showed that the addition of bacteriocins affected the crystallinity of starch matrix, whereas SEM images showed that peptides provoked surface irregularities. But adsorption approach proved to be a promise strategy to maintain a certain crystallinity and homogeneous morphology of films and consequently influencing other properties, like improving thermal performance. Mechanical resistance of films, measured by Young modulus and tensile strength, presented significant differences among films and justified the importance of the nanofiller incorporation. Elongation at break (EB) was maintained in treatments, except for samples N, P and those containing nisin plus halloysite (NH) in which EB increased significantly. Chemical interactions were stronger between additives and starch matrix, evidencing by band modifications via hydrogen bonding in FTIR spectra. Color measurements varied especially for pediocin samples and highlights peptide adsorption as an interesting way for keeping L*, a* and b* values near to those obtained for control films, although it affected the transparency. The results of this research suggest the potential use of developed antimicrobial nanocomposite films as food packaging, but more studies are needed to indicate adjustments and appropriate applications.

Introduction

Food-borne outbreaks are a global public health issue that increased concern for new approaches and technologies to control food-borne pathogenic microorganisms. On that basis, food packaging with new functions, known as active packaging, has been developed to provide more safety and quality of food products. Antimicrobial packaging is a type of active packaging which interacts with the product or the headspace inside to reduce, inhibit or retard

the growth of microorganisms that may be present on food surfaces (Appendini & Hotchkiss, 2002). This strategy is considered as an additional hurdle to food contamination, reducing the risk of pathogen growth and extending the shelf life of food. Also, the use of natural compounds for food preservation and currently demand for additive-free foods stimulate the incorporation of natural antimicrobials into packaging materials, which allows a gradual migration into a food matrix and eliminates the need for additional high concentrations of preservatives directly on the food product (Quintavalla & Vicini, 2002; Sung et al., 2013).

Bacteriocins are natural antimicrobials, ribosomally-synthetized bacterial peptides, especially isolated from lactic acid bacteria (LAB) since they are traditionally associated to food and regarded as safe (García, Rodríguez, Rodríguez, & Martínez, 2010). The most widely used bacteriocin in active food packaging is nisin due to its Generally Recognized As Safe (GRAS) approval by FDA. Nisin is applied worldwide in dairy products (especially cheese making) as well as sausages, canned and packaged meat and brewing (Sobrino-López & Martín-Belloso, 2008). Nisin is a small polypeptide (34 amino acids) produced by the bacterium *Lactococcus lactis* subsp. *lactis*. It shows antimicrobial activity against a wide range of gram-positive bacteria, such as the foodborne pathogens *Listeria monocytogenes*, *Staphylococcus aureus, Clostridium botulinum* and its spore (de Arauz, Jozala, Mazzola, & Vessoni Penna, 2009). Another important bacteriocin is pediocin, mainly pediocin PA-1. Pediocin are produced by *Pediococcus* strains and presents 44 amino acids (Papagianni & Anastasiadou, 2009). It exhibit bactericidal effect over some pathogenic and Gram-positive bacteria (Deegan, Cotter, Hill, & Ross, 2006). The use of pediocin as natural biopreservative to overcome the post-processing contamination of meat products (slicing, packaging, peeling and handling) has been reported and it is particularly effective against the food pathogen *L. monocytogenes* (Rodríguez, Martínez, & Kok, 2002; Santiago-Silva et al., 2009).

To develop antimicrobial films incorporating nisin and pediocin and, at the same time, to address environmental issues, one of the approaches is to use renewable biopolymers such as polysaccharides. Starches are a renewable resource widely available and can be obtained from different byproducts of harvesting and industrialization. However, these biodegradable polymers have not found extensive applications due to their poor stability in processing, high sensitivity to the environmental changes, weak mechanical and poor barrier properties (Sorrentino, Gorrasi, & Vittoria, 2007). The reinforcement of the starch matrix with the addition of low quantities of nanofillers, resulting in a nanocomposite material, has given rise to significant improvements in physical, mechanical, and barrier properties (He et al., 2012;

Schmitt, Prashantha, Soulestin, Lacrampe, & Krawczak, 2012; Xie, Chang, Wang, Yu, & Ma, 2011) .

Halloysite nanotubes (HNTs) are nanomaterials composed of double layered aluminosilicate minerals with a predominantly hollow tubular structure in submicron range. They are nontoxic in nature, have adjustable release rates and fast adsorption rates (Kamble, Ghag, Gaikawad, & Panda, 2012). Nisin and pediocin were able to adsorb on halloysite surface, revealing a potential carrier for these antimicrobial peptides, as previously performed by our work group (data not published).

Thus, the aim of this investigation is to develop active starch films incorporating nisin and pediocin and evaluate the effect of halloysite addition (adsorbed or not with the bacteriocins) on the polymer matrix.

Material and methods

Materials

Corn starch (Amisol 3408, Corn Products Brasil, São Paulo, Brazil) was used in this study. Glycerol PA was purchased from Nuclear (Diadema, Brazil). The nanoclay halloysite (HNT) was obtained from Sigma-Aldrich (St. Louis, USA). Commercial nisin product (Nisaplin®) was provided by Danisco Brasil Ltda (Cotia, Brazil) with 2.5% of pure nisin, whereas pediocin formulation used is ALTA™ 2345, provided by Kerry Ingredients & Flavours, USA.

Adsorption of nisin and pediocin on halloysite

Adsorption assays were carried out by adding 10 mL nisin solution (40 mg/mL or 1 mg/mL, considering respectively the commercial product or pure nisin within it) or 10 mL pediocin solution (100 mg/mL) to 100 mg of halloysite. These bacteriocin-nanoclay systems were maintained during 1 h at 25ºC and 80 rpm. After that, samples were centrifugated (5000 *g* for 5 min at 25ºC), the supernatant was thrown away and the pellets obtained (nanoclays adsorbed with bacteriocins) were washed twice and dispersed in distilled water. Bacteriocin concentrations used for adsorption reached halloysite surface saturation, based on preliminary tests.

Preparation of the films

The films were prepared using the casting method and the formulations were determined according to preliminary tests. Film-forming solutions were prepared by dispersing 4% w/v corn starch in distilled water (in treatments: 4 g of total solids/100 mL of water). The solutions were heated in a water bath until 74ºC for 15 min under stirring to promote gelatinization. After heating, the glycerol (1.8% w/v) was added as a plasticizer, the solutions were stirred for more 15 min and other components were added as required in each case. Samples containing only plasticized starch and glycerol were considered as control films.

Bacteriocins nisin and pediocin, after incorporated in film-form solution, are designated as N and P samples, at final concentrations of 0.4% and 1% w/v, respectively.

For nanoreinforcement, halloysite was added at 0.1% w/v (100 mg of halloysite /100 mL of film-form solution), resulting in treatment namely as H. When nisin and pediocin were included plus halloysite, samples are identified as NH and PH and components presented the same final concentrations of the other treatments. Also, nisin and pediocin were previously adsorbed in halloysite powder (as described in Section 2.2) and then incorporated in film-form solution (final volume of 100 mL in which the components were equilibrated and comparable with other treatments). In this last case, resulting samples were denominated as NA (films with nisin-adsorbed halloysite) and PA (films with pediocin-adsorbed halloysite).

Next, film-forming solutions were poured onto Petri plates and dried for 16 h at 40ºC in an oven. After this time, the dried film solutions were peeled-off the casting surface, cut into adequate samples and conditioned at 25ºC and 52% RH with saturated solution of $Mg(NO_3)_2$ for 48 h prior to characterization analysis.

In vitro antimicrobial properties

The films' antimicrobial activity was tested using the inhibition zone assay in agar medium. Pieces with 1 cm of diameter were cut from films and placed on Brain Heart Infusion (BHI) agar plates. Then, 10 ml BHI soft agar (7.5 g l^{-1}) inoculated with indicator strain *Listeria monocytogenes* ATCC 7644 and *Clostridium perfringens* ATCC 3624 (10^7 CFU ml 1^{-1}) was poured onto plates. Petri dishes were stored at 4°C during 12 h to initiate nisin desorption and after incubated at 37°C for 24 h. The antimicrobial activity is evidenced by clear zones (no micro-organism growth or survival) surrounding film pieces. The diameter of the inhibition zones was measured and expressed as mm.

Mechanical properties

Tensile tests were carried out using films with 20 mm X 70 mm of size using a TA.XT *Plus* Texture Analyzer (Texture Technologies Corp and by Stable Micro Systems, Hamilton, MA, USA) according to standard ASTM D-638. Samples were clamped between grips and force and deformation were recorded during extension at 20 mm/min, with an initial distance between grips of 60 mm. Tensile strength (TS), elongation at break (EB) and Young´s modulus (YM) were determined from five replicated for each film formulation.

Scanning electron microscopy (SEM)

The films surfaces were analyzed by using a JEOL model JSM-6060 microscope operated at a voltage of 5kV. To obtain fracture faces, nanocomposites were cooled in liquid nitrogen, and then broken. Samples were coated with gold layer prior to analysis in order to increase their electrical conductivity.

X-Ray diffraction (XRD)

XRD measurements were performed using a Siemens D-500 diffractometer. Nisaplin® powder and films were scanned in the reflection mode using an incident Cu K_α radiation (λ = 1.54 Å), at a step width of 0.05°min^{-1} from 2θ = 5° to 40°C.

FTIR

Fourier Transform Infrared (FTIR) spectra were measured using a FTIR Varian 640 IR thermo scientific spectrometer in attenuated total reflectance (ATR) mode with a diamond crystal. The scans were collected between 600 and 4000 cm^{-1} at a 4 cm^{-1} resolution.

Thermogravimetric analysis (TGA)

A thermogravimetric analyzer model QA 50 (TA Instruments) was used for the thermal stability evaluation. The samples were heated from 25 to 800°C at the rate 10°C min^{-1} under nitrogen atmosphere (50 mL min^{-1}).

Surface color measurement

Color values of films were measured with a colorimeter (Minolta, CR-400, Osaka, Japan). The measurements were done in the CIELAB scale, in which each measurement is expressed as L* (indicating the lightness, dark to light values range from 0 to 100), a* (positive in the

red direction and negative in the green direction), and b* (positive in the yellow direction and negative in the blue direction). Standard values refer to the white calibration plate (L= 94.23, a= -0.55, and b= 9.68). Results were expressed as the means of five measurements on different areas of each film.

Transparency value

Film specimens were cut into rectangles and directly placed in a spectrophotometer test cell (Shimadzu UV-1800, Kyoto, Japan), taking blank cuvette as the reference. The light transmittance was determined at wavelength of 600 nm and the transparent value was calculated from the following equation: Transparency = -log T_{600} / X, where X is film thickness (mm) (Han & Floros, 1997).

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, using the SAS software (version 9.3).

Results and Discussion

Antimicrobial activity

All starch films with bacteriocins in formulation can be considered active packaging materials because exihibited inhibition zones against the two target pathogens (Table 1).
As expected, control and H films did not show antimicrobial activity.

L. monocytogenes was the most sensitive strain and films containing nisin were the most effective. Samples with pediocin showed halos of inhibiton around film against *L. moncytogenes*, but not against *C. perfringens* which inhibition area was confined to the film perimeter (Table 1). Consequently, the chosen images of the antimicrobial activity results are the inhibition zones against *C. perfringens* and *L. monocytogenes* respectively caused by samples with nisin and by pediocin films (Fig. 1a and Fig. 1b, respectively).

Table 1. Inhibition zones of active starch films against food borne pathogens.

Samples	Inhibition zones diameter (mm)	
	Listeria monocytogenes ATCC 7644	*Clostridium perfringens* ATCC 3624
N	11 ± 1.0 a	5.33 ± 0.6 a
NH	9.33 ± 1.5 ab	4.67 ± 0.6 a
NA	8.33 ± 1.2 ab	3.83 ± 0.5 a
P	7.67 ± 0.6 b	+
PH	4.0 ± 1.0 c	+
PA	2.33 ± 0.7 c	+

Values are means ± standard deviation. Treatments followed by the same letter within the same column are not significantly different (P>0.05).

+ = the inhibition area was confined to the film perimeter

A B

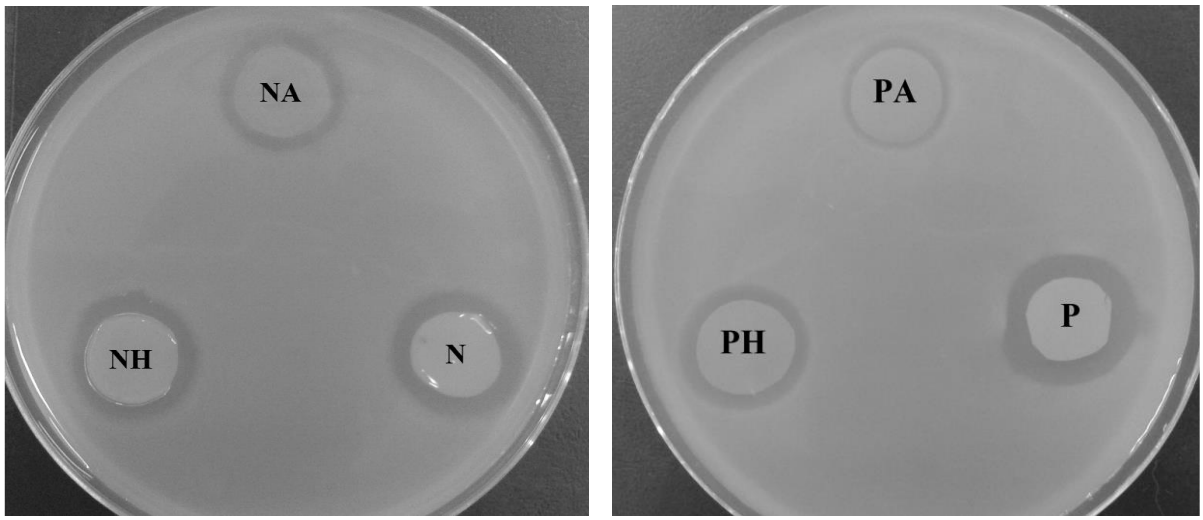


Fig. 1. Antimicrobial activity of the active starch films in BHI agar plates. (A) Nisin films against *Clostridium perfringens* ATCC 3624, designated as N (starch film with nisin), NH (starch nanocomposite of halloysite plus nisin) and NA (starch nanocomposite with nisin adsorbed on halloysite). (B) Pediocin films against *Listeria monocytogenes* ATCC 7644, designated as P (starch film with pediocin), PH (starch nanocomposite of halloysite plus pediocin) and PA (starch nanocomposite with pediocin adsorbed on halloysite).

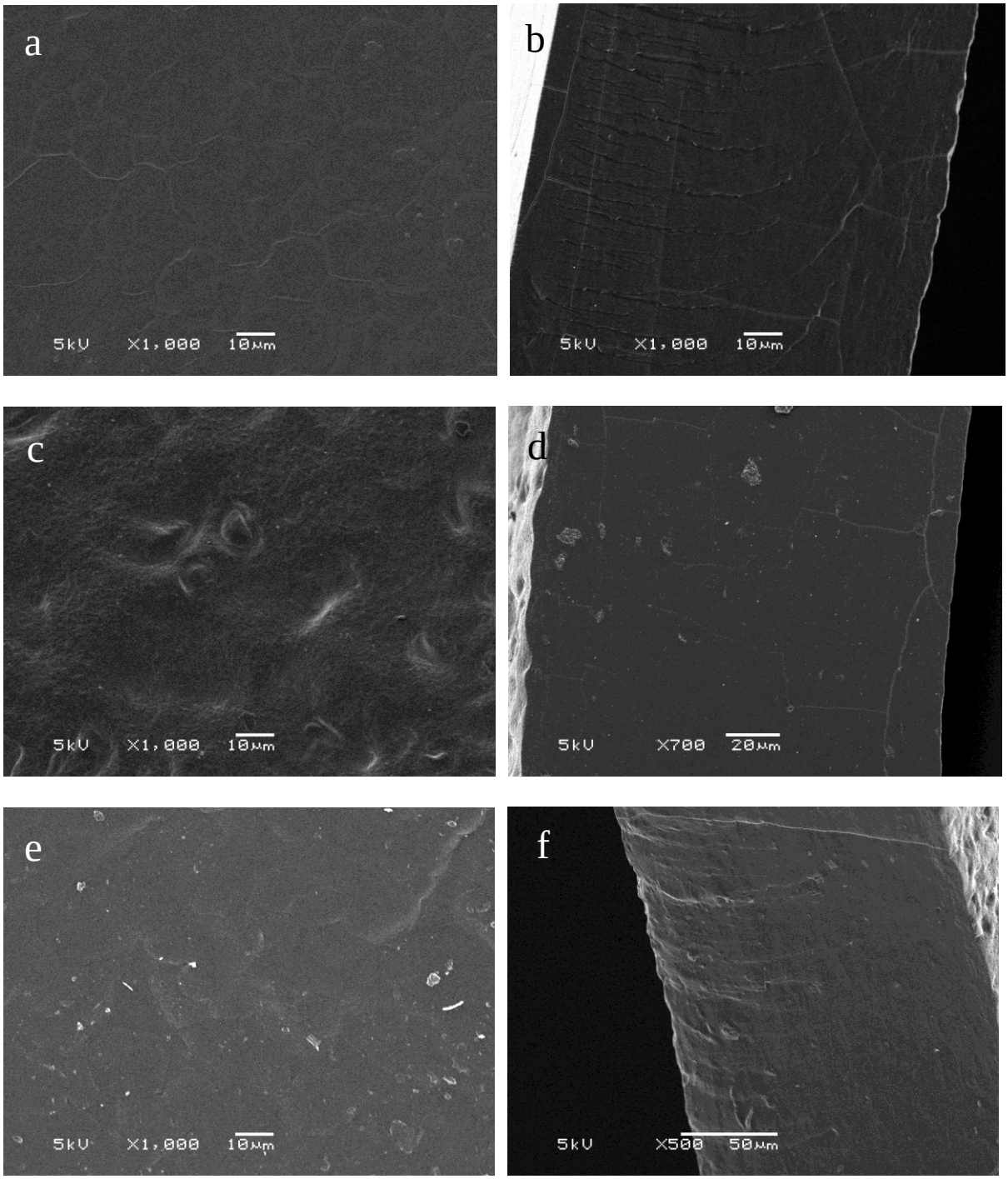
The casting method is largely used to prepare biopolymers (Sung et al., 2013). It was adequate for bacteriocin incorporation which ocurrered after starch gelatinization (temperature below 70ºC) and maintained antimicrobial activity. Nisin incorporated on starch-based films, made with tapioca starch and prepared by casting technique, were previoulsy reported as able to reduce *Listeria innocua* growth (Basch, Jagus, & Flores, 2013; Ollé Resa, Gerschenson, & Jagus, 2014; Sanjurjo, Flores, Gerschenson, & Jagus, 2006). However, the potential of pediocin in food packaging application has been little reported and, in relation to biopolymers, it has already been incorporated in cellulose (Paula Judith Pérez Espitia, Pacheco, Melo, Soares, & Durango, 2013a; Santiago-Silva et al., 2009) and poly(lactic acid) (Woraprayote et al., 2013) films with effective inhibition of *Listeria* species.

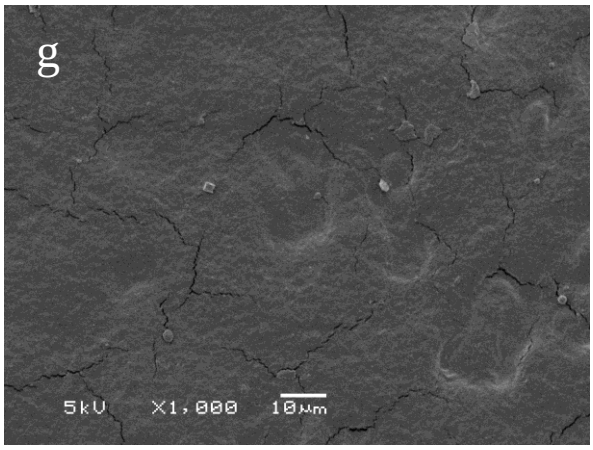
Active films with nanoclay in composition, especially those with nanoclay previously adsorbed with bacteriocins, presented lower inhibition zones but not at a significant level ($P>0.05$) for samples with nisin (Table 1). In antimicrobial films containing pediocin, the samples with halloysite (PH and PA) were significant different ($P<0.05$) from the one with just starch, glycerol and pediocin (sample P) and this behavior can be clearly observed in Fig.1b. Other researches previously reported effects of nanoclays in controlling diffusion or enhancing retention of antimicrobial agents by polymer matrices (Sanchez-Garcia, Gimenez, & Lagarón, 2008; Mascheroni, Chalier, Gontard, & Gastaldi (2010).

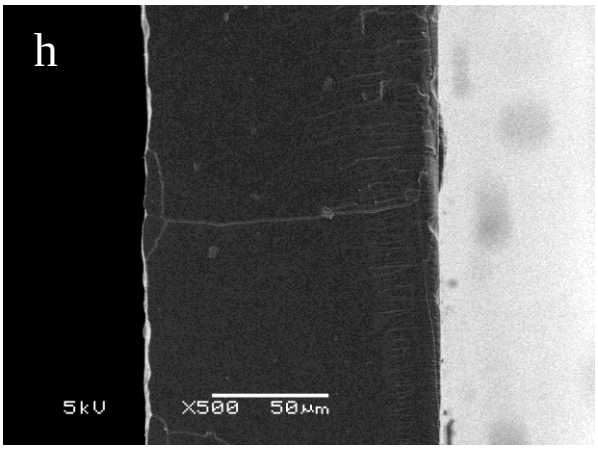
Structural properties

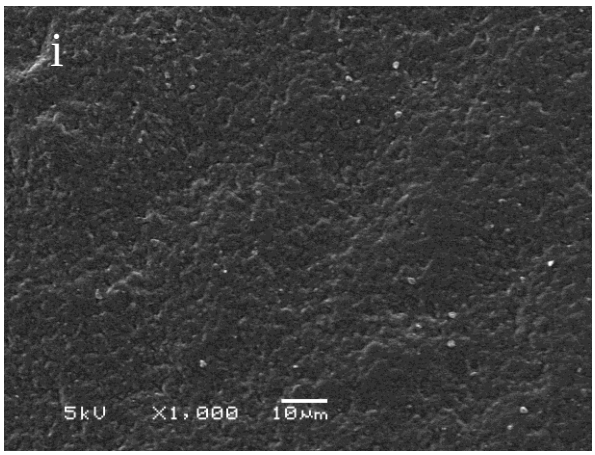
In order to evaluate whether the nisin and pediocin incorporation could modify starch films surface and cross-section properties, Scanning Electron Microscopy (SEM) were performed as can be seen in Fig 2. SEM micrographs of films containing bacteriocins showed uneven and rough surfaces with visible particles compared with control (Fig. 2a) and H films (Fig. 2c).

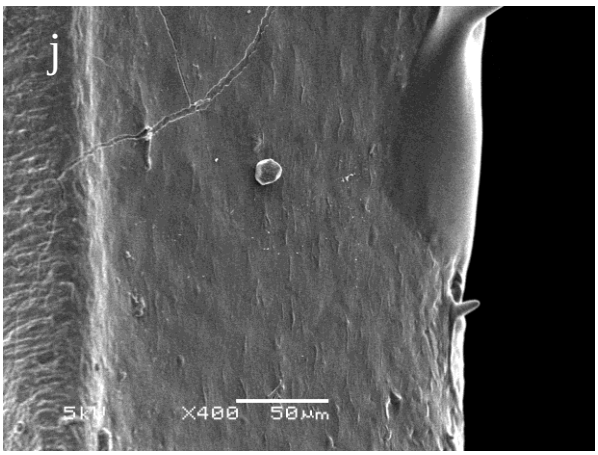
The aspect of these films can be justified by the commercial nisin powder used in this work that also contains milk proteins, carbohydrates and sodium chloride, whereas the pediocin applied on films is a fermented powder containing excipients, such as skim milk, whey and dextrose. Indeed, it is important to observe that films with bacteriocins adsorbed on halloysite (NA and PA, Fig. 2g and 2i, respectively) presented a more homogenous appearance, highlighting that just peptides were involved in adsorption process.

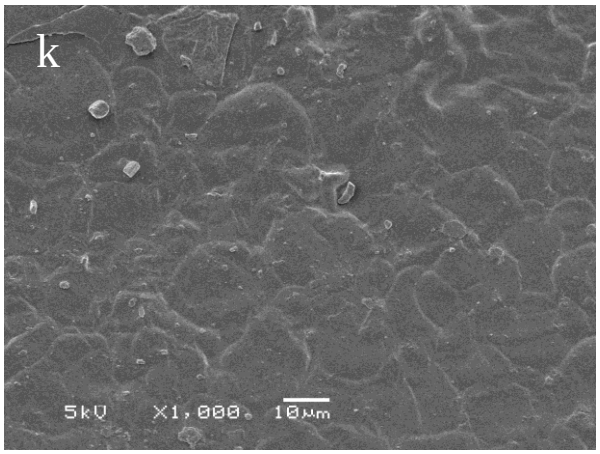

a
5kV X1,000 10µm
b
5kV X1,000 10µm
c
5kV X1,000 10µm
d
5kV X700 20µm
e
5kV X1,000 10µm
f
5kV X500 50µm

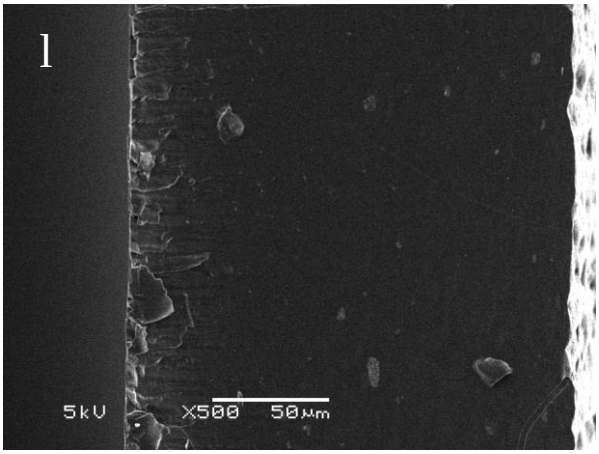

g
5kV X1,000 10µm


h
5kV X500 50µm


i
5kV X1,000 10µm


j
X400 50µm


k
5kV X1,000 10µm


l
5kV X500 50µm

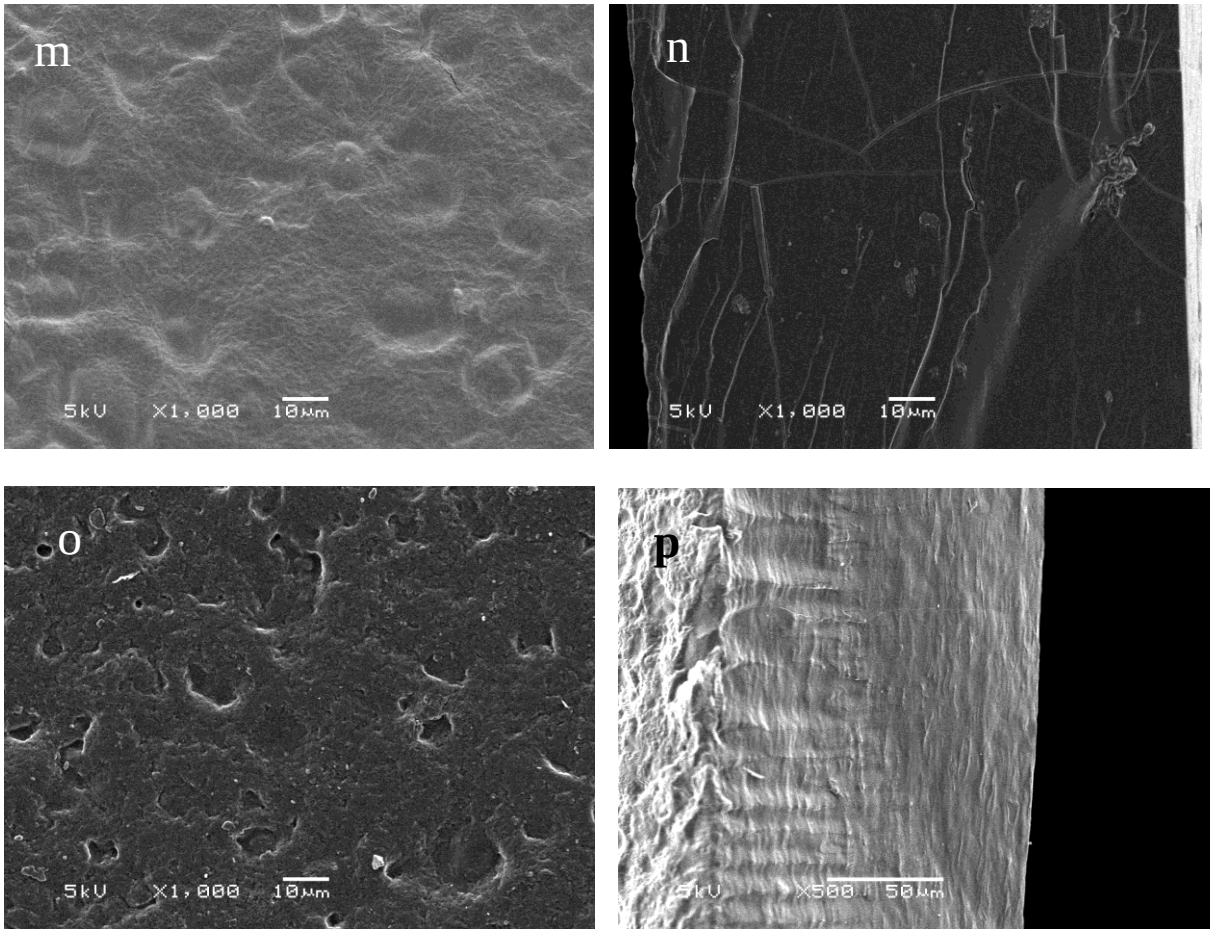


Fig. 2. SEM micrographs of surface (left column) and cross-section (right column) of starch films: (a) and (b) control; (c) and (d) H; (e) and (f) NH; (g) and (h) NA; (i) and (j) N; (k) and (l) PH; (m) and (n) PA; (o) and (p) P. Samples were identified as control film; starch films with nisin and pediocin, namely as N and P; and nanocomposites films containing halloysite (H), nisin and pediocin adsorbed on halloysite, respectively NA and PA, halloysite plus nisin (NH) and pediocin (PH).

A considerable increase of the surface irregularities with the presence of nisin was reported in tapioca starch films by Ollé Resa, Jagus, & Gerschenson (2014), as well as peptide crystals were observed in SEM images of cellulosic films with pediocin by Espitia et al. (2013a).

In cross section micrographs, the roughness and irregular aspect of samples with bacteriocins also appeared (Fig. 2 f, h, j, l, n and p). However, there are not any sign of debonding in starch matrix and pores were not observed, indicating that components had distribution and/or dispersion at an acceptable level during film preparation. When extrusion method was used to develop films with nisin, heat-sensitive components in antimicrobial

commercial product might have degraded, creating a porous structure (Bastarrachea et al., 2010; Meira et al., 2014).

XRD diffractograms of the starch films are shown in Fig. 3. Control and H samples presented a strong diffraction peak at 2θ = 19.8º and other two small peaks at around 2θ values of 17º and 21.9º. The amount of halloysite added did not alter the crystalline peaks of starch and the diffraction pattern of H film was very similar to control. He et al. (2012) reported that crystalline peaks of halloysite became apparent in X-ray spectra of starch-based films only at higher concentrations, especially at amount of 9 wt%.

The incorporation of nisin in starch films decreased the intensity of peaks, particularly samples NH and N. When nisin was adsorbed on nanoclay (sample NA) peaks became larger compared to control films and peak at around 2θ = 19.8º disappeared. In nanocomposite NH, a shift of the diffraction peaks occurred to lower angles. In X-Ray spectrum of nisin film without clay, sample N, no remarkable peaks were observed, showing a mainly amorphous behavior. This results are in agreement with Bastarrachea et al. (2010) that reported how the amount of nisin incorporated in a polymer matrix was able to block the crystal growth and hence the final crystallinity.

On other hand, films containing pediocin did not influence the crystallization of starch during the film preparation since their XRD diffractograms had no obviously difference comparing to control films.

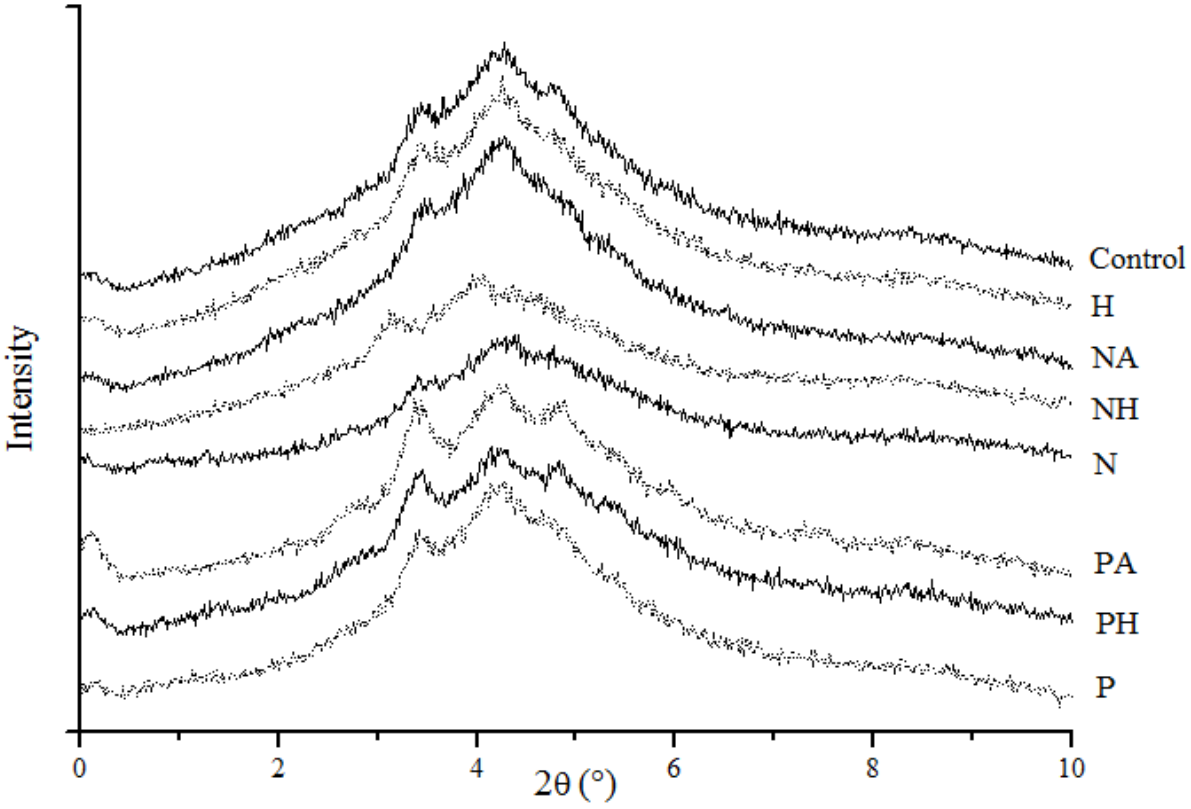


Fig. 3. X-ray diffraction (XRD) patterns of the control film; starch films with nisin and pediocin, namely as N and P; and nanocomposites films containing halloysite (H), nisin and pediocin adsorbed on halloysite, respectively NA and PA; halloysite plus nisin (NH) and pediocin (PH).

Mechanical Properties

The capacity of these starch films for preserving the integrity of food stuff was evaluated by measuring the Young's modulus (YM), tensile strength (TS) and elongation at break (EB) and results are show in Table 2.

Samples containing nanoclay (H) and nanoclay adsorbed with bacteriocins (NA and PA) displayed the best values of YM and did not differ significantly from each other ($P>0.05$). Active films without nanoclay (N and P) showed the lower values of YM but not at significant level comparing to NH, PH and control. These results demonstrated the positive effect of nanoreinforcement incorporation and in relation to the antimicrobial nanocomposites, the bacteriocin adsorption procedure revealed advantageous to increase film rigidity. After peptide adsorption, other compounds present in nisin and pediocin commercial products were eliminated by washing the bacteriocin-halloysite systems previously their

incorporation in film-form solutions. Thus, undesirable compounds not interacted with starch matrix and did not modify the films mechanical conduct as occurred to other antimicrobial films. NaCl, milk proteins and carbohydrates have high affinity for water, known to be very effective plasticizer for most biopolymers, which induced lowering of YM in N, P, NH and PH films.

Considering TS parameter, samples ranged from 0.47 to 2.05 MPa. The nanoclay addition significantly improved film resistance of H sample in relation to control. Also, the presence of HNT in nanocomposites NH and NA significantly increased TS when contrasted with N films that showed the lowest TS value. In a different manner, sample P did not differ significantly from PH and PA nanocomposites.

In relation to EB, no significant variation occurred between samples (EB around 14-28%), except for N, NH and P films. In the case of nisin samples, the increase in EB is probably caused by random break due to presence of salt crystals of the commercial antimicrobial product once the films were dried, as also observed by Imran, El-Fahmy, Revol-Junelles, & Desobry (2010). In turn, for pediocin films, the presence of HNT is responsible for the significant difference in EB between P films and PH and PA nanocomposites. Espitia et al. (2013b) evidenced that the addition of pediocin in cellulosic matrix increased values of elongation at break, indicating that this bioactive peptide acted as a plasticizer, which could possibly occurred in P samples.

Analyzing all parameters together, our results for N samples are in agreement with Ollé Resa, Jagus, et al. (2014) who reported that nisin presence in tapioca starch films, lowered YM and TS and increased the EB. In relation to pediocin, Espitia et al. (2013a) concluded that the interaction between an excessive amount of pediocin crystals and cellulosic chains lead to the weakening of the polymeric structure and decreased the resistance of the polymeric matrix. In general, mechanical properties of films with bacteriocins were improved by the presence of the nanoreinforcement.

Table 2. Mechanical properties and the thermogravimetric parameter T_{max} of starch films.

Samples	YM (MPa)	TS (MPa)	EB (%)	T_{max} (ºC)
Control	7.50 ± 0.31 bcd	1.13 ± 0.11 b	27.0 ± 3.7 b	306.0 ± 1 ab
N	2.83 ± 0.61 d	0.47 ± 0.15 c	80.9 ± 11.4 a	283.3± 6 e
P	3.76 ± 0.84 d	0.97 ± 0.33 bc	70.2 ± 13.9 a	293.2 ± 2 d
H	17.84 ± 5.03 a	2.05 ± 0.29 a	18.9 ± 1.0 b	307.2 ± 4 a
NH	5.51 ± 1.49 cd	1.04 ± 0.13 b	66.4± 10.1 a	294.2 ± 2 d
NA	11.12 ± 4.25 abc	1.16 ± 0.13 b	20.5 ± 1.7 b	295.0 ± 1 cd
PH	6.07 ± 0.23 cd	0.94 ± 0.09 bc	28.2 ± 0.9 b	297.6 ± 5 bcd
PA	14.04 ± 1.53 ab	1.15 ± 0.14 b	14.6 ± 1.6 b	304.1 ± 2 abc

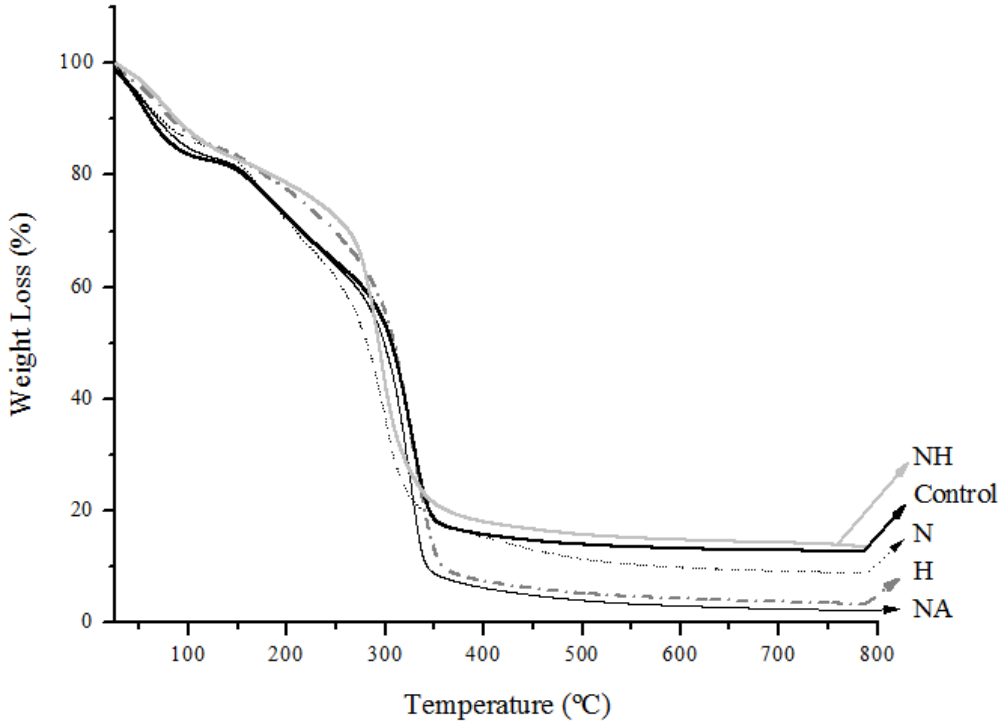
Values are means ± standard deviation. Treatments followed by the same letter within the same column are not significantly different (P>0.05).

YM =Young Modulus; TS = Tensile Strength; EB = Elongation at Break; T_{max} = Maximum rate of mass loss.

Thermal Properties

The thermogravimetric curves are shown in Fig. 5. It can be observed that control film displayed a similar thermal stability comparing to film H. These results are not in accordance with other works which reported high thermal stability of the matrix after addition of halloysite (He et al. 2012, Schmitt et al. 2012 and Xie et al. 2011), but it can be explained by smaller amout of nanoclay in our films. When nisin and pediocin were added to starch films the weight loss pattern is visible different between these samples (Fig. 5). Nisin samples were the most sensitive and exhibited the poor thermal properties comparing to pediocin films.

A



B

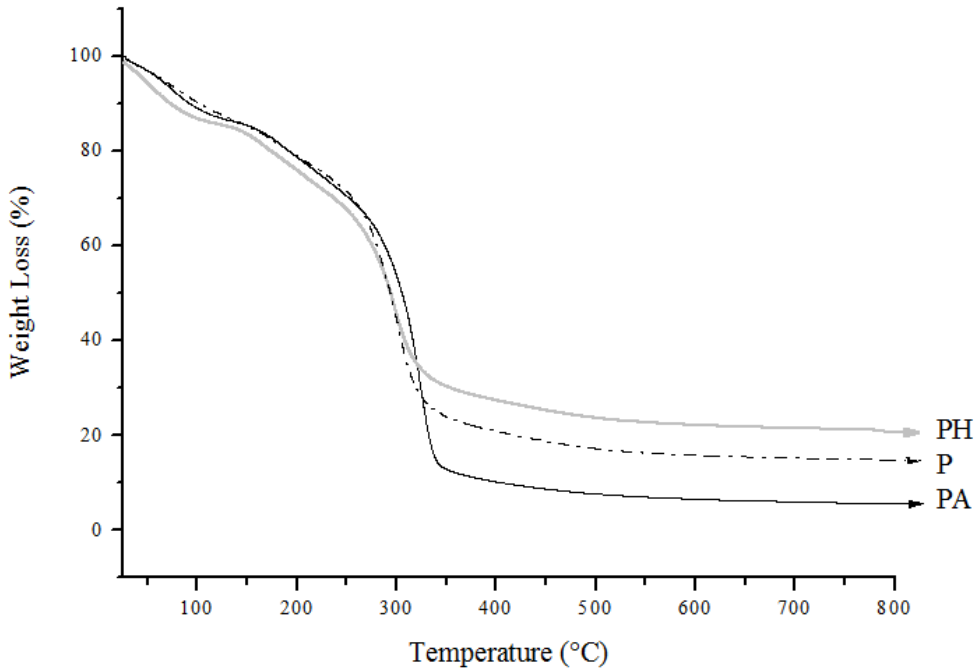


Fig. 4. Thermogravimetic analysis (TGA) of the starch films. (A) Control film; starch films with nisin, namely as N; and nanocomposites films containing halloysite (H), halloysite plus nisin (NH) and nisin adsorbed on halloysite (NA). (B) Starch films with pediocin, namely as P; and nanocomposites films containing halloysite plus pediocin (PH) and pediocin adsorbed on halloysite (PA).

Considering the temperature at maximum rate of mass loss (T_{max} – Table 2), samples without nanoclay displayed the lower T_{max} values, except for control which not differed significantly from sample H. Sample N differed significantly (P<0.05) from other films, exhibiting the lowest T_{max} (283.3ºC). Nanocomposites NH and NA displayed higher T_{max}, 294.2ºC and 297.6ºC, respectively, significantly higher from N samples. The maximum rate of mass loss for nanocomposite PA was at 305.1ºC which not differed significantly from nanocomposite H (307.2ºC). The T_{max} value of PH did not differ significantly from values obtained for PA and P films. Therefore, the presence of the nanoreinforcement and especially the adsorption of bacteriocins on halloysite previously their incorporation in film form solutions denoted improved thermal properties of the antimicrobial packaging materials.

Chemical interactions

FTIR analysis is a useful tool to analyze the interactions between starch molecules and plasticizers, clays or other molecules, like antimicrobials. Fig. 4 shows the FTIR spectra of control films, nanocomposites and starch films containing bacteriocins.

The control films exhibited the characteristic groups of starch: 3322 cm^{-1} related to hydroxyl groups; 2928 cm^{-1} attributed to –C-H and –C-H_2 bond stretching of the anhydroglucose ring; 1643 cm^{-1} relating to water bonding vibration; 1146 cm^{-1} and a shoulder in 1080 cm^{-1} attributed to –C-O bond stretching of the –C-O-H bonds; 1020, 925 and 855 cm^{-1} related to –C-O bond stretching of the C-O-C anhydroglucose ring (Schmitt et al., 2012; Xie, Chang, Wang, Yu, & Ma, 2011).

Compared to the characteristic peaks of starch, there were shifts to lower wave number in films added with bacteriocins and in nanocomposites. The peak frequency reduced indicates an increase of the molecular interaction (Gao, Dong, Hou, & Zhang, 2012). Based on the reduction extent of peak frequency near to the frequency of 3322 cm^{-1} (ascribed to free, intermolecular, and intramolecular bound hydroxyl groups) films with pediocin (PA, PH and P) had a stronger interaction with starch molecules via hydrogen bonds than other films. More stable hydrogen bond with the hydroxyl groups ocurred, so the frequency, related to the hydroxyl group of starch decreased a great deal in those films.

Also, the peak at 1020 cm^{-1} of the control film changed its style in the treatments and shifted to lower wave numbers (Fig. 4). This fact revealed that C-O-C group in starch formed hydrogen bond with film components (Ma & Yu, 2004), either halloysite or peptides.

HNT powder has a characeristic peak at 3622 cm^{-1} (data not shown) related to external hydroxyl groups, but it did not appeared in films H, NH, NA, PH and PA, indicating replacement of the free water in the interlayer of halloysite, and the formation of new groups with OH groups of the starch and/or glycerol (Slavutsky, Bertuzzi, & Armada, 2012). On the other hand, a new band appeared at 1740 cm^{-1} in H and NA films, characteristic of an ester group, indicating that some hydroxyl groups of halloysite or starch were esterified during film preparation.

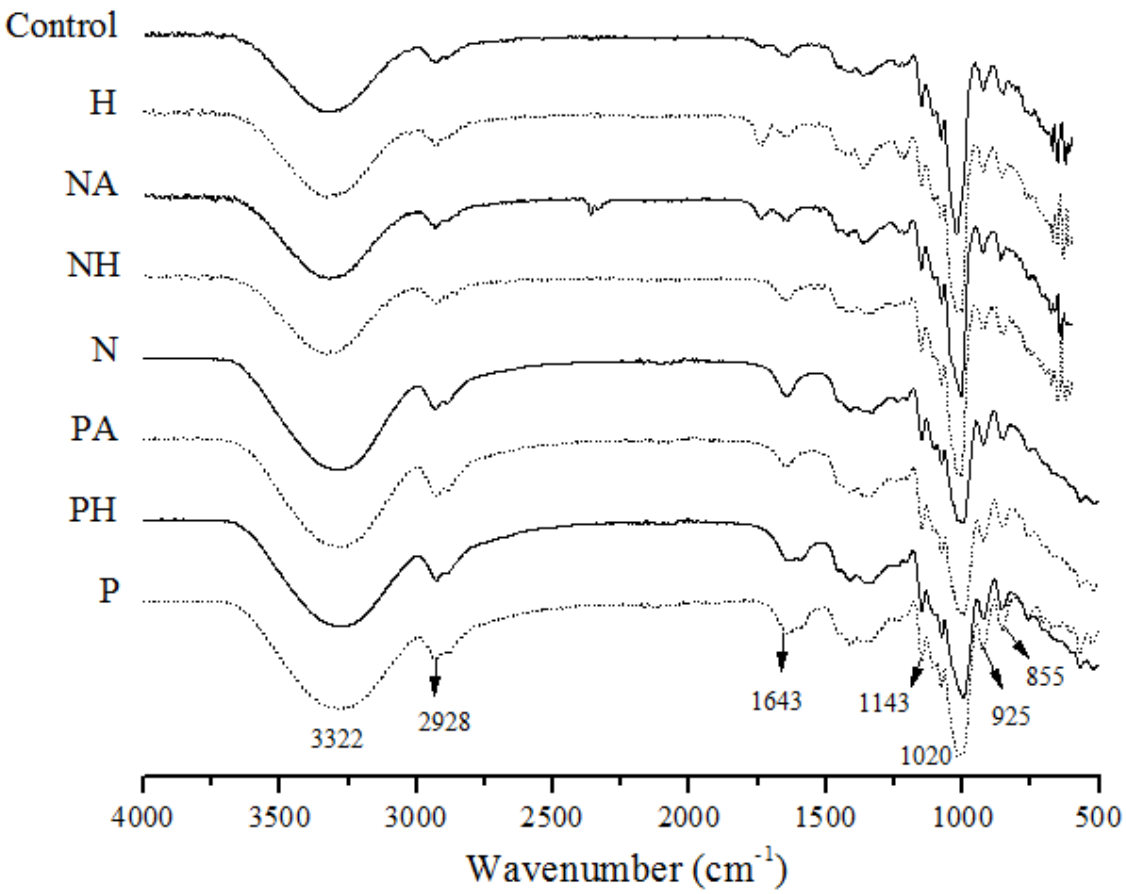


Fig. 5. FTIR spectra of the control film; starch films with nisin and pediocin, namely as N and P; and nanocomposites films containing halloysite (H), halloysite plus nisin (NH) and pediocin (PH), nisin and pediocin adsorbed on halloysite, respectively NA and PA.

Color and transparency of films

Color characteristics of the starch films determined instrumentally are shown in Table 3. For colorimetric parameter L*, control and H films did not differ significantly, as well as for nisin samples, which had no significant variation between them. On the other hand, PA exhibited the higher value of L* comparing to all other films, whereas PH and P samples presented the lowest values of lightness. In relation to a* parameter, nisin films did not differ from control and H films, however P and PH varied significantly from other samples and only PA presented the a* value comparable to control films. The b* values of nisin containing films and PA were approximated, but nonetheless significant different from control and H samples. PH and P samples showed much higher b* values, since this antimicrobial in the form of concentrated powder presents naturally a yellow color, thus, resultant films presented yellowish color. Our results are in accordance with (Basch et al., 2013; Espitia et al., 2013b). The first group of authors verified decrease of L* values and increase of b* caused by nisin incorporation in tapioca starch films. Also, the presence of pediocin in the formulation of nanocomposite films with ZnO produced slightly yellowish films and their luminosity was significantly diminished (Espitia et al., 2013b).

Besides color, transparency of the film is a relevant property since it has a direct impact on the appearance of packaged product. Control film presented the lower transparency value (1.75), closer to the one obtained for oriented polypropylene (1.67), a commercial film used for packaging purposes (Guerrero, Stefani, Ruseckaite, & de la Caba, 2011). Transparency of samples N and P did not differ significantly from control films. Meanwhile, the incorporation of HNT led to significant increased transparency value in the nanocomposites (H, NH, NA, PH and PA films), indicating higher opacity comparing to control samples. The decrease of transparency by the addition of halloysite had been previously reported in oxidized starch films (Kong, Wang, Gao, Liu, & Liu, 2011) and potato starch composites (He et al., 2012). Furthermore, Slavutsky, Bertuzzi, & Armada (2012) observed that opacity of starch-montmorillonite films was dependent on the nanoclay dispersion in the polymer matrix, revealing that a decrease in transparency is related to intercalated structure.

Table 3. Color measurements and solubility of the control film and starch/halloysite nanocomposites.

	L*	a*	b*	Transparency value
Control	90.98 ± 0.24 ab	-0.79 ± 0.03 b	3.63 ± 0.15 e	1.75±0.07 d
N	90.77 ± 0.49 bc	-0.73 ± 0.06 ab	4.76 ± 0.12 c	2.12±0.08 cd
P	88.80 ± 0.42 d	-1.43 ± 0.08 c	11.64 ± 0.48 a	2.07±0.06 cd
H	91.13 ± 0.13 ab	-0.68 ± 0.03 a	3.72 ± 0.17 e	2.59±0.13 ab
NH	90.24 ± 0.28 c	-0.74 ± 0.05 ab	4.81 ± 0.22 c	2.20±0.16 bc
NA	90.61 ± 0.46 bc	-0.76 ± 0.07 ab	4.62 ± 0.20 cd	2.32±0.09 abc
PH	89.38 ± 0.17 d	-1.43 ± 0.04 c	10.98 ± 0.08 b	2.45±0.04 abc
PA	91.63 ± 0.41 a	-0.78 ± 0.05 b	4.22 ± 0.24 d	2.68±0.16 a

Values are means ± standard deviation. Treatments followed by the same letter within the same column are not significantly different (P>0.05).

Conclusion

The addition of the peptides nisin and pediocin in starch films resulted in active packaging materials with antimicrobial activity against *L. monocytogenes* and *C. perfringens*, but it significantly modified the other properties. The development of antimicrobial nanocomposites by halloysite incorporation improved mechanical and thermal performance, specially when bacteriocins were adsorbed on the nanofiller. Using the peptides adsorption approach, the color measurements, morphology and crystalinnity were not significant altered comparing to control films. Neverthless, antimicrobial activity and transparency were negatively affected in films with halloysite-adsorbed bacteriocin, mainly in PA samples. In order to complete these investigations, further tests will be carried out with additional analysis and application of films on real food systems.

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ARTIGO 4 - Nisin incorporation on starch-halloysite nanocomposites: characterization and effectiveness against *Listeria monocytogenes* in Minas Frescal cheese

Abstract

Starch/halloysite (HNT)/nisin nanocomposite films were prepared to obtain active antimicrobial packaging materials. Nanocomposites without nisin and control films (only with starch and glycerol) were also prepared. The characterization of films samples by Scanning Electron Microscopy (SEM) revealed that cross sections of all samples depicted homogeneity, HNT was dispersed in starch matrix and film surfaces denoted aggregates as higher amount of nisin was added. X-ray diffraction (XRD) spectra displayed alterations in characteristic peaks of starch after HNT and nisin incorporation, denoting a decrease of polymer matrix crystallization. However, minimal chemical interactions were evidenced by Fourier Transform Infrared (FTIR) patterns between film components. The mechanical properties were improved when HNT was incorporated but were affected by nisin addition. The bionanocomposite containing 3 wt% HNT and 2 wt% nisin was the only sample that not differed significantly from control films in relation to all mechanical parameters – Young modulus, tensile strength and deformation at break. Thermal stability increased with increasing HNT concentration in film matrix without antimicrobial but not at a significant level, whereas bionanocomposites with nisin showed significant lower temperatures at the maximum rate of mass loss. The color value *L* was significantly lower with higher quantities of halloysite and nisin and the parameter *b* expressed the darker color acquired by nanocomposites because of these additives which approximately doubled their values comparable to control. Solubility was the unique property that not differed significantly between samples. The antimicrobial activities of films were tested against *Listeria monocytogenes, Clostridium perfringens* and *Staphylococcus aureus* in skimmed milk agar and all microorganisms were inhibited by bionanocomposites containing nisin. Therefore, these active samples were applied on Minas Frescal cheese surface previously inoculated with *L. monocytogenes* at refrigeration temperature. After 4 days, antimicrobial nanocomposite films with 2% nisin significantly reduced the initial counts of the bacterium and those with 6% nisin completely inhibited *Listeria* below the detection limit of the method. Results obtained showed that nisin supported in starch/halloysite films is active and a useful barrier to further food contamination, requiring more studies to improve overall bionanocomposite properties.

Introduction

Environmental concerns over non-biodegradable petrochemical-based plastics have raised interest in the use of biopolymers as packaging materials. Among the eco-friendly polymers, starch is one of the most promising candidates since it has easy availability, relatively low cost, and renewable natural polysaccharide obtained from a great variety of crops (Gao et al. 2012).

In food industry, when recycling is difficult and/or not economical, especially in short life-time application, starch films are a possible alternative. Unfortunately, the starch presents some drawbacks, such as the strong hydrophilic behavior and poorer mechanical properties than the conventional non-biodegradable plastic films used in the food packaging industries (Avella et al. 2005). To circumvent this fact, improvements in the functional properties of these films have been made with nano-sized fillers, such as nanoclays (especially montmorillonite), resulting in composite materials, namely nanocomposites. Recently, halloysite nanotubes (HNT) become the subject of research attention as a new type of nanofiller for enhancing the mechanical, thermal and the degree of crystallinity of thermoplastic polymers (Schmitt et al. 2012). Moreover, the functionality of packaging can be enhanced by incorporating active substances.

Active packaging mainly comprises the development of films capable of exercising an antimicrobial effect on food. Preservation of the food from microbial spoilage and contamination/proliferation of pathogenic microorganisms can be achieved by a food packaging material during storage (Dainelli et al. 2008; Meira et al. 2014). *Listeria monocytogenes* is one of the most important food-borne pathogens and many studies have been directed to the use of antimicrobial substances in order to inhibit its growth in food products. Bacteriocins produced by lactic acid bacteria have been often used to inhibit the development of *L. monocytogenes* (Loessner et al. 2003; Sobrino-López and Martín-Belloso 2008). Nisin exhibits antimicrobial activity against Gram-positive vegetative cells like *Listeria* and spores of *Bacilli* and *Clostridia* (de Arauz et al. 2009). It is the only bacteriocin recognized as safe for the food industry by the World Health Organization and it is approved for use in processes cheese by Food & Drug Administration. This bacteriocin is used as natural preservative against clostridial spoilage in processed hard and semi-hard cheese, cheese spreads and dairy desserts (Chollet et al. 2008).

Cheese is one ready-to-eat type of food that has been associated with food-borne listeriosis, especially cheeses with high and medium moisture content. Contamination by *L.*

monocytogenes is of special concern because of its psychrotrophic nature and its ability to grow at refrigeration temperatures (Kozak et al. 1996). This bacterium was previously isolated from Minas Frescal cheese, a fresh, soft, white cheese, which presents high pH (4.9-6.7), high moisture content (>55%), and low percentage of salt (1.4-1.6%) (Brito et al. 2008). Minas Frescal is a typical Brazilian fresh cheese and one of the most highly consumed lactic products in Brazil, with wide acceptance in the national market (Souza and Saad 2009).

Direct addition of nisin into cheeses results in an immediate reduction of bacterial populations. Meanwhile, if residues of the antimicrobial are rapidly depleted, the antimicrobial will not prevent the recovery of injured cells or the growth of cells that were not destroyed (Chi-Zhang et al. 2004). In this sense, nisin incorporated in antimicrobial nanocomposites films revealed a great potential to reduce post-process contamination of food pathogens (Meira et al. 2014; Salmieri et al. 2014).

Direct melt-extrusion is a more productive and efficient in industrial workshops than traditional solvent casting method used for biopolymers preparation. Therefore, the objective of this study was to prepare and characterize for the first time starch/halloysite nanocomposites by melt-extrusion incorporating nisin and further analyze their effectiveness against *Listeria monocytogenes* in a Minas Frescal cheese.

Material and methods

Materials

A commercial corn starch, Amisol 3408 (Corn Products Brasil, São Paulo, Brazil), was used. The nanoclay halloysite (HNT) was purchased from Sigma-Aldrich (St. Louis, USA). The non-volatile plasticizer was glycerol (99% purity) (Nuclear, Diadema, Brazil). Commercial nisin (Nisaplin®) was provided by Danisco Brasil Ltda (Cotia, Brazil). According to manufacturer, the formulation contains NaCl, denatured milk solids as fillers and 2.5% pure nisin.

Preparation of the bionanocomposites

Starch/halloysite nanocomposites were produced in two steps based on Schmitt et al. (2012). The control samples consisted of 75 wt% corn starch and 25 wt% glycerol and bionanocomposites materials were compounded with 3 and 6 wt% nanotubes (named as H3 and H6). Formulations containing nisin were added with two concentrations of the

antimicrobial (2 and 6 wt%), resulting in four samples designated as H*x*N*y*, *x* taking the value 3 or 6 accordingly to HNT concentration and *y* corresponding to nisin quantity in bio-nanocomposites.

Components were first dried at 40ºC for 48 h prior to physically blended at room temperature. Then, these mixtures were processed by melt mixing using a twin-screw extruder Haake H-25, model Rheomix PTW 16/25, L/D = 25, matrix with L/D = 3 (Thermo Scientific, Karlsruhe, Germany). The temperature setting from the feed to the die was 115–120ºC. After that, the samples were granulated in a Sagec SG-35 (Sagec Máquinas LTDA, Diadema, Brazil) and another extrusion was performed with a profile temperature of 135- 140ºC in a extruder Chill Roll AX 16:26 (AX Plásticos, Diadema, Brazil).

Scanning electron microscopy (SEM)

The films surfaces were analyzed by using a JEOL microscope, model JSM-6060 (Tokyo, Japan) microscope operated at a voltage of 5kV. To obtain fracture faces, bionanocomposites and the control were cooled in liquid nitrogen, and then broken. Samples were coated with gold layer prior to analysis in order to increase their electrical conductivity.

X-Ray diffraction (XRD)

XRD measurements were performed using a Siemens D-500 diffractometer (Siemens, Karlsruhe, Germany). HNT powder and films were scanned in the reflection mode using an incident Cu K_α radiation ($\lambda = 1.54$ Å), at a step width of $0.05°min^{-1}$ from $2\theta = 5°$ to 40°C.

FTIR

Fourier Transform Infrared (FTIR) spectra were measured using a FTIR Varian 640 IR thermo scientific spectrometer in attenuated total reflectance (ATR) mode with a diamond crystal. The scans were collected between 600 and 4000 cm^{-1} at a 4 cm^{-1} resolution.

Mechanical properties

Tensile tests were carried out using films with 20 mm X 70 mm of size using a TA.XT *Plus* Texture Analyzer (Texture Technologies Corp and by Stable Micro Systems, Hamilton, MA, USA) according to standard ASTM D-638. The samples were acclimatized for 24 h at 23°C ± 2 with humidity 50% ± 5 before analysis.

Thermogravimetric analysis (TGA)

A thermogravimetric analyzer model model QA 50 (TA Instruments, New Castle, DE, USA) was used for the thermal stability evaluation. The samples were heated from 25 to 800°C at the rate 10°C min^{-1} under nitrogen atmosphere (50 mL min^{-1}).

Surface color measurement

Color values of films were measured with a colorimeter (Minolta, CR-400, Osaka, Japan). The measurements were done in the CIELAB scale, in which each measurement is expressed as L* (indicating the lightness), a* (positive in the red direction and negative in the green direction), and b* (positive in the yellow direction and negative in the blue direction). Standard values refer to the white calibration plate (L=94.23, a = -0.55, and b= 9.68). Results were expressed as the means of five measurements on different areas of each film.

Film solubility in water

Film solubility in water was determined as the percentage of sample dry matter solubilized after 24 h immersion in distilled water (Pereda et al., 2011). Film samples were dried at 105°C for 24 h and weighed for initial dry mass (100–150 mg/piece). Films were immersed in 30 ml of distilled water with 0.02% sodium azide to prevent microbial growth. After 24 h in a shaker at 150 rpm and 25ºC, the specimens were recovered, gently rinsing with distilled water and the oven dried at 105 °C for 24 h, to determine the weight of dry matter not dissolved in water. Three measurements were taken for each treatment. Film solubility was calculated from the initial dry mass and the final dry mass using the following equation:

Film solubility (%) = [(W1 – W2)/W1)] x100

where W1 and W2 represent the film mass before and after solubility test, respectively.

In vitro antimicrobial properties

The films' antimicrobial activity was tested using the inhibition zone assay in agar medium. Pieces with 1 cm of diameter were cut from films and placed on skimmed milk agar (SMA) plates (Meira et al. 2014). Then, 10 ml SMA soft agar (7.5 g l^{-1}) inoculated with 10^7 CFU ml 1^{-1} indicator strain (*Listeria monocytogenes* ATCC 7644, *Staphylococcus aureus* ATCC 1901 or *Clostridium perfringens* ATCC 3624) was poured onto plates. Petri dishes were stored at 4°C during 12 h to initiate nisin desorption and after incubated at 37°C for 24

h. The antimicrobial activity is evidenced by clear zones (no micro-organism growth or survival) surrounding film pieces. The diameter of the zones of inhibition was measured and expressed as mm.

Application of bionanocomposites on Minas Frescal cheese

The antimicrobial effect of bionanocomposites was evaluated using commercial Minas Frescal cheese. Slices of the cheese of approximately 10 g were inoculated with a culture of *Listeria monocytogenes* ATCC 7644 to obtain an initial bacterial count on cheese of about 5 log UFC/g. The nanocomposite films were then brought in contact with upper surface of the inoculated cheese samples and stored at 4 ± 2ºC. Non-inoculated samples were also used as control.

After 0, 1, 4, 7 and 14 days, selective viable counts of *L. monocytogenes* were performed. For the analysis, the samples were placed in sterilized plastic bags in which 90 ml of sterile 0.1% (w/v) peptone solution were added, homogenized in a blender for 60 s and dilutions were plated on Oxford Listeria selective agar (Himedia) plates. All experimental treatments were tested in duplicates for three independent preparations, and averages were calculated for treatments at each time point.

Statistical analysis

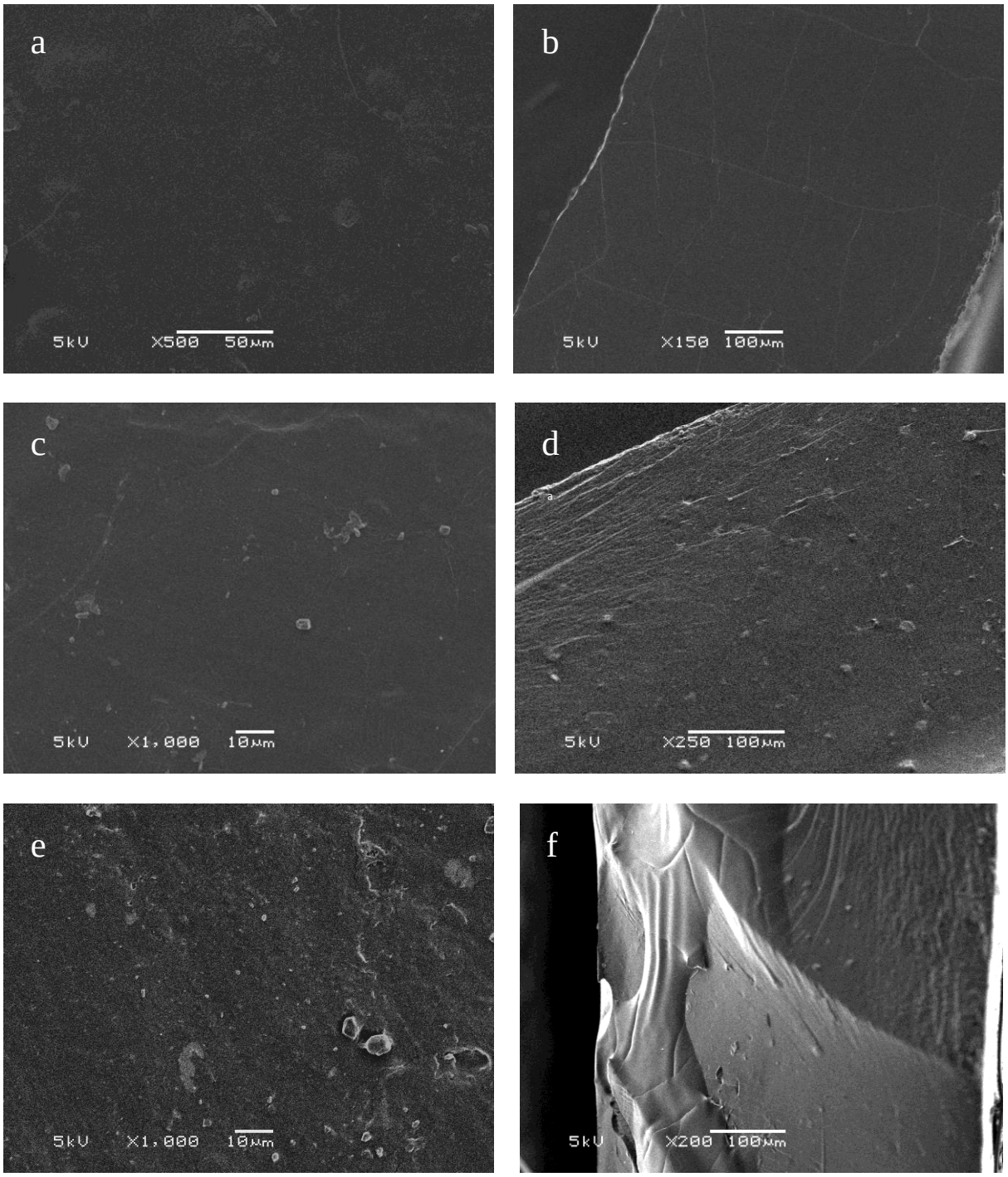
The results were subjected to variance analysis (ANOVA) and means were compared through the Tukey test at a level of 5% of significance, using the SAS software (version 9.3).

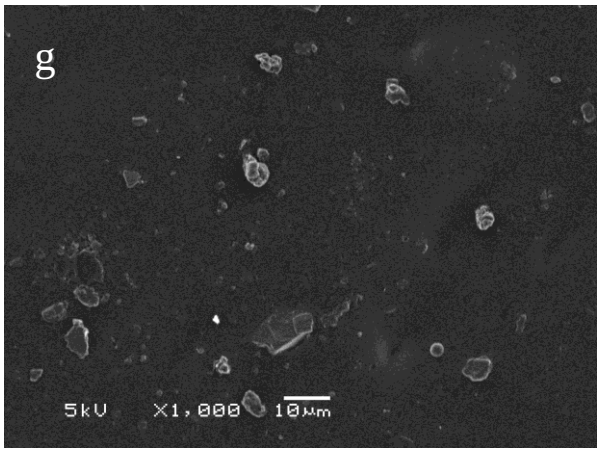
Results and Discussion

Structural Properties

The scanning electron micrographs of control films and bionanocomposites are shown in Fig. 1. The morphology of the control, that presents just starch and glycerol in its composition, is homogenous since granular characteristic of native starch was destructed after plasticization by melt-extrusion process. After addition of HNT in treatments H3 and H6, nanotubes are visible and uniformly dispersed in starch matrix. In bionanocomposites with nisin, agglomerates are evidenced, suggesting that during the preparation of the films some additive migrated to the film surface. A similar behavior was reported by Meira et al. (2014) during the preparation of polypropylene/montmorillonite nanocomposites containing nisin.

Fortunately, in the present study, voids or holes were not observed in nanocomposites with the antimicrobial agent, suggesting that the interface between halloysite and plasticized starch is of good quality without any sign of debonding.

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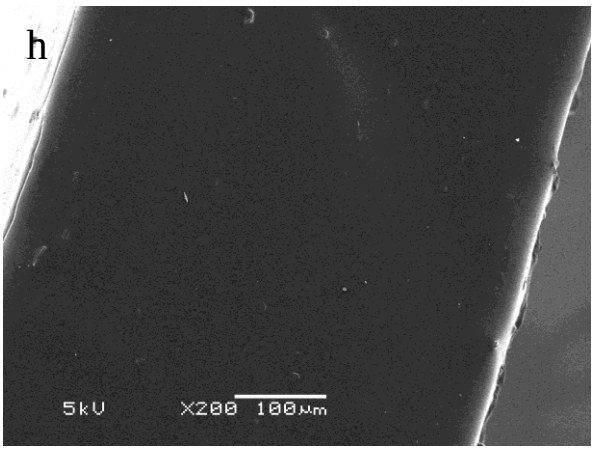
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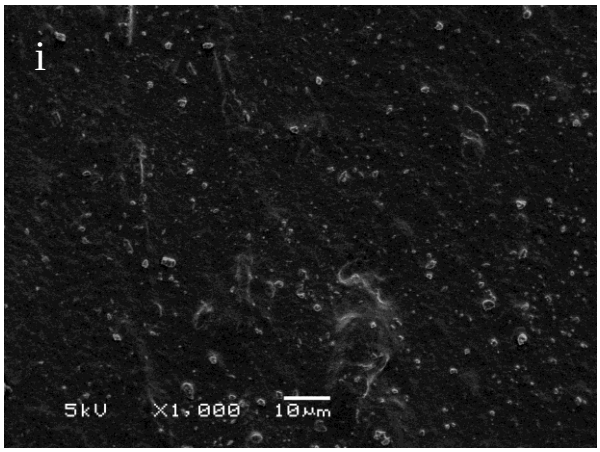
i
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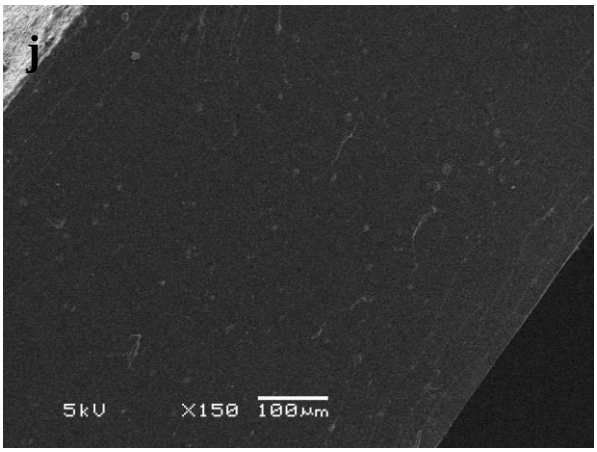
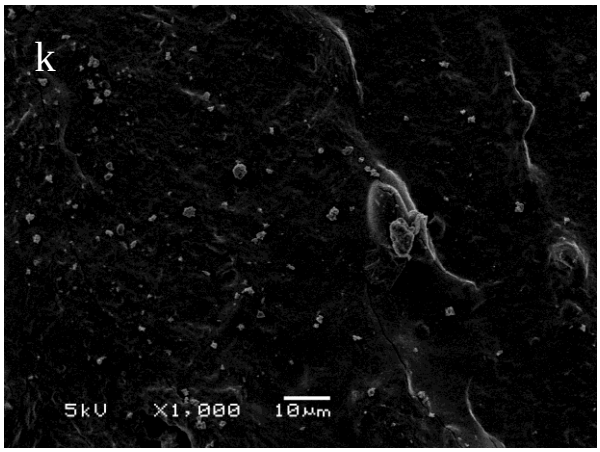
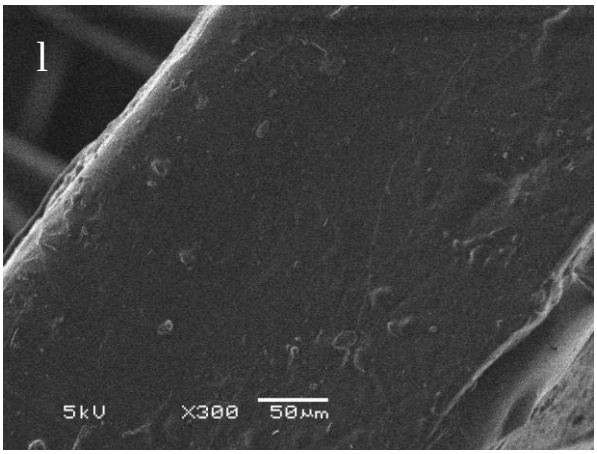

j
5kV X150 100µm


k
5kV X1,000 10µm


l
5kV X300 50µm

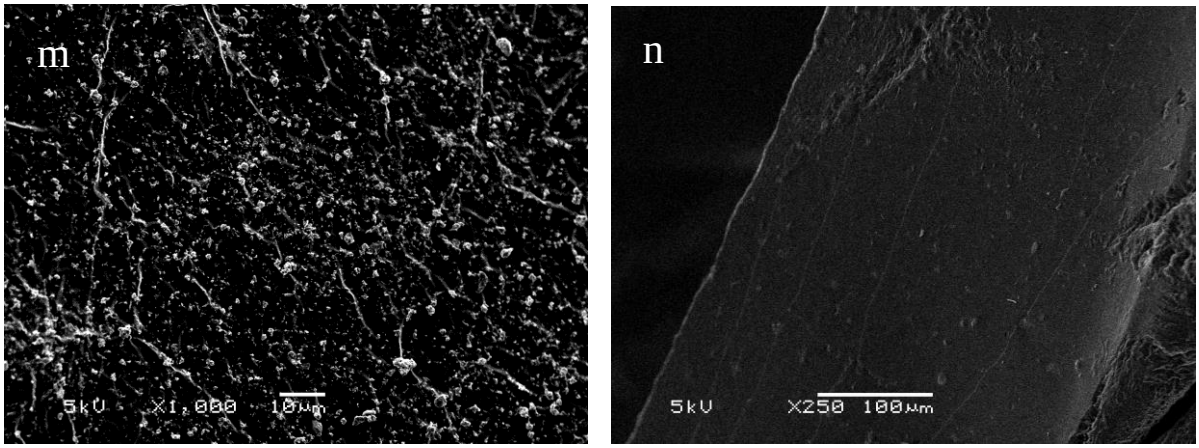


Fig. 1. SEM micrographs of surface (left column) and cross-section (right column) of starch films: (a) and (b) control; (c) and (d) H3; (e) and (f) H3N2; (g) and (h) H3N6; (i) and (j) H6; (k) and (l) H6N2; (m) and (n) H6N6.

Fig. 2 gives X-ray diffraction (XRD) spectra of control film, bionanocomposites and HNT powder.

The control films, that possesses just starch and glycerol, shows peaks at 2θ = 11.82°, 13.32°, 18.14° and 20.89°.

As a crystalline material, HNT powder has a peak at 2θ = 11.7°, corresponding to a basal spacing of 0.75 nm. It confirms the tubular structure at the nanoscale of halloysite (Joussein et al. 2005). Also, two peaks appeared at 18.2 and 20.0°.

Starch/halloysite nanocomposites without nisin, H3 and H6, demonstrated lower degree of crystallinity in characteristic peaks of starch compared to control films. Moreover, in these samples, the peak at 11.7° appeared larger and the peak at 19.8° is more intense which can be attributed to halloysite content.

Films with 2% nisin presented the peak at 18.14° less intense and it appeared almost invisible in films with 6% nisin. Films H3N6 and H6N6 evidence a peak at 31.7°-31.8° that belongs to the characteristic diffraction pattern of sodium chloride, which is a component of commercial nisin used in this work.

Meanwhile, addition of halloysite and nisin had obviously effect on the XRD spectra of starch and influenced its crystallization during the film preparation.

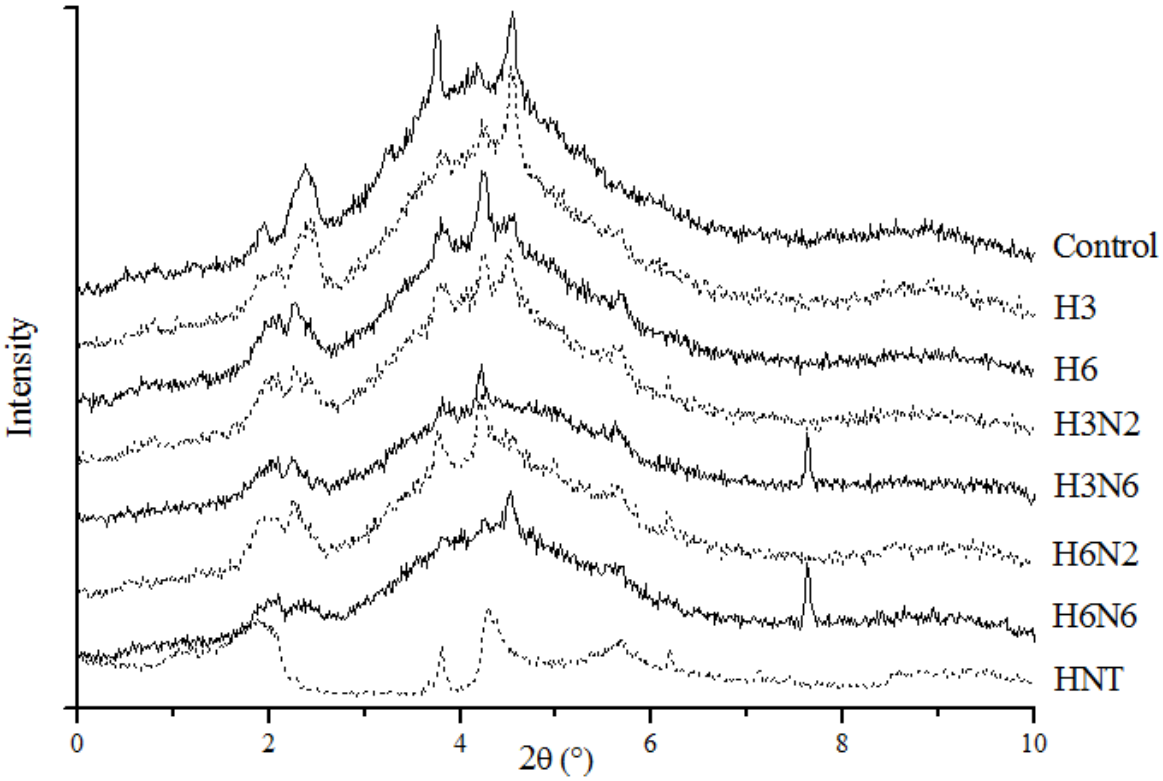


Fig. 2. X-ray diffraction (XRD) patterns of the control, halloysite powder (HNT) and starch/halloysite nanocomposites.

Chemical Interactions

FTIR spectra of control films and starch/halloysite nanocomposites are represented in Fig. 3. The films exhibited peaks with the same wavelength related to the characteristic groups of starch: 3322 cm^{-1} related to hydroxyl groups; 2928 cm^{-1} attributed to –C-H and –C-H_2 bond streching of the anhydroglucose ring; 1651 cm^{-1} relating to water bonding vibration; 1150 cm^{-1} and a shoulder in 1080 cm^{-1} attributed to –C-O bond streching of the –C-O-H bonds; 1020, 928 and 855 cm^{-1} related to –C-O bond streching of the C-O-C anhydroglucose ring (He et al. 2012; Schmitt et al. 2012; Xie et al. 2011).

FTIR spectra of HNT powder (data not shown) has characteristic bands around 3694 cm^{-1} indicating the presence of silanol groups forming hydrogen bonds; 3622 cm^{-1} attributed to the -OH stretching vibration of structural hydroxyl groups; 1640 cm^{-1} related to the -OH deformation of water; 1028 cm^{-1} assigned to Si-O stretching vibration; band around 910 cm^{-1} assigned to isolated Si-O groups and a shoulder around 1100 cm^{-1} attributed to Si-O deformation (Du et al. 2008).

The commercial nisin preparation presented the major bands at 3420 and 1634 cm^{-1} (data not shown). Absorption in this first area indicates stretching of the O-H and N-H bonds and the spectra in the region of 1720-1580 cm^{-1} are attributed to the amide bands (Kong and Yu 2007).

Salmieri et al. (2014) observed additional peaks related to peptides in the whole infrared region after addition of nisin in poly(lactic acid)-cellulose nanocrystals (PLA-CNC) film matrix. However, no peaks related to nisin and halloysite were observed in our samples. Moreover, starch and halloysite presented peaks in the characteristic regions of peptides, which could mask possible nisin interactions with the polymer matrix. A weak shift of the peaks at 3322 cm^{-1} to higher wavenumbers occurred in samples with 6% of halloysite suggesting interactions between clay and starch and/or glycerol.

Nevertheless, the FTIR spectra of the films are very similar, suggesting that the incorporation of halloysite and nisin in the polymer matrix did not alter its functional groups.

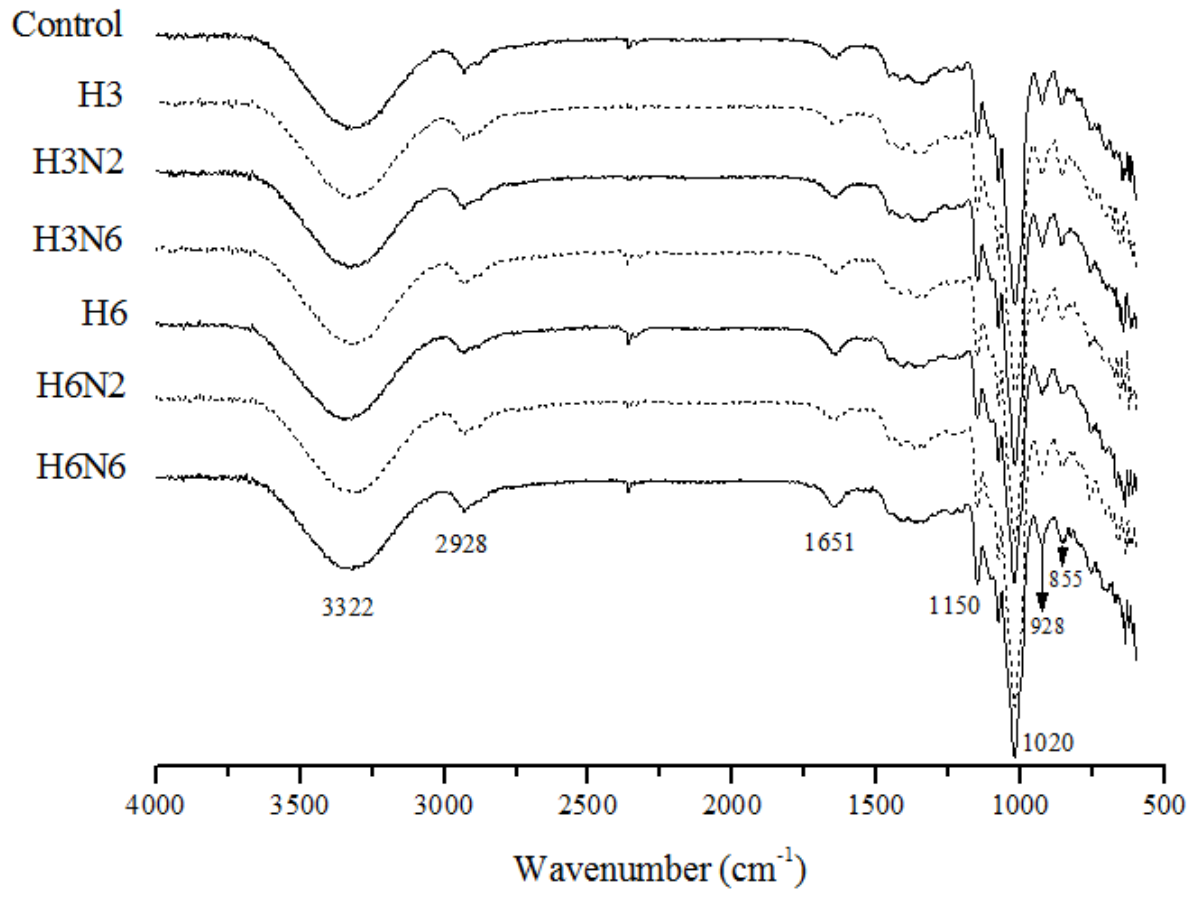


Fig. 3. FTIR spectra of control films and starch/halloysite nanocomposites.

Mechanical Properties

Young modulus (YM) and tensile strength (TS) increased significantly with increase of halloysite content in samples H3 and H6 compared to control (Table 1). Such performance is in agreement with Schmitt et al. (2012) that also prepared starch-halloysite nanocomposites by melt-extrusion. The high aspect ratio and high mechanical properties of halloysites combined to good compatibility between starch and such filler explain the enhancement of mechanical properties upon halloysite addition. Nevertheless, when nisin was added, lower values of these parameters were obtained (Table 1). Previous works demonstrated that a decrease in film strength and resistance was observed with increase in concentration of the nisin incorporated in the polymer (Basch et al. 2013; Bastarrachea et al. 2010; Pranoto et al. 2005). Sebti, Chollet, & Degraeve (2007) studied the effect of nisin addition on tensile strength of hydroxypropyl methylcellulose-based films and observed a strong decrease of this property, which was attributed to the disruption of the film forming matrix by the antimicrobial agent.

At the same time, elongation at break (EB) presented no significant differences between samples ranging 81.75% and 86.27%, except for treatments with the higher concentration of nisin, which values increased with the major HNT content, 119.0% and 166.98% for samples H3N6 and H6N6, respectively (Table 1).

Table 1. Mechanical properties and the thermogravimetric parameter T_{max} of starch films.

Films	YM (MPa)	TS (MPa)	EB (%)	T_{max} (ºC)
Control	26.29 ± 2.88 c	2.15±0.17 c	81.75 ± 6.34 c	316.2±0 a
H3	41.27 ± 1.37 b	3.85±0.23 a	74.49 ± 7.29 c	319.6±2 a
H6	55.33 ± 1.89 a	2.95±0.46 b	81.76 ± 6.52 c	323.5±6 a
H3N2	26.95 ± 4.08 c	1.98±0.11 c	85.11 ± 8.26 c	293.6±4 b
H3N6	24.48 ± 1.82 c	1.35±0.09 d	119.0 ± 11.69 b	298.1±2 b
H6N2	8.92 ± 1.82 d	1.04±0.21 d	86.27 ± 3.56 c	298.1±5 b
H6N6	2.21 ± 0.46 e	0.53±0.07 e	166.98 ± 3.38 a	299.8±1 b

Values are means ± standard deviation. Treatments followed by the same letter within the same column are not significantly different (P>0.05).

YM =Young Modulus; TS = Tensile Strength; EB = Elongation at Break; T_{max} = Maximum rate of mass loss.

Thermogravimetric Analysis (TGA)

The thermogravimetric curves are shown in Fig. 4. It can be observed that the addition of HNT in H3 and H6 samples increased the thermal stability of the films comparing to control. These results are in accordance with He et al. (2012), Schmitt et al. (2012) and Xie et al. 2011) that was related to the better thermal stability of HNT and the interaction between starch and HNT. Since halloysite has no weight loss at low temperature, the residue mass of the films increased as halloysite content raised. In contrast, when nisin is added to starch/halloysite films the weight loss pattern is visible different from other samples (Fig. 4). There was little weight loss below 200ºC, but samples with 6% of nisin showed the higher degradation at this step. This first mass loss may be caused by volatilization of the adsorption water and, for samples with nisin, the degradation of the antimicrobial. When the temperature reached about 290ºC, a massive decline appeared which was mainly caused by the thermal decomposition of starch and the volatilization of glycerol.

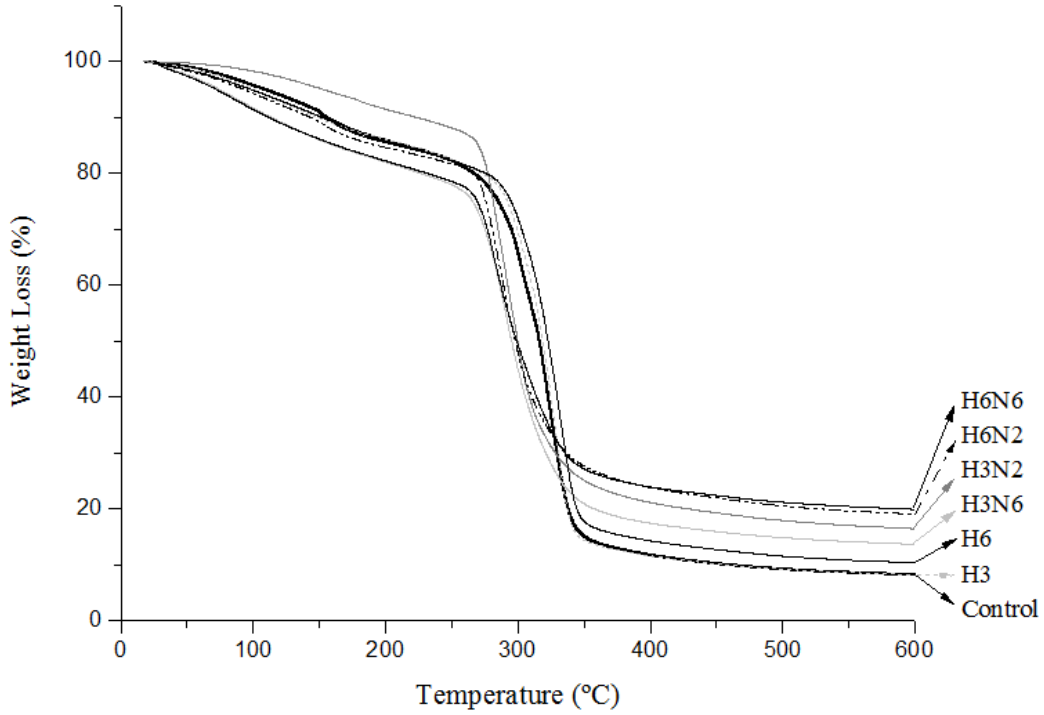


Fig. 4. Thermogravimetric analysis (TGA) of the control and starch/halloysite nanocomposites.

Considering the temperature at maximum rate of mass loss (T_{max}), samples with nisin demonstrated significant difference (P<0.05) comparing to other samples, with T_{max} ranging from 293.6ºC to 299.8ºC (Table 1). Contrary to this, Meira et al. (2014) observed no

significant differences in thermal properties of polypropylene/montmorillonite nanocomposites after nisin addition. Meanwhile, control, H3 and H6 films presented T_{max} of 316.2, 319.6 and 323.5ºC, respectively (Table 1), similar to Xie et al. (2011) that found 311, 315 and 321ºC, respectively, for control (without HNT), 3 and 9 wt% HNT starch films.

Color

Table 2 corresponds to color parameters for control films and bionanocomposites. For the parameter L* was observed that, in general, it decreased in the presence of 6% of HNT containing or not nisin. The a* color parameter took negative values in all cases, except for film H6N6, that showed a positive value. Significantly higher values of the b* were obtained in films containing 6% HNT and in treatment H3N6.

These results are in agreement with Flores et al. (2010) *apud* Basch et al. (2013) who reported that the addition of nisin to films made from tapioca starch, decreased the value of L* from 86 to 79 and increased the value of b* from 4 to 12 units, compared with systems without preservative.

Table 2. Color measurements and solubility of the control film and starch/halloysite nanocomposites.

	L*	a*	b*	Solubility (%)
Control	81.96 ± 0.86 a	-0.22 ± 0.05 b	5.20 ± 0.98 b	25.17 ± 2.83 a
H3	82.39 ± 0.83 a	-0.3 ± 0.02 c	5.50 ± 0.93 b	24.88 ± 1.61 a
H3N2	81.18 ± 0.89 a	-0.25 ± 0.08 bc	6.78 ± 0.99 b	28.61 ± 1.21 a
H3N6	82.28 ± 0.81 a	-0.59 ± 0.06 d	11.58 ± 1.12 a	28.17 ± 2.25 a
H6	75.36 ± 1.68 c	-0.28 ± 0.03 bc	12.40 ± 1.52 a	22.15 ± 1.01 a
H6N2	75.67 ± 1.47 c	-0.26 ± 0.04 bc	12.29± 1.23 a	21.50 ± 1.25 a
H6N6	78.56 ± 1.16 b	0.36 ± 0.03 a	12.68± 1.28 a	28.28 ± 4.39 a

Values are means ± standard deviation. Treatments followed by the same letter within the same column are not significantly different (P>0.05).

The color development in the films can be also attributed to the own color of the HNT and the antimicrobial incorporated, since films with more amount of these components produced slightly darker films. Moreover, browning reactions could be occurred during preparation of the films (Basch et al. 2013).

Solubility

Even though Table 2 shows a very slight decreasing trend of solubility with increasing HNT content in samples H3 and H6, Tukey's multiple comparison indicated no significant difference between these samples and control (P>0.05). Zhang, Liu, Hrymak, & Han (2013) described a similar trend on extruded thermoplastic starch reinforced by montmorillonite nanoclay.

Bionanocomposites with nisin presented higher values of solubility, except for treatment H6N2. However, no significant differences occurred between samples (Table 2). Basch et al. (2013) verified that the presence of nisin in tapioca starch-HPMC edible films formulation significantly increased solubility and attributed this trend to the polysaccharide matrix disruption caused by the antimicrobial. Otherwise, the introduction of nanorod-rich zinc oxide (ZnO) to sago starch matrix significantly decreased the solubility of the biocomposites, possibly by the formation of more hydrogen bonds the ZnO and the matrix components. Thus, free water molecules did not interact as strongly with antimicrobial films compared with control without ZnO (Nafchi et al. 2012).

In vitro Antimicrobial Activity

Starch-halloysite films added with nisin showed inhibition zones against growth of *L. monocytogenes, C. perfringens* and *S. aureus* as shown in Table 3. Inhibition zones varied from 8.0 to 10.0 mm of diameter for *L. monocytogenes*; from 15.0 to 17.5 mm for *Clostridium perfringens*; and from 1.0 to 3.0 mm for *S. aureus*. Control, H3 and H6 samples did not exhibited antimicrobial activity, as expected. The most sensitive strain was *C. perfringens*, and the inhibition depicted large zones of inhibition, illustrated in Fig 5.

Films made with tapioca starch containing nisin were effective against *Listeria innocua*, showing inhibition zones with 2.0 mm of diameter (Basch et al., 2013.). Sanjurjo, Flores, & Jagus (2006) studied the effect of 3000UI/ml of Nis contained in starch film and observed a low density of growth in the area of contact and a narrow clear zone of inhibition around the film. Also, Imran, El-fahmy, & Desobry (2010) studied coatings based on HPMC, glycerol and nisin. They found that these films were effective against different strains of foodborne bacteria, including *L. monocytogenes* and *S. aureus.*

Table 3. Inhibition zones of starch/halloysite nanocomposites containing nisin against food borne pathogens.

Samples	Inhibition zones diameter (mm)		
	Listeria monocytogenes ATCC 7644	*Clostridium perfringens* ATCC 3624	*Staphylococcus aureus* ATCC 1901
H3N2	8.5 ± 0.7 ab	16.0± 0 ab	1.25 ± 0.35 b
H6N2	8.0 ± 0 b	15.0 ± 1.4 b	1.0 ± 0 b
H3N6	10.0 ± 1.4 a	17.5 ± 0.7 a	3.0 ± 0 a
H6N6	10.0 ± 1.4 a	16.5 ± 0.7 a	2.75 ± 0.35 a

Values are means ± standard deviation. Treatments followed by the same letter within the same column are not significantly different (P>0.05).

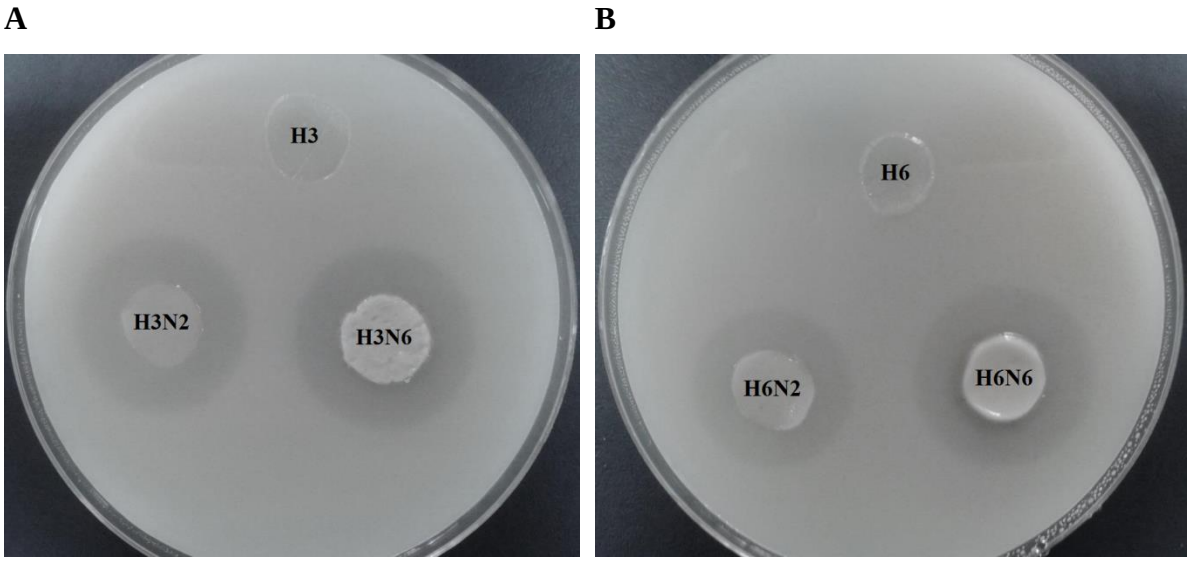


Fig. 5. Antimicrobial activity of the nanocomposites incorporated with nisin against *Clostridium perfringens* in skimmed milk agar plates. (A) Starch films containing 3% of halloysite and 2 and 6% nisin, namely as H3, H3N2 and H3N6. (B) Composite films containing 6% of halloysite and 2 and 6% nisin, namely as H6, H6N2 and H6N6. A sample without nisin addition was used as control.

The average clear zones of films with the same quantity of nisin did not differ significantly. However, samples with higher amount of nanotubes, H6N2 and H6N6, presented smaller zones of inhibition against all strains comparing to H3N2 and H3N6, respectively (Table 3). This observation is in accordance with some studies that have reported effects of nanoclays in controlling diffusion or enhancing retention of antimicrobial agents by polymer matrices (Sanchez-Garcia et al. 2008). Mascheroni, Chalier, Gontard, & Gastaldi (2010) showed that montmorillonite contents higher than 5% in a gluten matrix produced aggregated structures which entrapped carvacrol molecules, resulting in a higher carvacrol retention by both gluten film forming solutions and cast films.

Application of active bionanocomposites on Minas Frescal Cheese

Listeria monocytogenes is a widely distributed psychrotrophic pathogen that may survive and proliferate in cheeses and it is able to survive for long periods in the environment, on foods, and in processing plants (Salmieri et al. 2014). Thereafter, starch-halloysite nanocomposites containing nisin were applied in contact with one side surface of Minas Frescal cheese slices previously inoculated with this bacterium. *L. monocytogenes* counts were determined after 0, 1, 4, 7 and 14 days of storage at 4ºC as showed in Fig. 6.

As expected, control films did not show any inhibition against *Listeria* demonstrating an almost constant level of contamination (nearly 5 log, Fig. 6). Films H3 and H6 presented the same behavior of the control films (data not shown).

No significant difference occurred throughout the experiment between treatments H3N2 and H6N2, as well as between samples H3N6 and H6N6 (Fig. 6). Despite this, the values of viable cell counts of the pathogen were higher for samples with 6% of halloysite, reinforcing the behavior observed in antimicrobial activity assay in which the higher content of nanoclay was able to retain the diffusion of nisin.

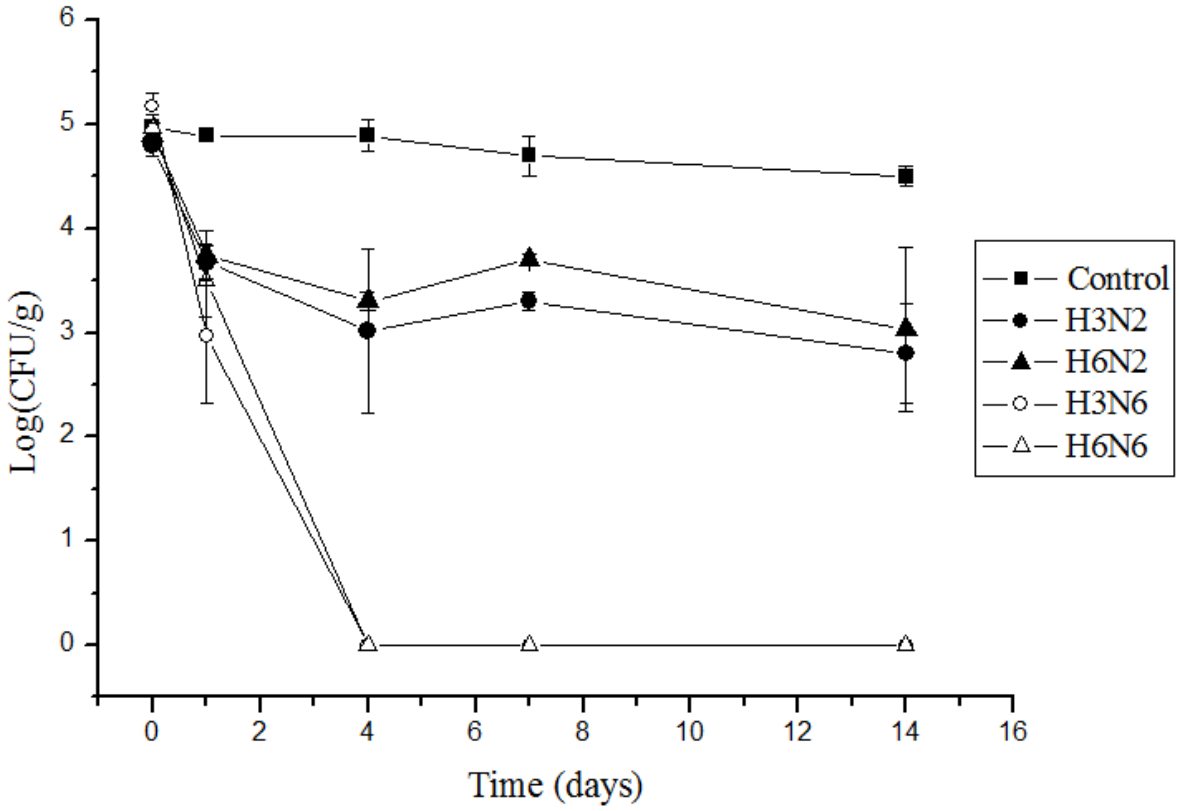


Fig. 6. Effects of antimicrobial packaging films against *L. monocytogenes* inoculated on slices of Minas Frescal cheese. Values are the means of three independent determinations ± S.E.M.

Bionanocomposites containing 2% nisin (H3N2 and H6N2) significantly inhibit *L. monocytogenes* cells after 4 days comparing to control (P<0.05), decreasing 1-2 log cycles until the last day of the storage period (Fig. 6). In a comparable way, sodium caseinate films containing nisin (1000 IU/cm^2) reduced *Listeria inoccua* in 1.1 log CFU/g after one week of storage at 4 ºC when applied on semi-soft cheese (Cao-Hoang et al. 2010).

On the other hand, cheeses packed in antimicrobial nanocomposites with 6% nisin presented a significant decrease in the population of *L. monocytogenes* immediately after 1 day comparing to cheeses packed in control films (P<0.05). After 4 days, samples H3N6 and H6N6 completely inhibited the bacterium below the detection limit of the method (Fig. 6). These results are in accordance with Salmieri et al. (2014) who inoculated sliced cooked ham with *L. monocytogenes* (3 log CFU/g) using nanocomposite films made of poly(lactic acid)-cellulose nanocrystals and nisin (aqueous solution of 1%) as packaging materials. These films showed a significant reduction of *L. monocytogenes* in ham from day 1 and a total inhibition from day 3. Otherwise, Ollé, Gerschenson, & Jagus (2014) evaluated the growth of *Listeria innocua* (initial counts of 4 log) at 25ºC on the surface of Port Salut cheese packed with

tapioca starch films containing nisin (2.31 mg nisin/dm^2 of film). After 24 h of storage, the bacterium counts were lower than the detection limit. But *L. innocua* reassumed its growth and, at the end of the storage (196 h), cheese packed with active films presented 7.66 log counts lower than the control cheese packed with antimicrobial free film.

Conclusions

Nisin incorporated in bionanocomposites prepared with starch and halloysite modified the structural, mechanical and thermal properties, as well as the appearance (darker color). At the same time, HNT addition demonstrated great importance to improve the functional properties of starch films, supporting the concept of bionanocomposite technology as a valuable tool for to expand the use of bio-polymers for food packaging purposes.

C. perfringens, *S. aureus* and *L. monocytogenes*, important food borne pathogens, were effectively inhibited by starch/halloysite nanocomposites containing nisin when antimicrobial activity assay was conducted. In respect to real food systems, cheeses are prone to spoiling on the surface and this study resulted in active films that can protect food, like Minas Frescal cheese, against post-process contamination with *L. monocytogenes* by contact packaging imbued with nisin. Indeed, to achieve a better combination of properties, further research is necessary to adequate adjusting the composition of these films.

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4. DISCUSSÃO GERAL

Os antimicrobianos naturais, nisina e pediocina, foram incorporados a embalagens de polímeros sintético e biodegradável para uso em alimentos. A abordagem inovadora consistiu em adicionar nanopartículas para melhoria do desempenho dos materiais e liberação controlada dos peptídeos antimicrobianos. As nanoargilas montmorilonita e haloisita são consideradas aditivos seguros para aplicação em sistemas alimentares e até o presente estudo não haviam sido empregadas em nanocompósitos poliméricos contendo bacteriocinas.

Materiais de embalagem à base de polipropileno apresentam utilidades domésticas e ampla aplicação em indústrias alimentícias, são de baixo custo e recicláveis. Neste trabalho, o polipropileno revelou-se adequado como matriz polimérica para o preparo de filmes com argila montmorilonita e incorporação direta de nisina. As etapas de extrusão e obtenção de grânulos de polímero e argila antecederam à moldagem por compressão para adição do peptídeo antimicrobiano. Apesar da alta temperatura empregada, os filmes resultantes demonstraram atividade frente a patógenos alimentares (*Listeria monocytogenes, Staphylococcus aureus* e *Clostridium perfringens*) em ágar leite, simulante de alimentos sólidos, bem como em soluções simulantes de alimentos ácidos. Este método de preparo mostrou-se vantajoso para ser reproduzido em larga escala e não requer uso de solventes, o que implica positivamente aspectos econômicos e ambientais. Ocorreram alterações no comportamento dos filmes quanto a propriedades morfológicas, mecânicas e térmicas, sendo mais intensas à medida que maiores quantidades de nisina foram adicionadas. As propriedades de barreira ao vapor d'água não foram afetadas, enquanto que à permeabilidade ao oxigênio aumentou após a adição do peptídeo. A migração da nisina a partir dos filmes foi completa após 48 h, resultado promissor para maiores estudos sobre sua liberação controlada em alimentos. Uma sugestão de aplicação destes nanocompósitos seria no produto lácteo requeijão, o qual é comercialmente embalado em potes plásticos de PP e geralmente adicionado de nisina como conservante.

Considerando aspectos ambientais, o amido de milho foi proposto como polímero biodegradável, obtido a partir de fonte renovável e de baixo custo para o desenvolvimento de bionanocompósitos antimicrobianos. Os dois métodos testados para o preparo dos materiais, *casting* e extrusão, foram satisfatórios para a obtenção de filmes os quais foram ativos frente à *L. monocytogenes* e *C. perfringens* quando adicionados de bacteriocinas e avaliados *in vitro*. Entretanto, os agentes antimicrobianos modificaram morfologia, cristalinidade, coloração e

resistências mecânica e térmica dos filmes biodegradáveis. A presença de haloisita foi importante para a melhoria e/ou manutenção das propriedades mecânicas e térmicas dos materiais.

O procedimento de adsorção das bacteriocinas em três argilas (bentonita, montmorilonita modificada com octadecilamina e haloisita) foi proposto como sistema de liberação de nisina e pediocina em alimentos. Estes peptídeos tiveram alta adsorção em bentonita, porém maior atividade antimicrobiana foi liberada após a adsorção nos nanotubos de haloisita. Diante destes resultados, os peptídeos adsorvidos em haloisita foram incorporados aos filmes de amido preparados pelo método de *casting*, considerando a conveniência dos componentes estarem em meio aquoso e a demanda por menor quantidade de material em relação ao método por extrusão. Os nanocompósitos resultantes mostraram-se mais interessantes quando comparados àqueles em que as bacteriocinas não tinham sido previamente adsorvidas na argila. O desempenho dos bionanocompósitos quanto às propriedades térmicas e mecânicas, bem como a cor dos filmes, obtiveram melhorias significativas devido à técnica de adsorção.

Como o principal propósito de embalagens antimicrobianas é evitar ou minimizar a contaminação pós-processamento, a aplicação dos nanocompósitos de nisina preparados por extrusão em queijo Minas Frescal demonstrou a viabilidade de sua utilização. Não apenas o controle do crescimento de *L. monocytogenes* foi alcançado, mas também houve redução significativa da população microbiana inicialmente inoculada nos queijos (aproximadamente 10^5 UFC/g) logo após 4 dias de estocagem a 4ºC. Este patógeno pode ser encontrado em queijos de alta e média umidade e a nisina é permitida no Brasil para uso nestes alimentos. Outra aplicação viável destes materiais seria na embalagem de queijos processados, fatiados e embalados individualmente, já que este tipo de produto requer tanto o emprego de uma embalagem primária, quanto de uma secundária, gerando após o seu consumo grande quantidade de resíduo plástico não biodegradável. No entanto, o potencial de aplicação é dependente das características do alimento a ser embalado e das condições de estocagem, sendo necessário testar caso a caso.

Bacteriocinas e nanoargilas como componentes de embalagens ativas constituem novos materiais promotores de alimentos seguros e de alta qualidade. Portanto, os nanocompósitos antimicrobianos desenvolvidos a partir de polipropileno e amido de milho oferecem inovação ao mesmo tempo em que revelaram potencial de aplicação em alimentos

prontos para consumo, com perspectivas de novos estudos para adequações de formulação e métodos de preparo a fim de aprimorar suas propriedades.

5. CONCLUSÕES

De acordo com os resultados obtidos neste estudo é possível concluir que:

- Os nanocompósitos de polipropileno/montmorilonita adicionados com nisina foram ativos, capazes de liberar nisina em simulantes de alimentos e as suas propriedades como embalagens foram satisfatórias.
- Nisina e pediocina foram capazes de adsorver nas nanoargilas bentonita, montmorilonita modificada com octadecilamina e haloisita. Em bentonita, houve a maior capacidade de adsorção. Porém, a maior liberação de atividade antimicrobiana ocorreu após a adsorção em haloisita.
- A técnica de adsorção das bacteriocinas nisina e pediocina em haloisita previamente à sua incorporação em solução filmogênica de amido mostrou-se promissora por manter determinada cristalinidade e morfologia homogênea comparáveis aos filmes de amido sem aditivos, o que influenciou positivamente as propriedades térmicas e mecânicas.
- O processo de extrusão foi efetivo para obtenção de bionanocompósitos de amido/haloisita contendo nisina com atividade antimicrobiana *in vitro* e capazes de controlar *L. monocytogenes* quando aplicados em queijo Minas Frescal. Entretando, as demais propriedades avaliadas foram afetadas pela adição do antimicrobiano.
- A incorporação de bacteriocinas em nanocompósitos de polipropileno e amido representa uma tecnologia promissora como uma barreira adicional ao controle de *L. monocytogenes* e possivelmente outros patógenos em alimentos.

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