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Tese

Anticorpos Monoclonais contra *Listeria* spp.: Produção, Caracterização e Aplicação em Métodos Diagnósticos

Marcelo Mendonça

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RESUMO

MENDONÇA, Marcelo. **Anticorpos Monoclonais contra *Listeria* spp.: Produção, Caracterização e Aplicação em Métodos Diagnósticos.** 2011. 109f. Tese (Doutorado) - Programa de Pós-Graduação em Biotecnologia. Universidade Federal de Pelotas, Pelotas.

Os métodos convencionais empregados para detecção de *Listeria monocytogenes* em alimentos são laboriosos e onerosos, requerendo vários dias para sua identificação final. A utilização de anticorpos monoclonais (MAbs) em imunoensaios para detecção rápida de bactérias tem como vantagem a alta especificidade e rapidez, principalmente quando direcionados para fatores de virulência conservados. Dentre os diversos fatores de virulência de *Listeria*, a proteína de membrana internalina A (InlA), é umas das mais bem caracterizadas, sendo um excelente alvo por ser altamente exposta na superfície e exclusiva de espécies patogênicas. Neste trabalho é relatado a produção, caracterização e utilização em métodos de diagnósticos de um painel de MAbs contra a InlA (2D12, 3B7, 4E4), e de um MAb (3F8) que reconhece especificamente todas as bactérias do gênero *Listeria*. Na produção dos MAbs, camundongos BALB/c foram imunizados com uma proteína recombinante InlA (rInlA) concomitantemente com *L. monocytogenes* inativadas por fervura. Os MAbs gerados demonstraram excelente reatividade por ELISA indireto, *Western blot* e imunofluorescência. O MAb anti-InlA 2D12 marcado com Cy5 foi usado como anticorpo de detecção de *L. monocytogenes*, no sistema tipo sanduíche de sensor de fibra óptica. Usando MAb-2D12 como anticorpo de captura nas fibras ópticas, obteve-se um limite de detecção de ~3 x 10^2 CFU.mL^{-1}, e um limite de detecção de ~1 x 10^5 CFU.mL^{-1} foi visualizado com MAb-3F8 como captura. Os MAbs anti-InlA 2D12 e anti-*Listeria* 3F8 foram posteriormente utilizados para sensibilizar esferas paramagnéticas e testados na separação imunomagnética (IMS) de *L. monocytogenes* em culturas puras, e em queijo e salsichas tipo hotdog artificialmente contaminados. Após a captura por IMS, as bactérias foram liberadas, incubadas com a fibra óptica ou plaqueadas em agares para contagem. Em paralelo, a confirmação da captura de *L. monocytogenes* foi realizada por PCR quantitativo em tempo real e por *light-scattering technology* (BARDOT). Utilizando IMS para separar e concentrar *L. monocytogenes*, seguido da utilização em plataforma de fibra óptica, foi possível realizar a detecção em menos de 22 horas, de aproximadamente 40 UFC/g de *L. monocytogenes* em presença de *L. innocua*, em

queijo e salsicha artificialmente contaminados. Além disso, a proteína alvo do MAb-3F8 foi identificado como frutose 1,6-bifosfato aldolase através de espectrometria de massa (MALDI-TOF-MS). Os resultados obtidos nesse trabalho indicam que a utilização em conjunto dos sistemas de IMS e fibra óptica com os MAb-2D12 e MAb-3F8, foram confiáveis e rápidos, e assim, podendo ser empregados em imunoensaios de rotina para detecção de *L. monocytogenes* em alimentos. Contudo, ambos MAbs possuem ainda grande potencial para serem mais explorados em outras plataformas de biossensores, assim como, em outros imunoensaios de detecção e funcionalidade de InlA e FBA em *Listeria*.

Palavras-chave: *L. monocytogenes*. internalina A. anticorpos monoclonais. imunoseparação magnética. sensor de fibra ótica.

ABSTRACT

MENDONÇA, Marcelo. **Monoclonal Antibodies against *Listeria* spp.: Production, Characterization and Application in Diagnostic Methods.** 2011. 109f. Tese (Doutorado) - Programa de Pós-Graduação em Biotecnologia. Universidade Federal de Pelotas, Pelotas.

The conventional methods used to detect the *Listeria monocytogenes* in foods are laborious and expensive, requiring several days for final identification. Monoclonal antibody (MAb) based immunoassays are highly specific and rapid to perform, especially when MAbs are raised to conserved virulence factors in the pathogen. Among diverse virulence factors of *L. monocytogenes*, the surface protein internalin A (InlA) is one of the most well-known and characterized protein, being an excellent target as it is highly exposed on the surface and exclusive of pathogenic species. In this work we report the production, characterization and use of a panel of MAbs against InlA (2D12, 3B7, 4E4), and a MAb (3F8) which specifically recognizes all bacteria belonging the genus *Listeria*. MAbs were produced by the immunization of BALB/c mice with a recombinant InlA together with heat killed *L. monocytogenes*. The MAbs produced showed excellent reativities by indirect ELISA, Western blot and immunofluorescence. A Cy5 conjugated anti-InlA MAb-2D12 was used as detection antibody for *L. monocytogenes* in a sandwich-like fiber optic immunoassay. Using MAb-2D12 as capture antibody on the waveguides, the limit of detection was ~3 x 10^2 CFU.mL^{-1}, and when MAb-3F8 was used for capture the limit of detection was ~1 x 10^5 CFU.mL^{-1}. Furthermore, MAbs 2D12 and 3F8 were used to coat paramagnetic beads and tested in the immunomagnetic separation (IMS) of *L. monocytogenes* from pure cultures, and artificially contaminated cheeses and hotdogs. After IMS capture, bacteria were released from the beads, used in the fiber optic assay or plated on agar for counting. In parallel, the capture of *L. monocytogenes* was confirmed by real-time qPCR and light-scattering technology (BARDOT). Using IMS to concentrate and separate *L. monocytogenes*, followed by a fiber optic platform, it was possible to detect in less than 22 h, approximately 40 CFU/g of *L. monocytogenesi,* even in the presence of *L. innocua* in cheese and hot dogs artificially contaminated. In addition, using mass spectrometry (MALDI-TOF-MS) the protein to which MAb-3F8 binds, was identified as fructose 1,6-bisphosphate aldolase (FBA). The results presented in this work indicate that using both systems together, the IMS and fiber optic immunosensor, were more reliable and faster, and could be applied in the routinely

for detection of *L. monocytogenes* in food. Moreover, both MAbs have the potential to useful in others biosensor platforms, as well as in other detection and functionality immunoassays for InlA and FBA in *Listeria*.

Keywords: *L. monocytogenes*. internalin A. monoclonal antibody. immunomagnetic separation. fiber optic sensor.

SUMÁRIO

1. INTRODUÇÃO GERAL

Listeria monocytogenes é um patógeno alimentar oportunista e invasivo que representa uma grande preocupação em saúde pública em todo o mundo. Este microrganismo foi descrito pela primeira vez por Murray et al. em 1926, através da constatação de um caso de septicemia em coelhos com intensa monocitose periférica (COSSART, 2007). Inicialmente, devido à monocitose apresentada por esses animais, esta bactéria foi nomeada como *Bacterium monocytogenes*. No ano seguinte, Pirie (1927) sugeriu *Listerella* como nome para o gênero, em homenagem ao lorde Joseph Lister, um reconhecido infectologista na época. Finalmente, em 1940 o nome foi mudado para *Listeria* (Pirie, 1940 apud STAVRU; ARCHAMBAUD; COSSART, 2011).

1.1 Características do gênero *Listeria* e *L. monocytogenes*

Atualmente, o gênero *Listeria* é constituído por oito espécies, mas apenas *L. monocytogenes e L. ivanovii* são consideradas potencialmente patogênicas para humanos e animais (GUILLET et al., 2010; LECUIT, 2007). Estas duas espécies patogênicas estão filogeneticamente ligadas com mais seis espécies, *L. seeligeri*, *L. welshimeri*, *L. innocua*, *L. grayi* e duas espécies recém documentadas, *L. marthii* e *L. rocourtiae* (GRAVES et al., 2010; LECLERCQ et al., 2010).

L. monocytogenes é o agente causador da listeriose, uma infecção grave geralmente adquirida pelo consumo de alimentos contaminados (HAAS et al., 2007). Este patógeno de origem alimentar, frequentemente se adapta e supera diversas condições hostis para se manter presente no ambiente e em alimentos, e assim provocar a infecção em humanos e animais (GANDHI; CHIKINDAS, 2007). Embora *L. ivanovii* seja mais reconhecida por causar infecção em animais, casos recentes de listeriose, demonstram que esse microrganismo pode atuar como patógeno oportunista para os seres humanos (GUILLET et al., 2010).

A sorotipificação pode diferenciar *L. monocytogenes* de acordo com sua diversidade de antígenos somáticos (O) e flagelares (H). Até o momento, são conhecidos 13 diferentes sorotipos de *L. monocytogenes* (1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4ab, 4b, 4c, 4d, 4e, 7) (TAMBURRO et al., 2010). No entanto, os sorotipos 1/2a, 1/2b e 4b estão relacionados com mais de 90% dos casos e surtos de listeriose (TORRES et al., 2005). Dentre esses três sorotipos, o sorotipo 4b é

predominantemente associado aos surtos e casos esporádicos dessa doença, demonstrando maiores taxas de mortalidade do que os outros sorotipos (VAZQUEZ-BOLAND et al., 2001).

Listeria spp. são bactérias Gram-positivas, e apresentam-se morfologicamente em forma de bastonetes curtos, com diâmetro de 0,4 - 0,5 µm e comprimento de aproximadamente 1 - 2 µm (VAZQUEZ-BOLAND et al., 2001). *Listeria* pode ocorrer como células únicas ou duplas, e ocasionalmente, podem demonstrar cadeias longas em forma de V ou Y, dependendo das condições e temperatura de desenvolvimento (BHUNIA, 2008a). Bactérias do gênero *Listeria* são desprovidas de cápsula e não possuem a capacidade de formar esporos. Contudo, esse gênero bacteriano é extremamente resistente aos efeitos de congelamento e descongelamento, assim como dessecação e aquecimento (CHEN; GUAN; HOOVER, 2006; SIMPSON et al., 2010). *L. monocytogenes* é capaz de multiplicar-se em uma ampla faixa de temperatura, incluindo temperaturas de refrigeração (1^{o}C até 45^{o}C), mas possui temperatura ótima de desenvolvimento entre 30 a 37^{o}C (WING; GREGORY, 2002). São bactérias móveis por flagelos peritríquios, apresentando de 5 a 6 flagelos, os quais são altamente expressos em temperaturas entre 20 e 25ºC. No entanto, a expressão desta proteína diminui ou cessa em temperaturas acima de 30ºC (FARBER; PETERKIN, 1991; O'NEIL; MARQUIS, 2006). *L. monocytogenes* é uma bactéria anaeróbia facultativa e intracelular facultativa, capaz de invadir e replicar-se em fagócitos e células epiteliais (DUSSURGET; PIZARRO-CERDA; COSSART, 2004). Além disso, possui habilidade de adaptação e sobrevivência a situações ambientais adversas, tolerando altas concentrações de sal (10%) e amplas variações de pH (4,1 a 9,6), o que a torna ainda mais resistente no ambiente, dificultando desta forma seu controle (HAIN et al., 2007). Contudo, a característica de *L. monocytogenes* de poder formar biofilmes e sobreviver sob baixa atividade de água (até 0,93), tem sido atribuída como uma das causas de contaminação de alimentos pós-processados por *L. monocytogenes* (CHEN; GUAN; HOOVER, 2006). Além disso, sua capacidade de suportar baixas tensões de oxigênio e temperatura de refrigeração a tornou um grande problema em produtos embalados a vácuo (SIMPSON et al., 2010).

1.2 Fontes de contaminação e surtos por *L. monocytogenes*

L. monocytogenes é um microrganismo ubíquo na natureza, que pode sobreviver e desenvolver-se em diversas condições ambientais e ser encontrado em várias fontes, incluindo solo, água, vegetação, silagem, e o trato intestinal de animais domésticos, principalmente ruminantes (ovinos, bovinos, caprinos) (NIGHTINGALE; WINDHAM; WIEDMANN, 2005; VAZQUEZ-BOLAND et al., 2001). Esta bactéria também se encontra amplamente distribuída no ambiente doméstico (ROCOURT et al., 2003), no ambiente de plantas de processamento de alimentos (AGUADO; VITAS; GARCIA-JALON, 2004; NALÉRIO et al., 2009; VON LAER et al., 2009) e, consequentemente, em diversos alimentos (SILVA et al., 2004; HOFER et al., 2006). Sua ampla disseminação, aliada à tolerância a altas concentrações de sal, acidez, atmosfera modificada, bem como sua capacidade de multiplicação sob temperaturas de refrigeração, torna difícil a obtenção de alimentos totalmente livres desse patógeno.

Os alimentos são as principais e primeiras fontes de infecção de *L. monocytogenes* para humanos. *L. monocytogenes* pode contaminar uma grande variedade de alimentos crus ou processados, incluindo produtos cárneos, leite e seus derivados, peixes e frutos do mar, além de alimentos minimamente processados como legumes e frutas (MCLAUCHLIN et al., 1996; RAMASWAMY et al., 2007). Devido à natureza psicrotrófica e a capacidade de formação de biofilmes de *Listeria* spp., geralmente é constatada a sua persistência em plantas de processamento de alimentos por longos períodos (BORUCKI et al., 2003; CARPENTIER; CERF, 2011). Isolados de *L. monocytogenes* podem persistir em uma planta de processamento mesmo após desinfecção, tornando-se parte da microbiota do ambiente por meses ou até mesmo anos (HEIR et al., 2004; GANDHI; CHINKINDAS, 2007). Por esta razão, *Listeria* spp. estão entre os microganismos que mais causam preocupação na contaminação cruzada pós-processamento de produtos prontos para consumo (*ready-to-eat* - RTE) (WHO/FAO, 2004).

Apesar do fato de uma grande variedade de alimentos poderem ser contaminados por *L. monocytogenes*, surtos e casos esporádicos de listeriose estão predominantemente associados com produtos prontos para consumo (RTE). Uma vez que alimentos RTE podem ser consumidos sem prévio aquecimento, estes apresentam um maior risco de doenças transmitidas por alimentos (ROCOURT et al., 2003, LONGHI et al., 2003). Por esse motivo, alguns países da Europa e

Estados Unidos, estabeleceram a política de ausência (*zero-tolerance policy*) de *L. monocytogenes* em alimentos RTE (WHO/FAO, 2004). Entretanto, apesar dos rigorosos esforços para eliminar a contaminação por *L. monocytogenes* em alimentos, surtos nos Estados Unidos e Europa têm sido relatados, e associados com altas taxas de mortalidade (23 - 44%) (CDC, 2011; DE VALK et al., 2001; OLSEN et al., 2005). Além disso, casos esporádicos associados com infecção sistêmica podem ter um logo período de incubação, de 10 até 70 dias, tornando difícil o rastreamento e isolamento da fonte de contaminação (LECUIT, 2007). Portanto, a educação continuada para consumidores, o reconhecimento e intensificação da fiscalização pelos órgãos reguladores e indústria de alimentos, são ações extremamente necessárias para prevenir e controlar casos e surtos de listeriose.

O primeiro surto reportado de *L. monocytogenes* ocorreu em 1981 no Canadá após o consumo de salada de repolho (*coleslaw*) contaminada e acometeu 41 pessoas, causando 18 mortes (SCHLECH et al., 1983). Desde então, diversos surtos têm sido reportados. Entre os anos 1998 - 1999, salsichas (hot dogs) contaminadas com *L. monocytogenes* foram a causa de listeriose em 108 pessoas, levando 14 delas à morte, além de provocarem quatro abortos em mulheres (GRAVES et al., 2005). Em outro surto relatado por Olsen et al. (2005) ocasionado pelo consumo de embutido de peru, 30 pessoas contraíram listeriose, quatro morreram e três sofreram aborto. Em 2008, no Canadá, um surto de listeriose pelo consumo de produtos de carne pronto para o consumo matou 22 pessoas, de 57 indivíduos acometidos (GILMOUR et al., 2010). Recentemente o *Centers for Disease Control and Prevention* (CDC) dos EUA, reportaram um grande surto de listeriose, ocasionado pelo consumo de melões contaminados, com casos distribuídos em 23 estados foram afetados durante os meses de agosto e setembro de 2011 (CDC, 2011). Os dados atualizados, devido ao longo período de incubação da bactéria, o número de indivíduos acometidos chegou a 123, acarretando um total de 25 mortes, o que fez com que fosse classificado como o segundo maior surto da doença nos Estados Unidos. Este número de pessoas afetadas somente permaneceu atrás do surto ocorrido com queijo feito no estilo mexicano (*mexican-style*) relatado por Linnan et al. (1988), onde 142 pessoas ficaram doentes e 48 vieram a óbito.

No Brasil, ainda não existem relatos de surtos de listeriose associados ao consumo de alimentos contaminados, no entanto, *L. monocytogenes* tem sido

isolada em uma ampla variedade de alimentos (BARROS et al., 2004; BUENO et al., 2010; NÁLERIO et al., 2009). Assim sendo, esses dados levam a presumir que casos isolados e surtos de listeriose no Brasil são subdiagnosticados, bem como subnotificados pelo nosso sistema de vigilância pouco eficiente.

1.3 Listeriose

Listeriose é um termo genérico para uma variedade de síndromes causada por *L. monocytogenes*. O CDC dos Estados Unidos estimou recentemente que casos fatais por listeriose (19%) foram a terceira causa de doenças acarretadas por patógenos alimentares naquele país entre os anos de 2000 e 2008 (SCALLAN et al., 2011). As complicações mais frequentes causadas por *L. monocytogenes* em humanos ocorrem na corrente circulatória, no sistema nervoso central e no útero em mulheres grávidas (VAZQUEZ-BOLAND et al., 2001). As manifestações mais graves em decorrência da listeriose geralmente são septicemia, meningite, meningoencefalite e aborto (COSSART, 2007). Embora seja uma doença que tenha uma baixa incidência, a listeriose apresenta alta taxa de mortalidade, a qual pode passar de 30% em grupos populacionais de alto risco (HAIN et al., 2007). Estes grupos incluem crianças, idosos, e indivíduos imunocomprometidos como pacientes com AIDS, pacientes com câncer, transplantados, assim como mulheres grávidas e seus bebês (COSSART, 2007; KUAR et al., 2007). Em contraste, a infecção de adultos saudáveis por *L. monocytogenes* pode ser assintomática ou manifestar-se de forma branda, como uma gastroenterite febril auto-limitante (OOI; LORBER, 2005).

Mulheres grávidas com listeriose podem apresentar sintomas parecidos com os de uma gripe, enquanto que, devido à migração transplacentária de *L. monocytogenes*, o feto pode desenvolver uma infecção sistêmica que pode culminar em aborto, morte fetal, parto prematuro ou nascimento de uma criança gravemente doente (GRAVES; SWAMINATHAN, 2001; SWAMINATHAN; GERNER-SMIDT, 2007). *L. monocytogenes* pode infectar mulheres gestantes em qualquer estágio da gravidez, no entanto a maioria dos casos de listeriose ocorre durante o terceiro trimestre devido provavelmente à baixa da imunidade celular (ALLERBERGER; WAGNER, 2010).

1.4 Mecanismos de patogenicidade de *L. monocytogenes*

L. monocytogenes infecta uma ampla variedade de espécies de hospedeiro, assim como diferentes tipos de células (VAZQUEZ-BOLAND et al., 2001). Para causar sua infecção, *L. monocytogenes* é capaz de cruzar três barreiras importantes: epitélio intestinal, hemato-encefálica e placentária (WERBROUCK et al., 2006). Entretanto, a primeira e principal rota de infecção por *L. monocytogenes* é a passagem através do epitélio intestinal, após o consumo de alimentos contaminados. Assim a translocação bacteriana através do trato intestinal é um pré-requisito para haver infecção (BHUNIA, 2008a).

A infecção primária envolve a colonização bacteriana no intestino e, em sequência, a translocação através da barreira da mucosa para a circulação sanguínea ou linfática, e assim, para uma disseminação sistêmica (DREVETS; BRONZE, 2008). Após a entrada na circulação, grande parte das bactérias atinge os linfonodos, baço e fígado, devido principalmente ao seu "livre" transporte por meio de macrófagos e células dendríticas (DREVETS; JELINEK; FREITAG, 2001; PRON et al., 2001).

A menos que a replicação seja controlada efetivamente por uma resposta do sistema imune inato, *L. monocytogenes* escapa e continua se espalhado para outros órgãos como cérebro e placenta (PLITAS et al., 2007). Nos casos em que *L. monocytogenes* consiga escapar da primeira frente de defesa, a sobrevivência do hospedeiro dependerá diretamente de uma resposta do sistema imune adaptativo, principalmente ligada à resposta de linfócitos T citotóxica CD8 (STAVRU; ARCHAMBAUD; COSSART, 2011). A habilidade de *L. monocytogenes* replicar-se no citosol da célula hospedeira e migrar para células adjacentes, sem sair para a circulação sanguínea, impossibilita a ação de uma resposta humoral efetiva contra essa bactéria (YU; DAN; LIN, 2008). A Fig. 1 mostra as diferentes etapas do processo infeccioso de *L. monocytogenes* através de eletromicrografias e de uma representação gráfica.

Uma grande variedade de proteínas de superfície e secretadas de *L. monocytogenes* têm sido reconhecidas como importantes fatores na virulência desta bactéria (BIERNE; COSSART, 2007; TROST et al., 2005). Muitas dessas proteínas representam um ponto crítico na persistência deste patógeno no trato intestinal, na aderência e entrada na célula do hospedeiro, na movimentação de célula para

célula, e no escape do sistema imune do hospedeiro (SCHUPPLER; LOESSNER, 2010). A maioria dos genes associados à virulência de *L. monocytogenes* estão agrupados em uma mesma região de 9 kb denominada de ilha de patogenicidade de *Listeria* 1 (LIPI-1) (VAZQUEZ-BOLAND et al., 2001). Em ordem, os genes estão organizados da seguinte forma: *prfA, plcA, hly, mpl, actA, plcB*. Esses genes, juntamente com os genes de internalinas A e B (*inlA* e *inlB*), *bilE* e *hpt,* são diretamente regulados e controlados pelo gene *prf*A que codifica a proteína PrfA (fator regulador positivo) (DE LAS et al., 2011; MILOHANIC et al., 2003).

Uma vez que *L. monocytogenes* é ingerida e passa pelo estômago, o aumento da temperatura e exposição ao baixo pH estimulam o aumento da produção de proteínas relacionados ao estresse, iniciando assim o estágio de virulência de *L. monocytogenes* (RAENGPRADUB; WIEDMANN; BOOR, 2008). Estas proteínas promovem a internalização da bactéria em células não fagocíticas, onde pode multiplicar-se no citoplasma como um patógeno intracelular facultativo e propagar-se para células vizinhas.

Ao chegar ao intestino *L. monocytogenes* utiliza principalmente as proteínas InlA e InlB para iniciar a aderência e a invasão dos enterócitos (CABANES et al., 2002; PENTECOST et al. 2010). A internalização da bactéria ocorre por um mecanismo progressivo de engolfamento chamado de mecanismo tipo zíper (*zipper mechanism*), em um processo de fagocitose que leva *L. monocytogenes* para o interior da célula hospedeira (COSSART; PIZARRO-CERDA; LECUIT, 2003). Dentro do fagossoma, as baixas concentrações de ferro e carboidratos reprimem a expressão das internalinas e fazem com que as proteínas listeriolisina O (LLO) e fosfatidilinositol fosfolipase C (PI-PLC) sejam expressas pela ativação dos genes *hly*a e *plc*A, respectivamente (GRAY; FREITAG; BOOR, 2006). Essas duas proteínas agem lisando a membrana do vacúolo fagocítico, o que faz com que a bactéria seja liberada no citoplasma celular. Neste compartimento celular é produzida uma alta quantidade de PrfA que promove a expressão da proteína polimerizadora de actina (ActA), a qual permite que *L. monocytogenes* polimerize a actina citoplasmática da célula hospedeira e seja impulsionada para células adjacentes (HAMON; BIERNE; COSSART, 2006). No processo de passagem para outra célula ocorre a formação de um vacúolo com dupla membrana, e é neste momento que *L. monocytogenes* expressa outra fosfolipase C, a fosfotidilcolina (PC-PLC, gene *plc*B), que quando é clivada por uma metaloprotease (Mpl) torna-se ativa.

Desta forma, o escape da dupla membrana é realizado novamente pela proteína LLO com o auxílio de PC-PLC e, assim, inicia-se o processo de multiplicação intracelular na nova célula (CABANES et al., 2002).

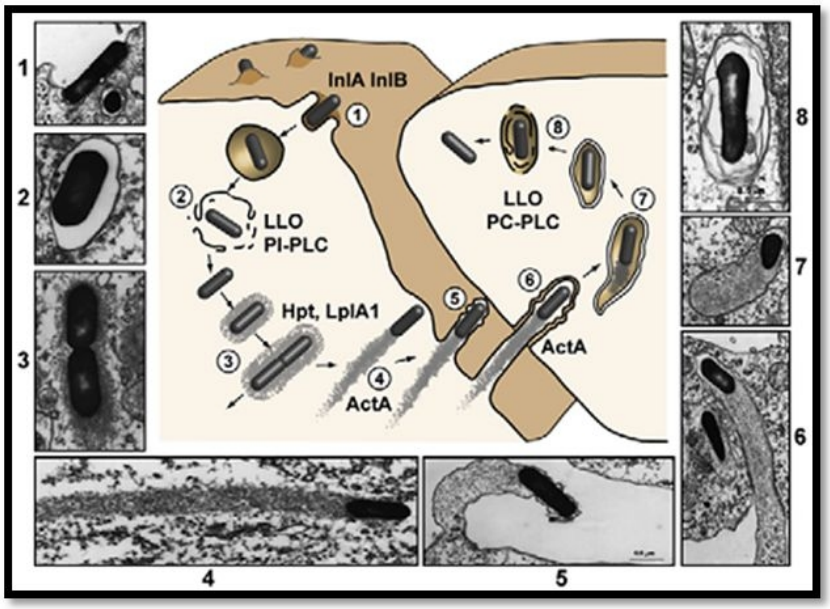

Figura 1. Eletromicrografias e representação esquemática dos sucessivos passos do processo infeccioso de *Listeria monocytogenes*. Fonte: COSSART; TOLEDO-ARANA, (2008).

1.5 Internalina A - a proteína chave de *L. monocytogenes*

A proteína internalina A faz parte da família multigênica das internalinas, a qual consiste em *inl*B, *inl*C, *inl*C2, *inl*D, *inl*E, *inl*F, *inl*G, *inl*H, *inl*I e *inl*J (BIERNE et al., 2007; BUBLITZ et al., 2008; TSAI et al., 2006; TSAI et al., 2011). No entanto, somente *inl*A e *inl*B estão localizadas no mesmo *locus* no genoma, enquanto as restantes situam-se em locais diferentes (BIERNE et al., 2007). A maioria das internalinas são membros da superfamília das proteínas com repetições ricas em leucina (LRR - Leucine Rich Repeat) (BONAZZI; LECUIT; COSSART, 2009). O domínio LRR consiste de uma repetição em *tandem* de 20-22 aminoácidos com resíduos conservados de leucina (CABANES et al., 2002). As internalinas podem ser classificadas em três tipos diferentes, de acordo com o modo de interação com a superfície bacteriana: 1) as que fazem ancoragem covalente com a parede celular por motivos LPXTG (Leu-Pro-X-Thr-Gly, onde X é qualquer aminoácido); 2) as que fazem ligação não-covalente por uma sequência de três dipeptídeos nomeados de

GW (Gly-Trp), onde somente InlB faz parte; 3) e as internalinas secretadas, que não possuem domínios de ancoramento à parede celular, representadas pela InlC (BIERNE et al., 2007; CABANES et al., 2002).

Dentre todas as internalinas, InlA e B são as melhores caracterizadas e as mais estudadas. A proteína InlA, que é ancorada à parede celular pela enzima sortase A (SrtA), foi a primeira proteína LPXTG identificada em *L. monocytogenes* (CABANES et al., 2002; GAILLARD et al., 1991). InlA é uma proteína de superfície de 800 aminoácidos, com aproximadamente 88 kDa, e que pode ser dividida em distintas regiões (BONAZZI; LECUIT; COSSART, 2009; WERBROUCK et al., 2006). Iniciando da porção N-terminal, InlA possui um peptídeo sinal seguido de um motivo alfa-hélice e de 15 repetições LRRs. Após a porção C-terminal da região de LRRs, existe uma região altamente conservada e flexível, chamada de região inter-repetição (IR), que separa os LRRs da denominada repetição B, que por sua vez possui três repetições consecutivas (duas de 70 aminoácidos e uma com 49 aminoácidos) (MENGAUD et al., 1996) (Fig. 2). O número de unidades repetidas LRR, leva a diferentes conformações das internalinas, como por exemplo, na proteína InlA. Nesta proteína, as quinze sequências LRR fazem com que essa proteína apresente uma estrutura com uma curva, a qual esta diretamente ligada com sua interação com as proteínas do hospedeiro (BIERNE et al., 2007).

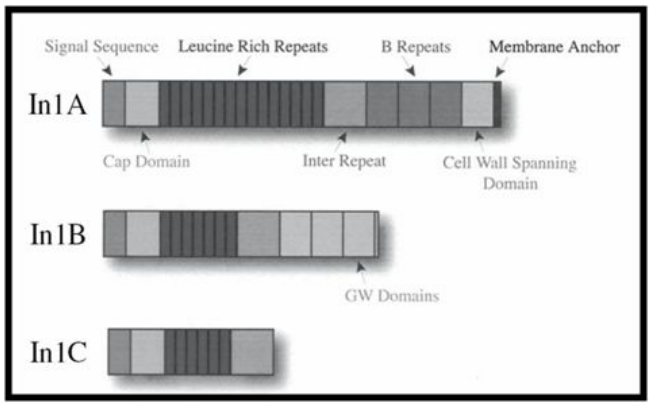

Figura 2 – Representação esquemática das proteínas InlA, InlB e InlC. Fonte: PIZARRO-CERDA et al. (2007).

Para a invasão das células epiteliais *L. monocytogenes* depende da interação da InlA com uma glicoproteína de membrana denominada E-caderina (E-Cad), que é uma molécula essencial para a adesão de células vizinhas e responsável pela

formação e integridade dos tecidos (SCHUBERT et al., 2002). A E-Cad está localizada na porção basolateral das células e não é exposta na superfície celular (HAMON; BIERNE; COSSART, 2006). Porém, recentemente foi provado que *L. monocytogenes* aproveita-se da constante extrusão e renovação do epitélio celular nas vilosidades intestinais para se ligar na E-Cad que é temporariamente exposta na superfície do lúmen e, assim, fazer sua internalização e infecção (PENTECOST et al., 2010).

A interação InlA com E-Cad é espécie-específica e a ligação inicia-se após o reconhecimento de um resíduo de prolina na posição 16 na E-Cad de humanos, cobaios, e coelhos (LECUIT et al., 1999). Em camundongos e ratos a prolina nesta posição é trocada por uma glutamina e, assim, InlA não consegue fazer a ligação na E-Cad, o que torna estas espécies mais resistentes à infecção oral por *L. monocytogenes* (LECUIT et al., 1999; MEGAUND et al., 1996). Após interação específica da InlA, a internalização de *L. monocytogenes* é auxiliada pela também específica ligação da InlB na célula do hospedeiro (VEIGA; COSSART, 2007). InlB é uma proteína de aproximadamente 65 kDa (630 aa) que, em comparação com InlA, também possui uma sequência sinal na região N-terminal, seguido por oito domínios de LRRs (BIERNE; COSSART, 2002). Na porção C-terminal, carrega três porções repetidas chamadas de módulo GW, que são fracamente associados ao ácido lipoteicóico presente na parede celular bacteriana (JONQUIERES et al., 1999; SEVEAU et al., 2007).

A proteína InlB liga-se ao receptor celular Met, um receptor de tirosina quinase (RTK) e ao receptor natural do fator de crescimento de hepatócitos (HGF) (SHEN et al., 2000). A aderência e internalização de *L. monocytogenes* através da InlB tem sido comprovada em vários tipos de células de mamíferos (MARINO et al., 2004). Entretanto, estudos recentes demonstraram que InlB não funciona como uma adesina, e, sim, promove aceleramento da invasão em células do intestino após a aderência da InlA na E-Cad (PENTECOST et al., 2006, PENTECOST et al., 2010). Seguindo a ligação InlB ao Met, inicia-se a ativação de uma cascata de sinalizações, a qual culmina com a alteração do citoesqueleto celular e uma endocitose localizada, promovendo a entrada da bactéria pelo mecanismo de zíper (PENTECOST et al., 2010).

1.6 Separação imunomagnética de patógenos em alimentos

Apesar de existirem técnicas rápidas para a detecção desse microrganismo em alimentos, a grande maioria dos laboratórios utiliza os métodos tradicionais. Estes são baseados no uso de meios de pré-enriquecimento seletivo, enriquecimento e crescimento em meios de cultura seletivo/diferenciais, para o isolamento de colônias, seguidos de testes bioquímicos para identificação da espécie (HEARTY et al., 2006). Os métodos tradicionais são bastante sensíveis e permanecem como padrão ouro, entretanto, apresentam custo operacional elevado e são demorados, requerendo vários dias para se obter a diferenciação entre as espécies (GASANOV; HUGHES; HANSBRO, 2005), além de não serem suficientemente sensíveis para detecção de baixa concentração de células presentes em alimentos (GRAY; BHUNIA, 2005).

Diversas estratégias têm sido utilizadas com o objetivo de melhorar a detecção de *Listeria* spp., a maioria baseadas na reação antígeno-anticorpo, como é o caso da separação imunomagnética (IMS), ou na amplificação do DNA através da Reação em Cadeia da Polimerase (Polimerase Chain Reaction, PCR) (HUDSON et al., 2001). Esses métodos apresentam maior facilidade de execução e rapidez na obtenção dos resultados, além de apresentarem excelente sensibilidade e especificidade quando comparados aos métodos tradicionais de cultivo.

A IMS permite a captura específica de *L. monocytogenes* em alimentos ou meios de cultivos, com concentração das células alvo e remoção de inibidores da reação de PCR (GRAY; BHUNIA, 2005; BILIR ORMANCI et al., 2008). As microesferas magnéticas são sensibilizadas com anticorpos, policlonais (PAbs) ou monoclonais (MAbs). Como os MAbs reagem com apenas um epítopo do antígeno, este tem sido cada vez mais utilizados devido a sua especificidade. Porém, a principal dificuldade na produção de anticorpos, é obter MAbs com uma afinidade adequada para detectar pequenas quantidades do antígeno (BHUNIA, 2008b). O alvo para produção de MAbs de captura de patógenos, deve ser uma proteína com distribuição uniforme na superfície do microrganismo, cuja localização facilite o acesso do anticorpo (TULLY et al., 2006). Para tanto, a proteína InlA atende a esses requisitos, principalmente por ser uma proteína extracelular específica de *L. monocytogenes*, que está covalentemente ancorada na parede celular, sendo um fator de virulência essencial para a adesão e internalização na célula hospedeira (BONAZZI; LECUIT; COSSART, 2009).

1.7 Utilização de anticorpos monoclonais em biossensores de fibra óptica

Nos últimos anos, houve um grande investimento em pesquisa e desenvolvimento na área de nanotecnologia em todo o mundo. Os biossensores são atualmente as ferramentas mais utilizadas para a detecção de variadas substâncias, incluindo um grande número de patógenos (TURNER, 2000). Um biossensor é um dispositivo no qual um material de origem biológica pode ser imobilizado junto a um transdutor físico-químico (SKOTTRUP et al., 2008), com o objetivo de produzir um sinal eletrônico proporcional à interação específica de uma determinada substância analisada com o sensor (BHUNIA, 2008b; TURNER, 2000).

A vantagem mais significativa da utilização de um biossensor é a redução do tempo para avaliar a presença de contaminantes (GENG; HAHM; BHUNIA, 2006), tempo este muito inferior ao requerido através de métodos convencionais de identificação de patógenos (GASANOV et al., 2005). Quando se considera a questão de segurança microbiológica, o uso de biossensores reduz a manipulação da amostra, consequentemente diminui também a possibilidade de contaminação humana (LEONARD et al., 2004), o que torna os biossensores altamente recomendados para os laboratórios microbiológicos, especialmente os de microbiologia de alimentos.

Os imunossensores, biossensores baseados na interação antígeno-anticorpo, têm demonstrado serem viáveis na detecção de patógenos (FURTADO et al., 2008), podendo até mesmo investigar a presença de mais de um patógeno em uma mesma amostra (DENG; YANG, 2007). Em vista disso, ficam claras as vantagens dos biossensores em relação às técnicas convencionais, principalmente daqueles que utilizam anticorpos que tornam as reações mais sensíveis e altamente seletivas. Além disso, os biossensores dispensam um elaborado pré-tratamento da amostra e proporcionam agilidade na obtenção dos resultados e redução nos custos financeiros.

Dentre os dispositivos biossensores, os que utilizam fibras ópticas têm-se revelado uma tecnologia promissora para a detecção rápida de patógenos alimentares (TAITT et al., 2005). Os mais utilizados são os sistemas RAPTOR^{TM} e Analyte 2000 da empresa Research International (Monroe, WA). Ambos são dispositivos automatizados e portáteis para detecção de agentes biológicos que utilizam um formato “sanduíche” com dois anticorpos, onde o primeiro realiza a

captura do antígeno e o segundo, marcado com substâncias fluorescentes, emite o sinal que revela sua presença para o aparato transdutor (GENG; HAHM; BHUNIA, 2006; BHUNIA, 2008b). O princípio básico do sensor de fibra óptica é a propagação de luz através do núcleo da fibra óptica (sonda), que gera um campo evanescente na superfície da sonda. A onda evanescente produzida por laser (635 nm), faz com que anticorpos marcados, quando ligados a patógenos ou toxinas na superfície das sondas, sejam animados e emitam o sinal fluorescente. Este sinal viaja de volta através da fibra óptica onde é então, capturado por um detector de fluorescência em tempo real (BHUNIA, 2008b; DENTON et al., 2009; TAITT et al. 2005).

Este formato sobre fibra óptica vem sendo aplicado com sucesso para identificar diversos microrganismos patogênicos, de origem alimentar ou não, além de toxinas bacterianas, incluindo *L. monocytogenes* (GENG; HAHM; BHUNIA, 2006; NANDURI et al., 2006; OHK et al., 2010), *Salmonella spp.* (KRAMER; LIM, 2004; VALADEZ et al., 2009), *Escherichia coli* O157:H7 (DEMARCO et al., 2002), *Bacillus anthracis* (JUNG et al., 2003), *Mycobacterium tuberculosis* (DENTON et al., 2009), assim como toxinas botulínica e estafilocócica (SAPSFORD et al., 2005). Contudo, para desenvolver ensaios sobre biossensores baseados na utilização de anticorpos, é extremamente necessário ter disponíveis anticorpos monoclonais (MAbs) ou policlonais (PAbs) de ótima qualidade (BHUNIA, 2008b).

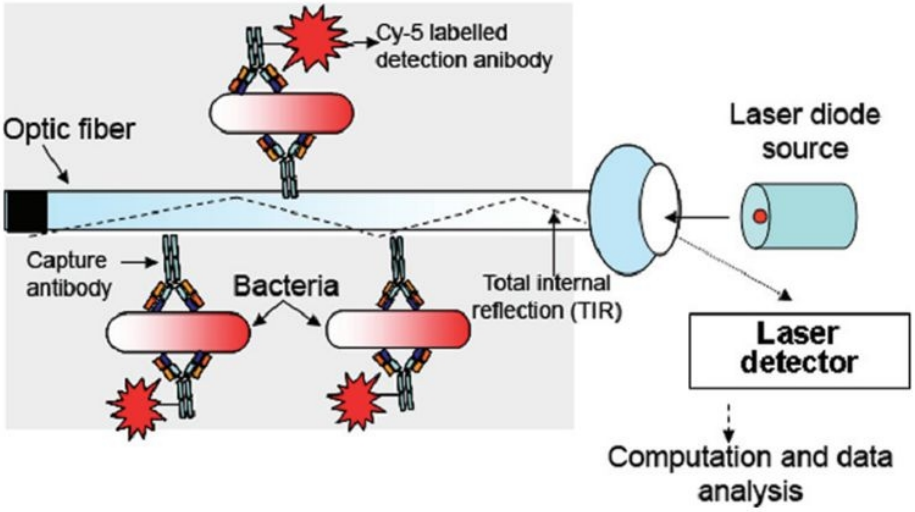

Figura 3 – Representação esquemática da detecção de bactéria pelo biossensor de fibra óptica. Fonte: BANADA; BHUNIA, (2008).

2. OBJETIVOS GERAIS

1- Produzir anticorpos monoclonais (MAbs) específicos contra *Listeria* spp. e *L. monocytogenes*;

2- Desenvolver um método para captura e detecção de *L. monocytogenes* em alimentos baseado na separação imunomagnética (IMS) com microesferas magnéticas sensibilizadas *in house* com MAbs e detecção através de amplificação por PCR;

3- Demonstrar a potencialidade de MAbs contra InlA ou contra o gênero *Listeria* usados como biossensores para detectar *L. monocytogenes* com sistema de captura em fibras ópticas.

3. OBJETIVOS ESPECÍFICOS

- Clonar e expressar o gene *inlA* de *L. monocytogenes*;
- Obter hibridomas secretores de MAbs anti-InlA;
- Caracterizar a reação dos MAbs produzidos com a proteína em sua forma nativa através de técnicas imunológicas;
- Utilizar os MAbs na sensibilização de microesferas magnéticas;
- Padronizar condições para imunoseparação magnética (IMS);
- Comprovar o desempenho do IMS-PCR;
- Utilizar os MAbs em sistema biossensor de fibra óptica.

4 ARTIGO 1

Monoclonal Antibody Anti-Internalin A Showed Improved for Capture and Detection of *Listeria monocytogenes* by Immunomagnetic Bead and Fiber-Optic Immunosensor

(Formatado de acordo com periódico Applied and Environmental Microbiology)

4 ARTIGO 1 - Monoclonal Antibody Anti-Internalin A Showed Improved for Capture and Detection of *Listeria monocytogenes* by Immunomagnetic Bead and Fiber-Optic Immunosensor

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Running Title: Immunomagnetic bead and fiber-optic sensor in the capture and detection of *L. monocytogenes*

ABSTRACT

Sensitive and specific methods for detection of the pathogenic *Listeria monocytogenes* or members of genus *Listeria* are highly desirable. Immunological methods including immunosensor approaches are attractive and widely used but for improved assay performance, highly selective antibodies are necessary. Here we generated monoclonal antibodies (MAbs) against *L. monocytogenes* targeting Internalin A and *Listeria* species targeting surface associated protein (p30) and used them on magnetic bead for capture after enrichment and fiber-optic immunosensor for detection. Western blot and ELISA assays revealed that anti-InlA MAb-2D12 (IgG2a subclass) was specific for *L. monocytogenes*, and the 30 kDA protein specific MAb-3F8 (IgM subclass) was specific for genus-*Listeria* and none had cross-reactions with any non-*Listeria* bacteria tested. In the immunomagnetic separation (IMS) assay, paramagnetic MyOne^{TM} streptavidin T1 (PMBT1) beads coated MAb-2D12 captured 49.2% of *L. monocytogenes* cells from an initial cell concentration of 1 × 10^5 CFU/mL, while PMBT1 coated 3F8 captured about 16.6% and these values were significantly greater than Dynabead anti-*Listeria* antibody. In the fiber-optic immunosensor, using MAb-2D12 as capture and reporter (labeled with Cy5), detection limit was established to be 3 x 10^2 CFU/ml for *L. monocytogenes.* Using MAb-3F8 as capture and MAb-2D12 as reporter, detection limit was 1 x 10^5 cells/mL. Moreover, we confirmed and quantified the *L. monocytogenes* capture from PMBT1-2D12 and PMBT1-3F8 from foods by qPCR and light-scattering assays, both systems showed more capture of *L. monocytogenes* using MAb-2D12 and MAb-3F8. Combining best performing IMS condition and fiber-optic immunosensor setup, we were successful in detecting *L. monocytogenes* in presence of *L. innocua* or other natural contaminants from artificially contaminated soft cheeses and hotdogs. These two steps showed detection in approx. 21 h with an initial inoculum of less than 50 cells/g of *L. monocytogenes* in food samples. Employing anti-InlA antibody in both IMS and fiber-optic immunosensor found to be suitable for specific detection of *L. monocytogenes* from foods.

INTRODUCTION

Listeria monocytogenes is a food-borne pathogen that causes human listeriosis, a severe illness that can range from a mild gastroenteritis to invasive infection (42, 76). The majority of deaths caused by listeriosis are frequently associated with immunocompromised people, neonates and the elderly (85). In pregnant women, the bacterium can cause premature births, miscarriages, and serious illness in newborns or death (40, 80). *L. monocytogenes* is ubiquitous and can be found in food-processing environments (1, 83), food products, including dairy (21, 72), meat and seafood (59, 68).

Sporadic cases and outbreaks of listeriosis are generally associated with Ready-to-Eat (RTE) food (17, 56). The Centers for Disease Control and Prevention (CDC) have recently estimated that fatality (19%) from listeriosis ranks third among all fatalities resulting from foodborne infections in the USA (76). Indeed, *L. monocytogenes* has become a huge public health concern as well as for the food industry, mainly due its capacity to grow and survive in many stress conditions such as high salt concentrations and low pH (4, 14). In addition, *L. monocytogenes* is a facultative anaerobic and psychrotrophic pathogen, thus allowing it to grow under vacuum environments and at very low temperatures, and may even survive during freeze-thaw process (41, 78). Food recalls due to *Listeria* contamination has been considerably increasing each year (18), exerting enormous economic burden to the food manufacturers and growers. Therefore, many countries have established the zero tolerance policy towards *L. monocytogenes* in RTE foods (25).

The genus *Listeria* consists of eight species, but just *L. monocytogenes* and *L. ivanovii* are considered potentially pathogenic for human and animals (34, 51). These two pathogenic bacteria are phylogenetically related to nonpathogenic *Listeria* species, including *L. seeligeri*, *L. welshimeri*, *L. innocua*, *L. gray* and recently discovered *L. marthii* (32) and *L. rocourtiae* (50).

An arsenal of virulence and structural gene products are exclusive of the genus *Listeria* and *L. monocytogenes*, and these could be used as target for antibody- or nucleic acid-based assay development (6). In order to cause infection, *L. monocytogenes* expresses several proteins such as invasion-associated proteins internalin A (InlA) and B (InlB), virulence protein (Vip), invasive-associated protein (p60), *Listeria* adhesion protein (LAP) and autolysin amidase (Ami); along with

proteins related with the bacterium escape and spread into the mammalian organism such as listeriolisin O (LLO), actin-based motility (ActA), phospholipases (PLC) and the flagellar protein (6, 11, 66, 77).

Among those, one of the most important and well characterized proteins is the InlA, a molecule that is essential in the adhesion and internalization of the bacterium into the host cells (12). Moreover, InlA is one of the most abundant surface protein found in pathogenic *Listeria* (35). Structurally, InlA is characterized by its covalent anchoring to bacterial cell wall peptidoglycan, and the presence of a N-terminal domain containing leucine-rich repeats (LRRs) that binds the most distal extracellular domain of E-cadherin, a transmembrane protein that play an essential role in cell-cell adhesion and maintenance of tissue architecture (52, 58).

Generally, both pathogenic and non-pathogenic *Listeria* species can be found in the same environment or food (65). However, when an enrichment step is used the non-pathogenic species, at most situations, can overgrow and suppress *L. monocytogenes* growth (5, 47), leading to increased chances for obtaining false-negatives results. In addition, *L. innocua* is the most frequent bacteria found in *Listeria*-contaminated foods (57, 69), therefore raising a great challenge for specific capture and detection of pathogenic *Listeria* from contaminated foods. Hence, methods that are capable of detecting the pathogenic species in presence of others are extremely crucial.

The traditional methods used to detect this bacterium in foods are generally based on its enrichment in selective media and subsequent isolation, biochemical and serological confirmation (26). These methods can take several days to complete and usually are laborious and highly expensive. Therefore, sensitive and specific detection methods that are capable of detecting *Listeria* in less time are always desirable (7, 20). Several rapid methods of isolation and identification of *L. monocytogenes* in food have been reported but still there is a great need for improved detection methods with high specificity and selectivity.

Immunological approaches to detect pathogens in food are simple, easy to use and are widely acceptable. In most cases, monoclonal antibodies (MAbs) are employed for the assay development (7). Researchers have used many strategies to produce highly specific antibodies that react only with *L. monocytogenes*. However, in many cases, the antibody reaction profile with different serotypes and cross-reaction with nonpathogenic *Listeria*, made them undesirable for assay development.

For instance, Bhunia and Johnson (10) reported the production of a MAb that failed to detect two of the 13 serotypes of *L. monocytogenes*. Heo et al. (36) also reported similar results with their MAbs. A MAb that recognizes both *L. monocytogenes* and *L. innocua* was also produced by Bhunia et al. (8). MAbs against *L. monocytogenes* serotype 4b were also produced by Lin et al. (53, 54). Using purified flagellar antigen, Kim et al. (46) produced MAbs that were specific for all species in the genus *Listeria*. In an effort to generate MAbs specific to *L. monocytogenes*, Hearty et al. (35) used formalin-inactivated *L. monocytogenes* as immunogen and selected one MAb that recognized specifically all serotypes of *L. monocytogenes*; in the sequence they found that the reactive protein was InlA. Antibody phage display technology also has been used for production of single-chain antibody (scFv) for specific detection of *L. monocytogenes* in food (70, 71).

To address regulatory compliance food industry first use commercial kits that can detect all *Listeria* species. Based on the initial findings, *L. monocytogenes*-specific assay kits to confirm presence of this pathogen are employed. Therefore, antibodies that are genus-specific or *L. monocytogenes*-specific are essential for assay development. Immunomagnetic separation (IMS) system use magnetic particles coated with antibodies to separate and concentrate a variety of target microorganisms (27, 29, 60, 71). In most applications, IMS are used to capture *Listeria* from food followed by culturing on selective or differential media (3, 84) or detection/confirmation by PCR (13, 38), flow cytometry (37), and cytotoxicity (33).

Fiber-Optic Sensors utilize a laser excitation to generate an evanescent wave to quantify biomolecules immobilized on the optical waveguide (9). The fundamental lay out of the assay is based on sandwich immunoassay where the capture antibody is immobilized on the waveguide and fluorescence (Cyanine 5 or Alexa-Fluor 647) labeled second antibody is used as a reporter for target analyte (7). Once the laser beam (635 nm) travels through the optical waveguide, undergoes total internal reflection (TIR) and the fluorophore on reporter antibody bound to analyte is excited generating an evanescent wave. The signal is propagate back up to the fiber and is detected in real time by a fluorometer (7). This format has been successfully applied to many food-borne microorganisms, including *L. monocytogenes* (30, 30, 61, 67), *E. coli* O157:H7 (22, 30, 31), *Salmonella* (49, 82), as well as staphylococcal and botulinum toxins (74). In this paper, we first demonstrate the production and characterization of a panel of MAbs against InlA from *L. monocytogenes* and *Listeria*

spp; after we report on the use of two MAbs for capture and concentration of *L. monocytogenes* from foods using paramagnetic beads; and finally these MAbs were used on the fiber optic sensor system to detect *L. monocytogenes* from inoculated food samples.

MATERIALS AND METHODS

Cultures and growth conditions

All organisms, including all serotypes of *L. monocytogenes* used in this study are listed in Table 1. All bacterial cultures were maintained on brain heart infusion (BHI, Acumedia, Lansing, MI) agar plates at 4°C with an exception for lactic acid bacteria, which were maintained on De Man Rogosa Sharpe agar (MRS, Becton Dikinson, Sparks, MD, USA). When necessary, fresh cultures of *Listeria* spp. were grown at 37^{o}C for 16-18 h in Tryptic soy broth (TSB; BD, Sparks, MD) containing 0.6% yeast extract (TSB-YE; Acumedia) or *Listeria* enrichment broth (LEB, BD). Other non-*Listeria* organisms were grown in TSB-YE and lactic acid bacteria were grown in MRS broth at 37^{o}C for 16-18 h. Other individual selective enrichment broths and plating agars were purchased from BD such as Fraser Broth (FB) and modified Oxford agar (MOX). All bacteria were also maintained in BHI broth with 20% glycerol at -80^{o}C for further use.

Cloning of *inlA* gene and Immunogen Preparation

The *inlA* specific primers were designed using Vector NTI 10.0 software (Invitrogen) aiming to amplify the whole gene except the c-terminal portion (2332 bp, 67-2379 aa) and purchased from MWG-Biotech (USA). To insert the *inl*A gene in the pAE expression vector, the restrictions sites for *Bam*HI and *Kpn*I enzymes were incorporated in the primers sequence for forward primer, For-inlA (‘5-CGGGATCCGTATGGATTAACACGA-3’) and reverse primer, Rev-inlA (‘5-GGGGTACCCTAAGTAAGAACCATTGCAGT-3’). The *inl*A gene was amplified from genomic DNA of *L. monocytogenes* (ATCC 19114) by PCR in an Eppendorf thermocycler (Mastercycler EP gradient S) and standardized under the following conditions 94°C for 7 min; 94°C for 1 min; 45°C for 1 min; 68°C for 2 min, with a final extension of 68°C for 7 min. The PCR product was cloned into *Bam*HI and *Kpn*I sites of pAE vector and transformed by eletroporation into *Escherichia coli* Top10 (Invitrogen). After confirming the insertion of inlA gene in pAE vector by sequencing, the recombinant plasmid containing the *inlA* gene was transformed into *E. coli* BL21 (DE3) pLysS (Invitrogen) competent cells. The transformed cells were grown to reach the log phase ($D.O_{600}$= 0.5-0.7) and then induced with 1 mM IPTG for an additional 3 h at 37 °C. Cells were harvested, suspended in lysis buffer (100 mM NaH_2PO_4, 10 mM Tris HCl and 20 mM imidazole, pH 8.0) and lysed by sonication (three cycles).

The recombinant InlA (rInlA) containing poly-histidine tag (6x-His) was purified by using Ni-NTA affinity chromatography system (GE Healthcare). Finally, column eluted proteins were dialyzed against PBS for 24 h and concentrated with polyethylene glycol (MW 20.000).

Immunization and Hybridoma Production

Six-week old BALB/c female mice were inoculated intraperitoneally (i.p.) with approx. 1×10^8 cells/mL of heat-killed *L. monocytogenes* serotype 4b diluted in PBS and mixed 1:1 with complete Freund's adjuvant (CFA). Two weeks after the first immunization, a mixure of heat-killed *L. monocytogenes* and 50 µg of rInlA prepared with incomplete Freund's adjuvant (IFA) were administered i.p. every week for eight weeks. Four days before the last immunization, the mouse showing the highest antibody titer against rInlA in indirect ELISA received a booster immunization with rInlA intravenously and again via i.p. The splenocytes were harvested from mouse and fused with murine Sp2/O-Ag14 myeloma cells in the presence of 50% (w/v) PEG 1450 (Sigma Aldrich) as described by Harlow and Lane (1988). Fused cells were cultivated in Dulbecco's modified Eagle's medium (DMEM; Sigma Aldrich) containing hypoxanthine-aminopterin-thymidine (HAT; Sigma Aldrich) and 20% of fetal bovine serum (FCS; Cultilab, Brazil). Hybridomas screening were performed by indirect ELISA using rInlA or viable *L. monocytogenes* 4b cells as antigen. Wells presenting hybridomas producing specific antibodies against rInlA and/or *L. monocytogenes* were cloned at least twice by the limiting-dilution method. Once established, the hybridoma lines were expanded to tissue culture flasks and supernatants re-tested by ELISA with rInlA and *L. monocytogenes* (4b, 1/2a, 1/2b). As negative control, *L. innocua*, *L. selligeri*, *Salmonella enterica* serovar Enteritidis and *E. coli* O157:H7 were used. Hybridomas producing MAbs that recognized any rInlA, and/or *L. monocytogenes* as well as other *Listeria* species were administered in pristane primed mice to produce ascitis fluid for antibody production. MAbs were purified by affinity chromatography using a protein A-Sepharose 4B column (GE Healthcare, Piscataway, NJ) and the class and subclass of each MAb were determined by ELISA with a mouse subisotyping kit (Sigma Aldrich). Selected hybridoma clones were stored in liquid nitrogen for future use.

Characterization of MAbs by Indirect ELISA

L. monocytogenes, *L. innocua* and *L. seeligeri* were cultured in LEB, other non-*Listeria* bacteria were grown in BHI, and then harvested by centrifugation and washed three times with PBS. Cell concentrations were adjusted to achieve OD_{600} of 1 (approx. 10^9 CFU/mL^{-1}) in 0.1 M sodium carbonate coating buffer (pH 9.6). Then, 96-well ELISA plates (Nunc, Maxisorp) were coated with 100 µL of each cell suspension or 10 ng/ well of rInlA for 16 h at 4°C. After washing the plates three times with PBS containing 0.5% Tween 20 (PBST), plates were then blocked with a 5% solution of dry skimmed milk for 1 h at 37°C. Ascites of each hybridoma or purified antibody were diluted in PBST and 100 µL added to the wells and incubated for 1 h at 37°C. After washing with PBST, a goat anti-mouse polyvalent antibody conjugated to horseradish peroxidase (HRP; Sigma) diluted in PBS-T (1:4000) was added and incubated at 37°C for 1 h. The wells were then washed five times to remove excess of secondary antibodies. To develop color, 100 µL of the chromogenic solution containing 10 mg of ortho-phenylenodiamine (OPD; Sigma) in 10 mL of 0.1 M citrate buffer (pH 5.0) and 10 µL of 30% H_2O_2.

Protein preparation, SDS-PAGE and Western blot

Bacterial proteins were prepared following the method described by Jonquières et al. (43) with some modifications. For isolation of cell wall-associated protein, 100 ml of 18-h grown bacterial cultures were centrifuged (7000 *g* for 10 min), and the cell pellets were resuspended by gently pipetting up and down with 250 µL of protein extraction buffer (0.5% SDS, 10 mM Tris, pH 6.9), followed by 30 min of incubation at 37°C. Following centrifugation (16100 *g*, 10 min at 4°C), the supernatant was collected and placed in a new tube. The remaining cell pellets were resuspended in sample solvent (4.6% SDS, 10% β-mercaptoethanol, 0.124 M Tris, 20% glycerol, pH 6.9), sonicated 4 times for 15 seconds, and centrifuged (16100 *g* for 20 min at 4°C) to collect the supernatant with the intracellular proteins fractions. Protein concentrations were adjusted using BCA method (Pierce) and separated in 10% or 12% SDS-PAGE (Bio-Rad, Hercules, CA). The proteins were blotted onto Immobilon-P membranes (Millipore Bedford, MA, USA) and blocked with 5% skimmed milk for 1 h at room temperature (RT). The membranes were washed with PBST, immunoprobed with the MAbs and incubated as above. After washing, the membranes were incubated with a HRP-conjugated goat anti-mouse polyvalent

antibody (Sigma). Antibody-reactive bands were visualized following treatment with chemiluminescence substrate system (ECL kit: Thermo Fisher Scientific, Rockford, IL) or DAB (6 mg of 3.3-diaminobenzidine tetrahydrochloride; 10 µL of H_2O_2 30%; 9 mL of 50mM Tris-HCl pH 7.6; 1 mL of 0.3% sulphate Nickel). Two MAb producing clones were selected for further studies: *L. monocytogenes* (InlA-reactive)-specific MAb-2D12 and *Listeria* genus specific (p30 reactive) MAb-3F8.

Immunofluorescence Microscopy

L. monocytogenes (serotypes 4b, 1/2a, 1/2b and 4d) and *L. innocua* were grown in 10 mL of LEB and centrifuged (7000 *g* for 3 min). Pellets were washed twice with PBS and resuspended in 1 mL of PBS containing 5% BSA (PBS-BSA 5). Then 20 µL of cells were incubated with MAbs diluted in 500 µL PBS-BSA for 1 h at 37^{o}C. After PBS washing (2X), the cell pellets were resuspended in 250 µl of FITC-conjugated goat anti-mouse IgG (1:100; Sigma) and incubated at 37^{o}C for 1 h. After three sequential washing with PBS, the pellets were stained with Hoechst 33258 (for nuclear staining) for 15 min and one drop of the suspension was examined by epifluorescence microscope (Olympus BX 51).

Antibody Labeling

Antibodies were biotinylated with the EZ-Link NHS-Biotin kit (Pierce, USA) for use with magnetic bead and fiber optic sensor that are pre-coated with streptavidin. First, 10 mM biotin stock solution was prepared in dimethylformamide (DMF) and known volume of biotin was added into each MAb solution. Then, the solution was incubated at room temperature for 30 min in a rotary shaker. Thereafter, unbound biotin was removed using a Zeba spin desalting column (Pierce). The biotinylated MAbs were tested by ELISA in avidin coated microtiter plates and the ratio of biotin binding on MAbs was calculated using HABA assay (4′-hydroxyazoben-zene-2-carboxylic acid; Pierce).

MAb anti-InlA was labeled with Cy5 using the Cy5-Ab labeling kit (Amersham Biosciences, NJ, USA) for use with fiber-optic sensor. Briefly, purified MAb-2D12 (1 mg.ml^{-1} in 1 mL of PBS) was added to vials containing coupling buffer provided in the kit and then mixed by inverting the vials ten times manually. The antibody suspension was then transferred to a vial containing Cy5, covered with aluminum foil to protect from photo bleaching and incubated at RT for 30 min by manually mixing the

contents every 10 min. The unbound dye was separated by gel filtration column and the ratio of dye to antibody was estimated from spectrophotometer (Beckman DU800) measuring at 280 nm and 650 nm.

Paramagnetic beads preparation

Two different paramagnetic beads (PMBs), Dynabeads streptavidin M-280 (2.8 µm diameter) and MyOne^{TM} streptavidin T1 (1.0 µm diameter) from Invitrogen (Carlsbad, CA, USA), were used for immunomagnetic separation. Bead preparation involved mixing of streptavidin-coupled PMBs with 200 µg.ml^{-1} of biotinylated MAbs for 30 min under constant rotation at RT. The unbound biotinylated MAbs were separated by removing the PMBs with a magnetic particle concentrator (MPC-S; Dynal) followed by washing the beads three times with PBS containing 1% BSA. The beads were stored at 4^{o}C until use.

Bacterial Capture using PMB Coated with Antibodies

Immunomagnetic separation was performed as follows. First, bacteria were grown in different media at 37^{o}C for 18 h, harvested (7000 *g* for 3 min) and washed twice with PBS, and resuspended in PBS containing 0.1% bovine serum albumin. Then, 20 µL of MAb coated PMBs were added to 200 µL of bacterial cell suspension containing variable cell counts (approximately 10^3 to 10^8 CFU.mL^{-1}) and mixed in a rotary incubator for 30 min at 25^{o}C. PMBs were recovered using MPC-S. After magnetic separation, beads were washed three times using 1 mL of PBST and then resuspended in 200 µL of PBS. Finally, PMBs were subjected to vigorous vortexing to release the captured bacteria and then 100 µL of each sample was surface plated onto BHI or Modified Oxford (MOX) agar plates for enumeration (48). In some experiments, Dynabeads anti-*Listeria* (Invitrogen) was used in parallel as control.

Immunomagnetic Capture of Bacteria from Contaminated Food Samples

The ability of PMBs coated with Anti-InlA or Anti-p30 MAbs to capture *L. monocytogenes* from food matrices was examined. Ready-to-eat soft cheese made from goat milk and hotdogs were purchased from local grocery stores (West Lafayette, IN). The procedures to contaminate artificially the food samples were modified from Kim and Bhunia (44). Briefly, 10 g of each sample was weighed individually and inoculated with approximately 4 × 10^2 CFU.g^{-1} of *L. monocytogenes*

and *L. innocua* (40 CFU/g) and then held for 15 min at 25^{o}C to allow intimate association of the bacteria with the food matrices. The samples were placed in stomacher bag containing a filter layer inside (Whirl-Pak; Nasco, Fort Atkinson, USA) and 90 mL of FB or LEB was added to each bag, blended for 2 min in a stomacher and incubated at 37^{o}C for 18 h. Uninoculated food samples were processed as above and served as negative controls. 10 mL of each enriched culture was placed into a 15 mL tube, centrifuged (7000 *g*, 10 min) and washed twice with PBST, and resuspended in 10 mL of PBST. Samples were diluted 10-fold in PBS, and IMS was performed using 200 µL of the inoculated sample. The IMS procedures were carried out exactly as described above. The precise levels of inoculums and growth after enrichment were enumerated on BHI agar after 24 h and MOX agar after 48 h at 37^{o}C. Bead captured bacteria were further tested by using fiber optic sensor, light scattering sensor and by qPCR (see below).

Fiber Optic Sensor

Optical waveguides (fibers) were prepared according to the method described by Ohk et al. (67) with slight modifications. Briefly, polystyrene waveguides (Research International) were pre-cleaned by immersing in 50% isopropyl alcohol and followed by sonication for 280 sec at a setting of 20W using a ultrasonic bath (Jeken, CD-4800). Waveguides were then placed inside pipette tips (200 µL maximum capacity tips) with the dispensing end sealed containing 100 µg.ml^{-1} of streptavidin (NeutrAvidin, Pierce) for 2 h at 4^{o}C. Streptavidin-coated waveguides were then blocked with SuperBlock blocking buffer (Pierce) for 1 h and incubated overnight at 4^{o}C with each of biotinylated-MAbs (200 µg.ml^{-1}). The waveguides were rinsed gently with PBS-Triton (PBS containing 0.05% Triton X-100) and then reacted with biotinylated-BSA (100 µg.ml^{-1}; Pierce) for 1 h at RT to block unbound streptavidin sites.

Selectivity and sensitivity analysis of fiber optic sensor

Fresh cultures of *Listeria* species were grown in TSB-YE, LEB or FB, and other non-*Listeria* bacteria (table 1) were grown in TSB-YE or MRS. After harvesting by centrifugation, bacterial pellets were washed three times with PBS and used with the fibers. Cultures of *L. monocytogenes* 4b and *L. ivanovii* were serially diluted to adjust for concentrations ranging from 1 × 10^{8} to 1 × 10^{2} CFU.mL^{-1}. Other bacterial

cultures were used exclusively at a concentration of 1 × 10^8 CFU.mL^{-1}. The fibers pre-coated with capture antibody were immersed in 100 µL of bacterial suspensions and incubated for 2 h at RT. Following gentle washing with PBS, the fibers were allowed to react with a Cy5-labeled anti-InlA antibody for 2 h at 4^{o}C, and fibers were washed again with PBS-Triton before acquiring the signals by using Analyte 2000 Fiber Optic Fluorometer (Research International Co., Monroe, WA, USA). The fluorescence intensity signals were recorded for each fiber for 30 sec. For each treatment, 3-5 waveguides were used and average values ± standard deviation for each experiment was presented.

Confirmation of captured bacteria by Optical Light Scattering Sensor

An automated light-scattering sensor, BARDOT (BActerial Rapid Detection using Optical light-scattering Technology: Advanced Bioimaging Systems, LLC, West Lafayette, IN, USA), was used to identify colonies of *Listeria* captured by IMS (mentioned above) on BHI or MOX agar plates (2, 47). The system collects scatter images of bacterial colonies (diameter, 1.3 ± 0.2 mm) through a diode laser (635 nm) in a noninvasive manner and the bacteria are identified by comparing the scatter images with images stored in the database library (2). Before conducting the food sample testing experiment, initial experiments were done to determine the capture rate of IMS for *L. monocytogenes* and *L. innocua* present at 10^6 CFU.mL^{-1} in a mixture in PBS, followed by BARDOT analysis.

Quantitative PCR

PMB captured bacteria were also analyzed by qPCR. To avoid PCR inhibitors, primarily the salts from the PBS washing buffer after IMS, the DNA was purified from captured bacteria using the DNeasy blood and tissue kit (Qiagen, Valencia, CA) following instructions for DNA isolation of Gram-positive bacteria. Briefly, 100 µL of the PMB-bacteria complexes were resuspended directly in 180 µL enzymatic lysis buffer (20 mM Tris-HCl pH 8.0; 2 mM sodium EDTA; 1.2% Triton X-100; 20 mg/mL Lysozyme) and incubated for 30 min at 37^{o}C; then the steps described in the Instruction Manual (Qiagen) were followed. PMBs were removed from the solutions by using MPC-S (Dynal) and the supernatant was pipetted onto the columns. DNA was eluted in 100 µL of elution buffer and used for real time quantitative PCR assay (qPCR).

Primers specific for *hly*A (hlyA-For: 5'-TGCAAGTCCTAA GACGCCA-3' and hlyA-Rev: 5'-CACTGCATCTCCGTGGTATACTAA-3') of *L. monocytogenes* were used for detection (64). Primers for 16s (Lis-16s-For 5'-CACGTGGGCAACCTGCCTGT-3' and Lis-16s-Rev 5'-CTAATGCACCGCGGGCCCAT-3') were used as internal control. The qPCR was performed using Power SYBR green master mix (Applied Biosystems; Foster City, CA, USA) with 5 µL of DNA template in a 20 µL total of reaction, ran in triplicate. The PCR amplification were carried out in a StepOnePlus Real-Time PCR system (Applied Biosystems) as follows: denaturation, one cycle of 95^{o}C for 10 min, followed by 40 cycles of 95^{o}C for 20 sec, 58^{o}C for 1 min and 95^{o}C for 1 min for the dissociation curve. To construct the standard curves, DNA of *L. monocytogenes* F4244 was quantified and a serial dilution was prepared to give a concentration curve. In all qPCR assays the DNA template of *L. monocytogenes* and *L. innocua* were used as internal controls. For unknown samples, Ct values were compared with the standard curves to obtain the number of cells and efficiency of PCR. The number of the *hly* gene copies were estimated as described previously (73), which is based on the molecular weight of the genome of *L. monocytogenes* where 1 ng of DNA is estimated to have 3.1 × 10^5 copies of the entire genome, i.e., 3.1 × 10^5 cfu. The estimated cell number was calculate as described in Yang et al. (86), the copy number of the *hly* gene was calculated by multiplying the copy number per PCR by a factor of 20, as the DNA isolation was performed from a volume of 100 µL and 5 µL out of this were subjected to real-time quantitative PCR.

Statistical analysis

Data are expressed as mean ± SD from at least three independent experiments run in duplicate samples unless noted otherwise. Mean values were compared by ANOVA using GraphPad Prism version 5.0 program (GraphPad Software, USA) and the differences in mean values were compared using Tukey's multiple comparison test at $P < 0.05$.

RESULTS

MAb production and characterization

Mice were immunized with whole cells of *L. monocytogenes* and protein rInlA, and the sera were screened for reactivity with both live *L. monocytogenes* cells and rInlA. The mouse with the highest serum titer was selected for the cell fusion aiming to obtain hybridoma against the native InlA of *L. monocytogenes*. From initial screening of the hybridomas secreting antibodies by indirect ELISA, we selected 11 stable hybridomas, where 7 (2F2; 2A2; 3B3; 3B7; 4E8; 2D12; 4E4) reacted with both rInlA and *L. monocytogenes*, and 4 (4E5; 4C1; 2A12; 3F8) reacted to *L. monocytogenes*, *L. innocua and L. seeligeri*. Furthermore, MAbs that showed cross-reactions in the initial screening with other non-*Listeria* bacteria such as *Salmonella* or *E. coli* were not selected for further characterizations. From all clones, only anti-InlA MAb-2D12, MAb-3B7 and MAb-4E4, and anti-*Listeria* MAb-2A12 and MAb-3F8 were chosen for further characterization. Additional analysis of anti-*Listeria* MAb-2A12 revealed that besides *Listeria*, it also showed a weak reaction with *Bacillus cereus*, thus this MAb was not selected for further characterization. Immunoglobulin subtypes of each antibody were determined to be as follows; MAb-2D12 and MAb-3B7 are IgG2a, MAb-4E4 is IgG1, and the MAb-3F8 is IgM.

Characterization of MAbs by ELISA and Western blot

All MAbs were tested in an indirect ELISA against rInlA protein, or cells of *L. monocytogenes* serotypes 4b, 4a, 1/2a, 1/2b, *L. innocua*, *L. seeligeri*, and other bacteria (Fig. 1). Among the anti-InlA antibodies, MAbs-2D12 and 3B7 had strong reactions (A_{450} = 1.0 or higher) with *L. monocytogenes* 4b cells, while the MAb-4E4 had slightly lower reaction values (A_{450} = 0.75 - 0.9). *Listeria* genus-specific MAb-3F8 gave strong ELISA values ($A_{450,}$ = 0.8 - 1.5) when tested against all *Listeria* species without any significant cross-reactions with other bacterial species (Fig. 1).

In the Western blot assay, all anti-InlA MAbs reacted with a protein band from *L. monocytogenes* comparable to the native InlA (~80 kDa). Further Western blot analysis of protein preparations from all eight *Listeria* species, along with all 13 serotypes of *L. monocytogenes*, showed that MAb-2D12 reacts with all serotypes of *L. monocytogenes* and *L. ivanovii* and did not react with proteins from other *Listeria* spp. (Fig. 2). Interestingly, MAb-2D12 showed relatively weak reaction with two

strains of serotype 1/2c (ATCC 19112 and ATCC 7644), and reacted strongly with InlA protein variant (truncated form) from the serotype 3c (SLCC 2479) (Fig. 3).

The MAb-3F8 showed a strong reaction with a single protein band of apparent molecular mass of approximately 30 kDa (p30) (Fig. 4). This MAb showed strong reactions with protein preparations from all 13 *L. monocytogenes* serotypes, as well as with all species from the genus *Listeria*, including the two new species *L. marthii* and *L. rocourtiae* (data not shown). Based on the above results, InlA specific MAb 2D12 and p30 specific MAb-3F8 were used in subsequent experiments.

Immunofluorescence Microscopy

Immunofluorescence microscopy also revealed specific binding of anti-InlA antibody (MAb-2D12) to the surface of cells of *L. monocytogenes* 4b, 1/2a, 1/2b, 4d (Fig. 5). This antibody did not show any binding to the cells of *L. innocua* used as negative control in the immunofluorescence assay (Fig. 5).

Bacterial Capture using PMB Coated with Antibodies

Application of antibodies to capture target bacteria on paramagnetic beads (PMB) was examined by using two different beads, Dynabeads® streptavidin M-280 (PMB280) and MyOne™ streptavidin T1 (PMBT1), coated with MAbs-2D12 or 3F8 in the capture of pathogenic *L. monocytogenes*, *L. ivanovii* and two non-pathogenic strains of *L. innocua* and *L. marthii*. The results showed that PMBT1 had higher capture rate than the PMB280 (Fig. 6, 7, 8 and 9). PMBT1 coated MAb-2D12 captured 49.2% of the *L. monocytogenes* cells compared to 33.7% for PMB280 coated MAb-2D12, from an initial cell concentration of 1×10^5 CFU.mL^{-1}. Furthermore, as shown in the Fig. 9, the PMBT1 coated 3F8 captured less (16.6%) *L. monocytogenes* than PMBT1 coated MAb-2D12 (49.2%), from a cell suspension containing 10^5 CFU.mL^{-1}. The limit of detection for PMBT1-MAb-2D12 was estimated to be about 1×10^3 CFU.ml^{-1} for *L. monocytogenes* and 2×10^4 CFU.ml^{-1} for *L. ivanovii*.

After these preliminary experiments, all further IMS experiments were performed using PMBT1. The specificity of PMBT1 coated with both MAbs was evaluated using cell suspensions (10^6 CFU.mL^{-1}) of different *Listeria* species grown in two selective enrichment broths, LEB and FB. PMBT1-MAb-2D12 showed significantly higher ($P < 0.05$) capture of *L. monocytogenes* and *L. ivanovii* than other

Listeria species, and capture efficiency was similar for *Listeria* when grown on LEB or FB. Capture efficiency for PMBT1-MAb-2D12 was very good for all pathogenic *L. monocytogenes* serotypes tested including 4b (36.9%), 1/2a (27%) and 1/2b (28%), and a strain of *L. ivanovii* (21.6%) (Fig. 10). On the other hand, as expected, PMBT1-MAb-3F8 did not show significant difference in the capture rate among all *Listeria* species tested, irrespective of the enrichment broth used. However, capture of *L. monocytogenes* by PMBT1-MAb-3F8 was relatively lower compared to the capture by PMBT1-MAb-2D12 (Fig. 11). Furthermore, when the performance of PMBT1-MAb-3F8 was compared with the commercial Dynabeads anti-*Listeria*, both had similar capture efficiency for all bacteria tested (Fig. 12). Even though Dynabeads anti-*Listeria* captured more *L. monocytogenes* than PMBT1-MAb-3F8, it was not statistically significant ($P < 0.05$) (Fig. 12).

The capacity of all PMBs to capture *L. monocytogenes* in a co-culture with *L. innocua* was also determined (Fig. 13). The bacteria were grown in FB and mixed 1:1 (100 µL) in PBS to achieve concentrations of ~1 x 10^5 CFU.mL^{-1} for each one, and the efficiency of the capture was determined by plating and BARDOT light-scattering detection. The PMBT1-MAb-2D12 captured about 10^4 CFU.mL^{-1} (9.5 %) of bacteria, out of which most colonies (~80%) were confirmed to be *L. monocytogenes* by BARDOT (Fig. 24). When PMBT1-MAb-3F8 was used, it captured total of ~2.1 x 10^3 cells (2.75%) and approximately 50% of which was confirmed to be *L. monocytogenes*. Dynabeads anti-*Listeria* captured about 2.9 x 10^3 CFU.mL^{-1} (3.3%) and 40% of which were *L. monocytogenes*.

Experiments were also conducted to investigate the capture of bacteria from inoculated food matrices. Hotdogs were inoculated with 10 CFU.g^{-1} of each *L. monocytogenes* 4b and *L. innocua* either together, as a co-culture, or separately, as a monoculture. PMBT1-MAb-2D12 showed higher capture of *L. monocytogenes* (12%) than *L. innocua* (1%) in the monocultures, but in the co-culture experiment the total of bacterial capture dropped to 3.5%. PMBT1-MAb-3F8 captured 3.7% of the *L. monocytogenes* cells in the monoculture experiment while the Dynabeads anti-*Listeria* captured only 1.8% (Fig. 14). Also, the Dynabeads captured a high percentage of *L. innocua* (4.2%) cells compared to *L. monocytogenes* (1.8%). Collectively these data showed that PMBT1-MAb-2D12 captured 10-fold more *L. monocytogenes* cells than *L. innocua*, while PMBT1-MAb-3F8 captured 1.5-fold more

L. monocytogenes than *L. innocua* and Dynabeads anti-*Listeria* had the highest capture rate for *L. innocua* from hotdog samples.

The capture of *Listeria* was also investigated with another food matrix, a soft cheese made from goat milk, in a co-culture experiment. Cheese samples were inoculated with *L. monocytogenes* 4b (270 CFU.10 g^{-1}) and *L. innocua* (315 CFU.10 g^{-1}) and enriched in FB for 18 h and the total count reached ~1.7 x 10^8 CFU.mL^{-1}. The bacterial capture rate using PMBT1-MAB-2D12 was 7.41 ± 0.69 x 10^6 CFU.mL^{-1} (4.67 ± 0.46%) as determined on BHI plates and 1.84 ± 1.44 x 10^6 CFU.mL^{-1} (1.27 ± 1.14%) on MOX agar plates. PMBT1-MAb-3F8 and Dynabeads anti-*Listeria* showed similar capture rate, with bacterial counts being higher on BHI than on MOX plates (Fig. 15).

Specificity and Sensitivity of Fiber Optic Sensor

We tested the specificity and sensitivity of the fiber optic sensor using MAb-2D12 or MAb-3F8 as capture antibody and bacterial cell concentrations of ~10^8 CFU.mL^{-1}. The Cy5-labeled MAb-2D12 was used as detection (reporter) antibody in all experiments. With MAb-2D12 as capture molecule, the sensor generated strong signal against *L. monocytogenes* and *L. ivanovii*, with the maximum peak signal of 22561 pA. On the other hand, all non-pathogenic *Listeria* species had a maximum signal of 3000 - 4200 pA and other non-*Listeria* bacteria such as *E. coli* O157:H7, *Staphylococcus aureus*, and *Samonella* Typhimurium, generated signals of about 2500 pA. Similar results were obtained with the MAb-3F8 sensor (Fig. 16 and 17). When these sensors were used with mixed cultures containing *L. monocytogenes*, *L. innocua* and *E. coli* O157:H7, the signal for MAb-2D12 was 15440 ± 1764 pA, and for MAb-3F8 was 8440 ± 569 pA, which were significantly ($P < 0.05$) higher than the values obtained for *L. innocua* (2725 ± 2227 pA) or *E. coli* (1589 ± 662 pA) alone (Fig. 16). The background control (buffer only) values ranged from 504 - 650 pA. These data indicate that both fiber optic sensor configurations, 2D12 - 2D12 or 3F8 - 2D12, are highly specific for pathogenic *Listeria*.

The limit of detection (LOD) for these sensors was also evaluated using pure culture of *L. monocytogenes* and *L. ivanovii* serially diluted in PBS (Fig. 18 and 19). Using MAb-2D12 as capture molecule, the signals increased proportionately as the bacterial concentration increased until a cell concentration of 1 x 10^6 CFU.mL^{-1}, which gave the maximum fluorescence signal (22561 pA) that the Analyte 2000 fluorometer

can acquire. The lowest cell concentration that generated signals that were significantly different from other bacteria were about 3 x 10^2 CFU.mL^{-1} for *L. monocytogenes* (6252 ± 1213 pA) and at 1 x 10^3 CFU.mL^{-1} for *L. ivanovii* (8657 ± 4019 pA), and thus considered to be the detection limit for this sensor using MAb-2D12. When MAb-3F8 was used as capture antibody, the LOD for *L. monocytogenes* (16156 ± 6382 pA) and *L. ivanovii* (13882 ± 5250 pA) was about 1 x 10^5 CFU.mL^{-1} (Fig. 19).

IMS-based concentration followed by fiber optic sensor-based detection of *L. monocytogenes*

A two-step method (IMS followed by fiber optic sensor) was used to detect *L. monocytogenes* rapidly from buffer or food. In buffer solution, the PMBT1-2D12 and PMBT1-3F8 captured bacteria were released from the beads through vigorous vortexing and 100 µL of the cell suspension was directly tested with the fibers coated with MAbs 2D12 or 3F8. Cells released from PMBT1-2D12 or PMBT1-3F8 and captured by MAb-2D12 fiber optic sensor yielded signals of 18230 ± 1837 pA and 13280 ± 2885 pA, respectively, whereas MAb-3F8 fiber optic sensor gave signals of 11225 ± 2860 pA (PMBT1-2D12) and 8890 ± 1901 pA (PMBT1-3F8) (Fig. 20).

For food sample testing, first IMS was applied to enriched goat cheese samples that were inoculated with *L. monocytogenes* and *L. innocua* in a co-culture environment and followed by capture with fiber coated with MAb-2D12. The PMBT1-2D12 captured bacteria gave a fiber optic signal of 13026 ± 2713 pA, while PMBT1-3F8 captured cells emitted a slightly lower fiber optic signal of 12619 ± 4554 pA. Although Dynabeads anti-*Listeria* captured bacteria had the lowest fiber optic signals 7273 ± 1007 pA, there was no statistically significant difference ($P < 0.05$) than the PMBT1-2D12 and PMBT1-3F8 used for capture (Fig. 21). Likewise, from hotdog samples, PMBT1-2D12 captured bacteria generated fiber optic signal from co-culture food was 8376 ± 2448 pA while, from *L. monocytogenes* inoculated food was 8552 ± 4363 pA and from *L. innocua* inoculated food was 2549 ± 1358 pA. These data indicate that the two step method of IMS and fiber optic sensor can be used for detection of *L. monocytogenes* from food samples in about 21 h, even in presence of other *Listeria* or other bacteria in food.

Real-time quantitative PCR

The bacterial capture rate using PMBT1-2D12, PMBT1-3F8 and Dynabeads anti-Listeria from goat cheese and hotdogs artificially contaminated with *L. monocytogenes* and *L. innocua* were also quantified by real-time qPCR using *hly*A primers. Capture rate for *L. monocytogenes* had a similar trend for all biomolecules as visualized in the plating method and BARDOT (Fig. 24). When IMS was applied in the cheese samples followed by qPCR, PMBT1-2D12 and Dynabeads anti-*Listeria* showed 4 times higher cell counts than those derived from plate count, and a 10-fold increase in the capture rate with PMBT1-3F8 (Fig. 23). While in hotdog samples, the combination of PMBT1-3F8 based capture and real-time PCR showed 2-fold more capture than cells enumerated by plate count method, whereas PMBT1-2D12 and Dynabeads anti-*Listeria* demonstrated 3 times higher capture than the plating method (Fig. 14).

DISCUSSION

L. monocytogenes has been implicated in a large number of food outbreaks and several product recalls (18, 68). One of the drawbacks of the conventional culture technique is the time-consuming required to detect this pathogenic bacteria in food, which take usually 5 to 10 days. Therefore, rapid and sensitive methods are required to detect this pathogen in foods. A recovery of low numbers of pathogenic *Listeria* cells from a complex food matrix is a huge challenge for any sensitive detection method. For that reason, immunomagnetic separation using paramagnetic beads (PMB) have been extensively used as a potent tool to separate and concentrate the target bacteria from food samples (7). In many cases, separation of target organisms by IMS following pre-enrichment from food is combined with culture and/or PCR-based detection (48). Fiber-optic biosensor has been also widely used to detect food pathogens, but it generally depends on sample preparation and isolation of the target microorganism from the food matrices previously the application in the sandwich system (31, 67). In the present work, we have produced MAbs against the protein InlA of pathogenic *Listeria*, as well as an IgM-MAb (3F8) that recognize specifically the genus *Listeria*. Further, we investigated the use of paramagnetic beads coated with MAb-2D12 (InlA) and MAb-3F8 (p30) in the capture of *L. monocytogenes* from foods and the rapid detection of this pathogen on fiber-optic

immunosensor for the specific detection of *L. monocytogenes*. To the best of our knowledge, this is the first demonstration of these two approaches together.

The specific detection of *L. monocytogenes* using immunological methods relies fundamentally on the availability of highly specific antibodies with sufficient affinity for bacterial surface antigens (7). Moreover, the protein target for the production of capture MAbs should be uniformly distributed on the surface of the organism, and its location would be easy to the accession of the antibody (81). The InlA protein is one of the best characterized proteins of *L. monocytogenes* that fills all these requirements, being an extracellular protein and highly specific of *L. monocytogenes* (12). Our first and main objective was to produce MAbs against InlA. For that, we used as immunogen whole cells of *L. monocytogenes* and using rInlA we aimed to select and produce B-lymphocytes secreting antibodies with more affinity against the native InlA. With this approach, we could easier select hybridomas secreting MAbs against InlA and also one MAb (3F8) that recognized the genus *Listeria*.

The InlA MAb-2D12 here produced reacted with all *L. monocytogenes* serotypes, differently of the most MAbs documented by other groups that failed in recognize all 13 serotypes (36, 53). Although the serotype 1/2c has shown less reaction in the Western blot (WB), it still can be detected by the MAb-2D12. The fact of 1/2c strain demonstrate less expression of InlA could give an explanation why few sporadic cases of listeriosis is implicated by this serotype (15, 39, 62). Our data also has shown that MAb-2D12 reacted strongly in WB with 3c strain that clearly revealed as a truncated InlA. It is already well-known that some *L. monocytogenes* strains can produce a mutated InlA, and due to it, these strains significantly reduce the ability to invade human epithelial cells or cause systemic infection in animal models (19, 63). In the same way, Jacquet et al. (39) has shown that none of 25 strains belonging to serotype 1/2c evaluated, expressed a functionally full-length InlA. This also could explain the low recognizing by the MAb-2D12 against 1/2c strains tested.

Taking the advantage that we used as immunogen whole cells of *L. monocytogenes*, we also had a chance to select the MAb-3F8 against the genus *Listeria*. The IgM MAb-3F8 shown extremely specific for all eight *Listeria* species, differently of most MAbs until now documented. It makes this MAb a powerful probe to trail *listerial* contamination in many food segments. Here we have shown that the

MAb-3F8 recognizes an exquisite and unique epitope of approximately 30 kDa in the *Listeria* species.

Separation of target organisms following pre-enrichment using IMS is considerably quicker than using selective enrichment and may assist in the recovery of injured cells (7). In this study, we performed an initial magnetic-separation with two different commercial PMBs, and demonstrated that MyOne streptavidin T1 (PMBT1) coated MAbs 2D12 and 3F8 had a better capture when compared with Dynabeads streptavidin M-280 (PMB280). The same trend was observed by Foddai et al., (28) in the capture of *Mycobacterium avium*, in their work they evaluated six different magnetic beads, when compared the same two streptavidin-coated beads here used, PMBT1 had better capture efficiency over PMB280, but PMBT1 still showed a capture considered low (< 10%) for this microrganism. Our results has shown an efficiency capture of approximately 50% using PMBT1 coated MAb-2D12 (InlA) and 16% when used MAb-3F8 (p30). Paoli et al. (71) using PMB280 coated scFv antibodies had a maximum capture of 19% of *L. monocytogenes*. In comparison when we used PMB280 our efficiency recovery for *L. monocytogenes* was 33.7% for MAb-2D12 and 8.5% using PMB280 coated MAb-3F8.

Afterwards, we tested PMBs in a co-culture of *L. monocytogenes* and *L. innocua*. Taking in consideration that food contaminated with *L. monocytogenes* generally contain at least one other species of *Listeria*, as well as the background competitive microflora (45, 67). Moreover, *L. monocytogenes* is a slow grower and poor competitor, and lower cell numbers are expected in food samples (29). Thus, in a mixed population, *L. monocytogenes* can be outgrown by the other species of *Listeria* during enrichment (16). In fact, it was visualized in our experiments, but IMS system used here has shown efficient and even in the presence of many foods debris and other competitor bacteria could capture *L. monocytogenes* cells. PMBT1-MAb-2D12 has shown to overcome the fact that less *L. monocytogenes* is present in the sample and captured more *L. monocytogenes* than *L. innocua*. However, it was not observed with the MAb-3F8 and Dynabeads, where seems to capture more *L. innocua* cells.

It is worthwhile to note that *L. ivanovii* may poses as an opportunistic pathogen and it has been recently associated to gastroenteritis and bacteremia in humans (34, 79), therefore, methods to detect this pathogen also would be necessary. The MAb-2D12 here presented was able to recognize *L. ivanovii*, and due to it, we conducted

others experiments such as IMS and fiber-optic sensors using this pathogenic bacteria. However, Hearty et al. (35) reported the MAb2B3 against InlA that was unable to identify *L. ivanovii*; it may due to this MAb do not recognize an epitope in this bacteria similar finding in *L. monocytogenes* strains.

In order to confirm the results from immunomagnetic separation, BARDOT and qPCR were also used. BARDOT is capable of rapid detection and identification of several bacterial colonies with a high degree of precision on a single agar plate because each species has distinctive fingerprint (2). It was possible to differentiate *L. monocytogenes* and *L. innocua* on BHI plates and MOX plates because their scatter patterns were unique. Moreover, using BARDOT, it was possible to access the number of *L. monocytogenes* captured by PMBs coated MAbs. Light scattering images were also important because we could verify that after release the captured cells we had a satisfactory number of bacteria, confirming this way that it would work at fiber-optic biosensor. Nonetheless, qPCR here carried out also supported the IMS capture and the utilization on the fiber-optic assays. As we observed by counting plate, BARDOT and qPCR, the captured *L. monocytogenes* from artificially contaminated food were ~10^5 - 10^6 CFU.mL^{-1}. The sensibility of fiber-sensor verified in the present study was 10^3 CFU.mL^{-1}, thus easily detected by this system.

The qPCR assay permits specific identification and quantification of the bacteria targeted, eliminating a step such as gel electrophoresis of the conventional PCR (48). However, the quality of the DNA is essential to avoid poor and unreliable amplifications in the PCR. For real-time qPCR used in this study, four different DNA isolation methods after IMS were tested. The bacteria captured by PMBs were boiled for 15 min in either PBST, TE buffer, or lysis solution (0,125% of SDS; 0,05 M NaOH) and the supernatant was directly used for PCR amplification (48, 60). However, none of these DNA extraction methods yielded satisfactory results (data not shown). We attributed the low PCR efficiency using these methods to the high concentration of salt from the PBS washing steps after IMS. Therefore, we decided to use the DNeasy blood and tissue kit (Qiagen), which yielded satisfactory PCR results. Although qPCR with cells captured by all PMBs showed 2-fold more recovery, problems inherent with expensive materials, DNA purification and unsatisfactory performance can lead to a lower demand for real-time PCR. Other studies have reported the advantages of using IMS to recover *L. monocytogenes* from food samples and use in the real-time PCR (23, 24, 86). Yang et al., (86) using IMS nanoparticles obtained a slightly better

capture and detection with real-time PCR (9%) compared to plate counts (6%) at ~10^7 CFU.mL^{-1} of *L. monocytogenes* in milk and also showed an increase of 133.4% for samples with 10^2 CFU.mL^{-1}. The qPCR assay can detect DNA from nonviable or viable but non-culturable *Listeria* cells that might not be otherwise detected by traditional plating method (24, 55), thus showing higher bacterial counts than the actual plate counts values.

In the fiber-optic biosensors experiments here carried out, we used the MAb-2D12 against InlA labeled with Cy5 as antibody reporter, this way our system was very specific for pathogenic *Listeria*, *L. monocytogenes*, as well as *L. ivanovii*. Fiber-optic biosensor using both MAbs-2D12 and -3F8 as capture antibody successfully detected *L. monocytogenes* when tested separately with pure cultures of different species and genus, and the mixture of *L. monocytogenes*, *L. innocua* and *E. coli*. When MAb-3F8 was used as capture antibody, although it is genus *Listeria*, due to the monoclonal antibody 2D12-InlA used as reporter, the signals were very specific to *L. monocytogenes* and also *L. ivanovii*, and no cross-reacts were visualized. Geng et al. (30) using MAb-C11E9 had also reaction with *L. innocua* strain, due this MAb also reacts with some *L. innocua* strains. Using a polyclonal antibody anti-*Listeria* for capture and an InlA aptamer as signal molecule in a fiber-optic system, Ohk et al. (67) had a specificity for *L. monocytogenes* with a sensitivity of 10^3 CFU.mL^{-1}. In our study was observed a limit of detection for *L. monocytogenes* of 3 x 10^2 CFU.mL^{-1} when used MAb-2D12 and 1 x 10^5 CFU.mL for MAb-3F8.

Many IMS assays reported depend on bacterial confirmation by other rapid method such as PCR assays. In the present study, once we established the IMS and fiber-optic immunosensor in separately assays, both systems were tested in sequence to rapid detection of *L. monocytogenes* in food samples. For this, following IMS with the PMB coated MAbs, the captured bacteria were released and directly applied on the waveguides coated MAb-2D12. Thus, the use of IMS followed by fiber-optic analysis proved to be an efficient approach to rapidly detect low numbers (40 cells/g) of *L. monocytogenes* cells from food samples in presence of other bacteria in less than 22 h. In summary, we have produced two antibodies that combined with powerful tools such as IMS and fiber-optic immunosensor-based provides an excellent method to a rapid and specific detection of *L. monocytogenes* in contaminated foods.

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TABLE

Table 1. Description of bacterial strains tested

Species	Serotype	Strain / Isolated	Source[a]
L. monocytogenes	1/2a	V7	FDA
L. monocytogenes	1/2b	F4260	CDC
L. monocytogenes	1/2c	7644	CDC
L. monocytogenes	4a	19114	ATCC
L. monocytogenes	4b	F4244	CDC
L. monocytogenes	4b	19115	ATCC
L. monocytogenes	4c	19116	ATCC
L. monocytogenes	4d	19117	ATCC
L. monocytogenes	4e	19118	ATCC
L. monocytogenes	4ab	Murray B	FDA
L. monocytogenes	3a	19113	ATCC
L. monocytogenes	3b	2540	ATCC
L. monocytogenes	3c	2479	SLCC
L. monocytogenes	7	2482	SLCC
L. monocytogenes	4b	ScottA	FDA
L. monocytogenes	1/2c	19112	ATCC
L. innocua	6a	11288	ATCC
L. innocua		F4248	CDC
L. innocua	6a	Li01	UFPel
L. welshimeri		35897	ATCC
L. seeligeri		3954	ATCC
L. seeligeri		Ls02	UFPel
L. ivanovii		19119	ATCC
L. ivanovii		SE98	USDA
L. grayii		19120	ATCC
L. marthii		BAA-1595	ATCC
L. rocourtiae		----	----
Salmonella enterica ser. Typhimurium		DUP-1167	MFM-Purdue
Salmonella enterica ser. Enteritidis		13076	ATCC
Bacillus subtilis		6633	ATCC
Bacillus thuringiensis		DUP - 6044	MFM-Purdue
Escherichia coli	O157:H7	EDL933	CDC
Lactococcus lactis		11454	MFM-Purdue
Enterococcus aerogenes		DUP-14591	MFM-Purdue
Lactobacillus paracasei		DUP-13076	MFM-Purdue
Klebsiella pneumonia		---	MFM-Purdue
Enterococcus faecalis		---	MFM-Purdue
Lactococcus lactis subsp. *lactis*		HK21	MFM-Purdue
Enterobacter cloacae		HK8	MFM-Purdue
Staphylococcus epidermidis		HK7	MFM-Purdue
Bacillus cereus		11778	ATCC
Staphylococcus aureus		13301	ATCC
Pseudomonas aeruginosa		10145	ATCC

[a] FDA: Food and Drug Administration, Washington, D.C.; CDC: Centers for Disease Control and Prevention, Atlanta, GA. ; ATCC: American Type Culture Collection, Rockville, MD.; SLCC: Special Listeria Culture Collection, Institute of Hygiene and Microbiology, Univ. of Würzburg, Germany; USDA: National Center for Agricultural Utilization Research, Peoria, Illinois, U.S.A.; MFM-Purdue: Molecular Food Microbiology Lab. Collection, Purdue.; UFPel: Laboratório de Microbiologia de Alimentos Collection, FAEM-UFPel.

FIGURES

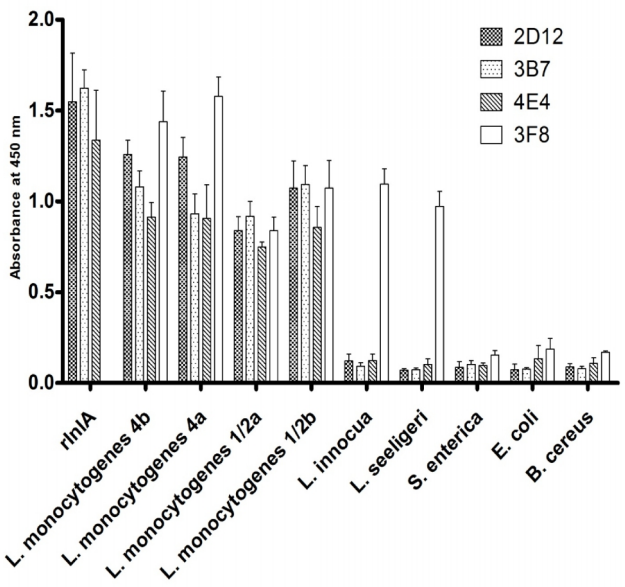

FIG. 1. Indirect ELISA of MAbs 2D12, 3B7, 4E4 and 3F8 with different bacterial strains and rInlA. MAb-3F8 was not tested with rInlA. Plates were coated with live bacteria for 16 h at 4°C. Values are average of three independent assays in duplicate. Data are shown as mean ± SD.

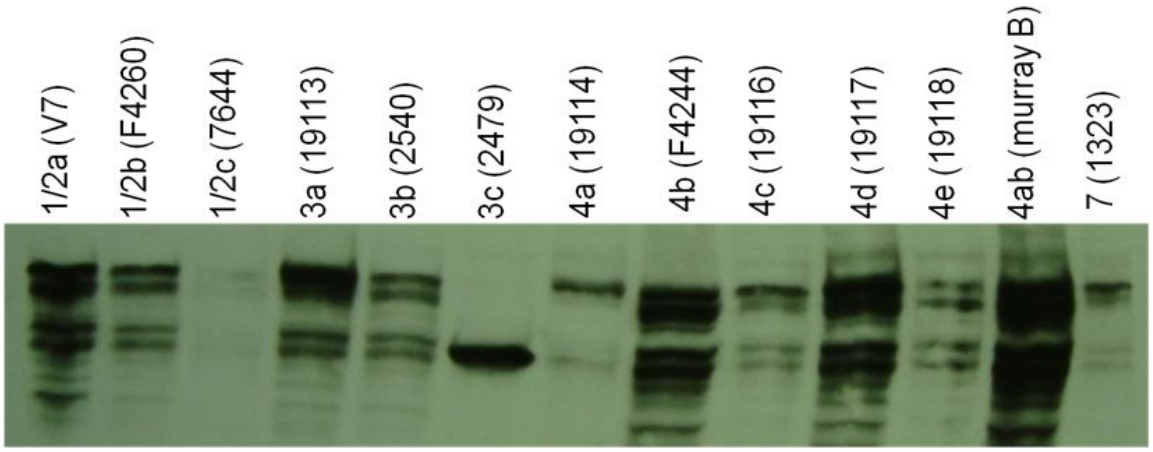

FIG. 2. InlA analysis from all 13 serotypes of *L. monocytogenes* strains by Western blot. Bacteria were grown in TSB-YE and cell wall extractions were separated in 10% SDS-PAGE, transferred to PVDF membranes and probed with MAb-2D12.

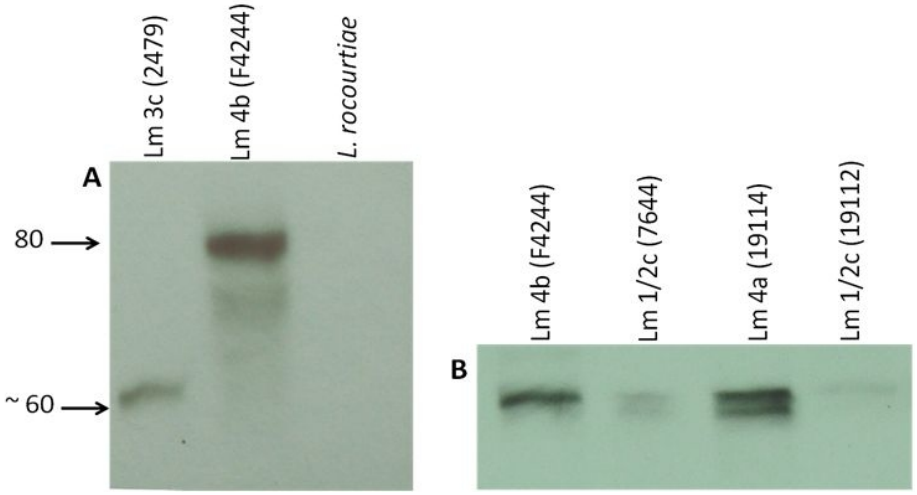

FIG. 3. Western blot analysis of InlA expression from *L. monocytogenes* strains (Lm) using MAb-2D12. Cell wall-associated protein extractions were separated in 10% SDS-PAGE, transferred to PVDF membranes. (A) Lm 3c (ATCC 2479) showed a truncated InlA, Lm 4b (F4244) and *L. rocourtiae* used as controls; (B) Lm 1/2c CDC 7644 and ATCC 19112 showed less expression of InlA.

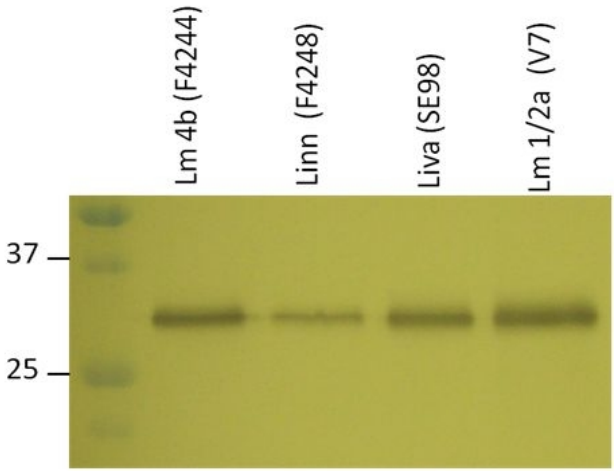

FIG. 4. Western blot analysis of *Listeria* spp. strains using MAb-3F8. Cell wall-associated protein extractions were separated in 15% SDS-PAGE and transferred to PVDF membranes. MAb-3F8 binds to a 30 kDa protein (p30) in the strains *L. monocytogenes* (Lm) 4b F4244 and 1/2a (V7), *L. innocua* F4248 (Linn), and *L. ivanovii* SE98 (Liva).

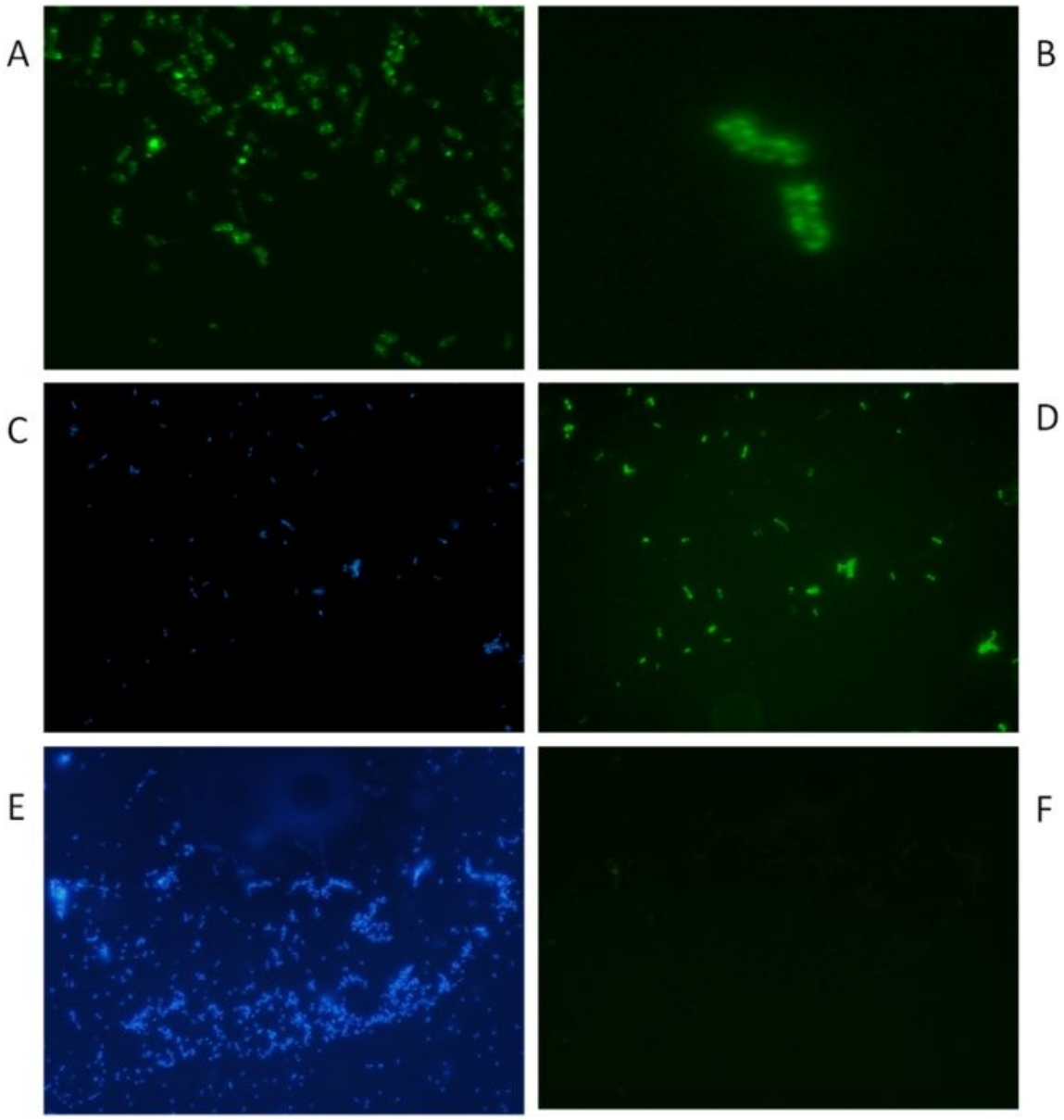

FIG. 5. Indirect immunofluorescence of *L. monocytogenes* and *L. innocua* using MAb-2D12 and staining with anti-mouse conjugated fluorescein isothiocyanate (FITC). Panels (A) *L. monocytogenes* 4b (F4244); (B) *L. monocytogenes* 4b (F4244); (C) *L. monocytogenes* 4d (ATCC 19117) DNA stained with Hoechst 33258; (D) *L. monocytogenes* 4d (ATCC 19117) stained with FITC; (E) *L. innocua* 6a (Li01) DNA stained with Hoechst 33258; (F) *L. innocua* 6a (Li01) not stained with FITC.

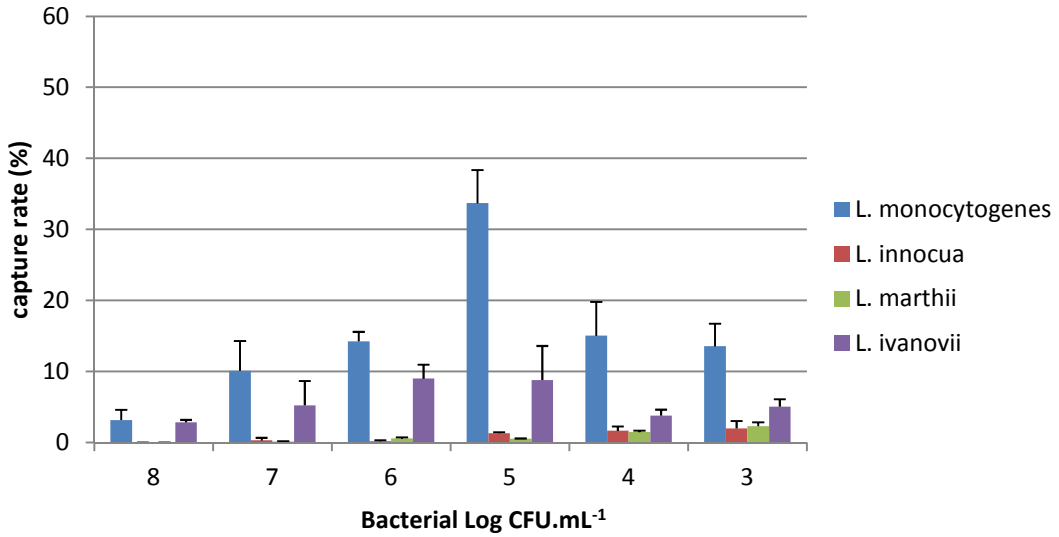

FIG. 6. Capture efficiency of Dynabeads® streptavidin M-280 coated with MAb-2D12 (InlA). Different *Listeria* species were grown in TSB-YE for 18 h at 37°C. The capture rate was compared by different initial concentrations. Values are average ± SD of three independent experiments.

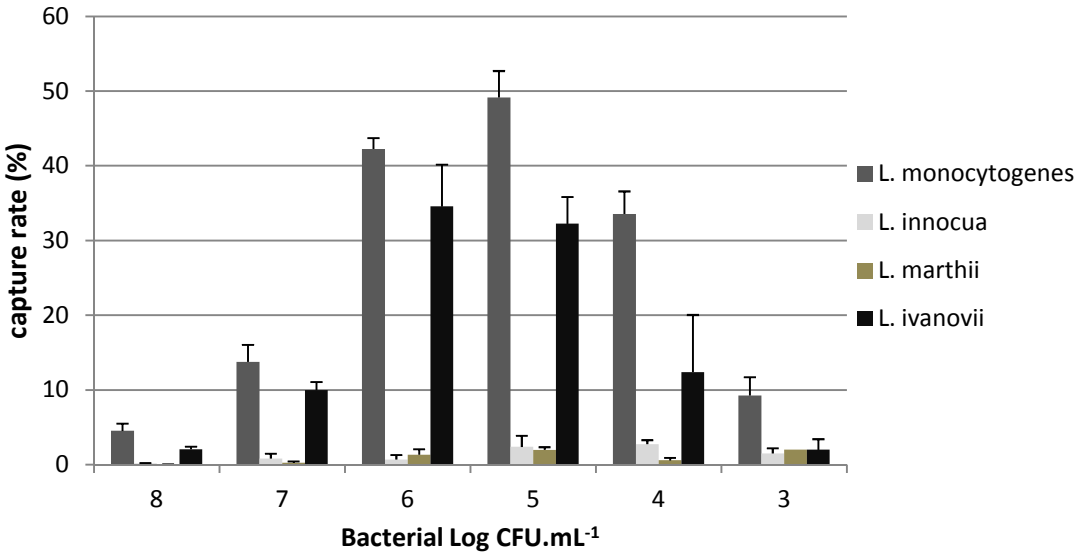

FIG. 7. Capture efficiency of Dynabeads® MyOne™ streptavidin T1 coated with MAb-2D12 (InlA). Different *Listeria* species were grown in TSB-YE for 18 h at 37°C. The capture rate was compared by different initial concentrations. Values are average ± SD of three independent experiments.

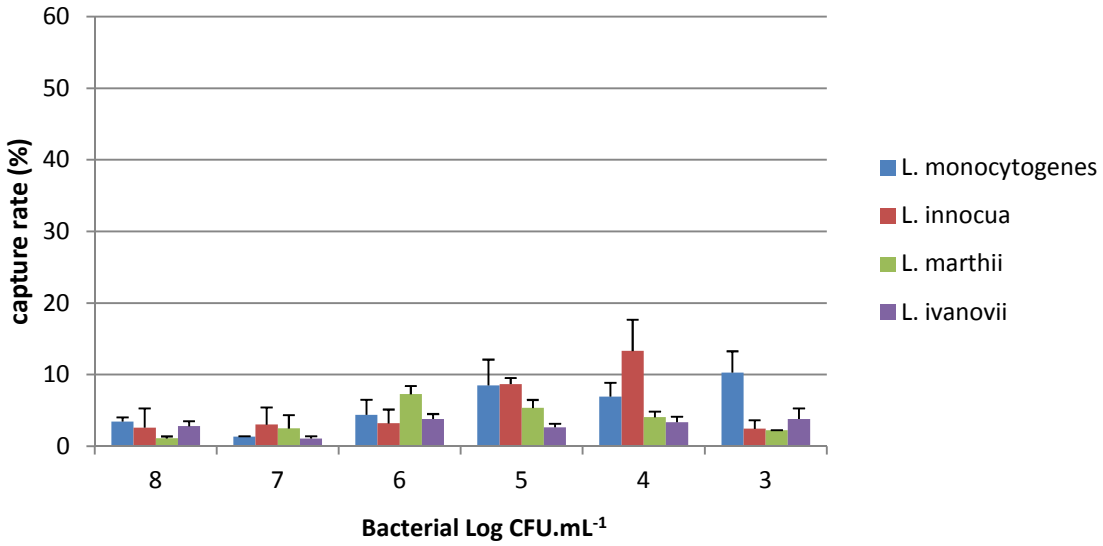

FIG. 8. Capture efficiency of Dynabeads® streptavidin M-280 coated with MAb-3F8 (p30). Different *Listeria* species were grown in TSB-YE for 18 h at 37°C. The capture rate was compared by different initial concentrations. Values are average ± SD of three independent experiments.

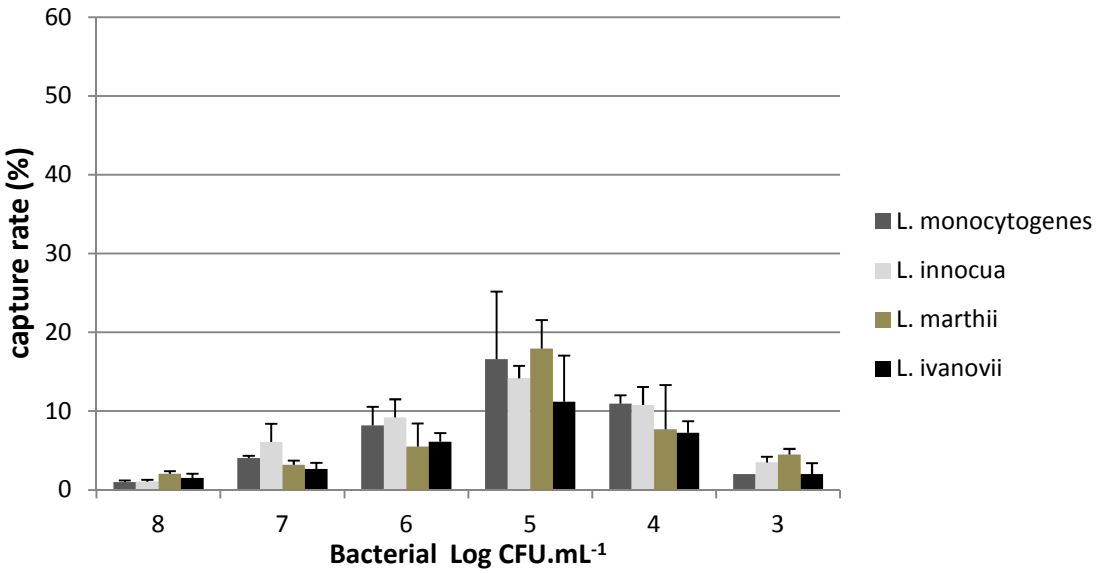

FIG. 9. Capture efficiency of Dynabeads® MyOne™ streptavidin T1 coated with MAb-3F8 (p30). Different *Listeria* species were grown in TSB-YE for 18 h at 37°C . The capture rate was compared by different initial concentrations. Values are average ± SD of three independent experiments.

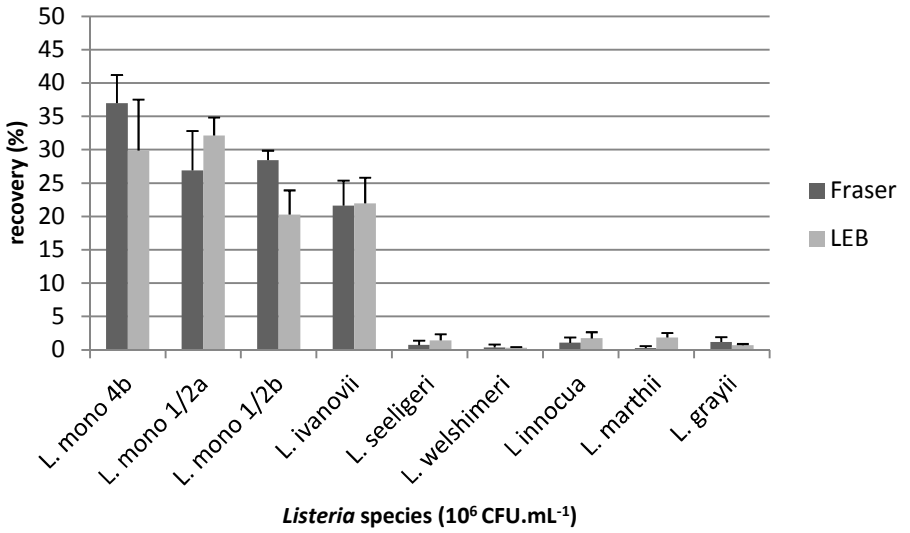

FIG. 10. Determination of specificity of capture using PMBT1 coated with MAb-2d12 (InlA). Different *Listeria* spp. were cultivated in Fraser broth or LEB, and the capture profile were compared. Initial bacterial concentration were aprox. 10^6 CFU.ml^{-1}. Values are average ± SD of three independent experiments.

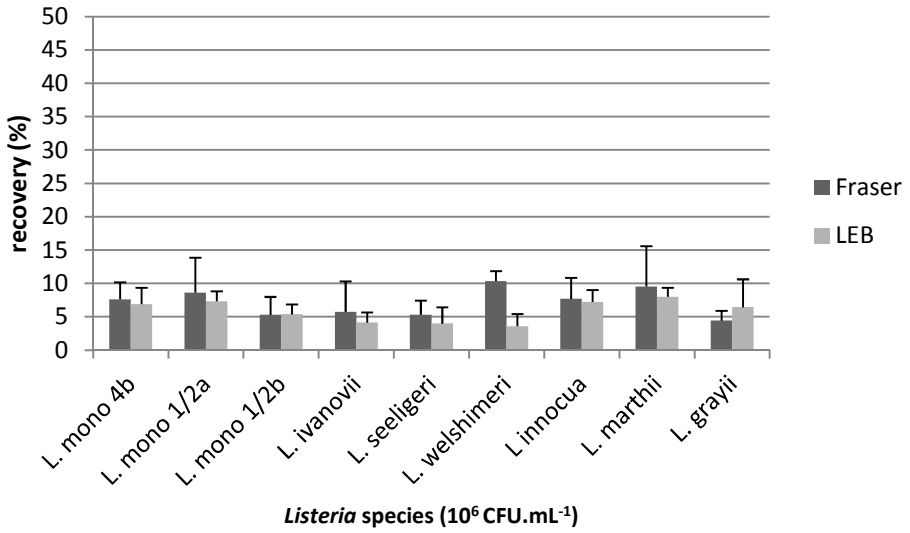

FIG. 11. Determination of specificity of capture using PMBT1 coated with MAb-3F8 (p30). Different *Listeria* spp. were cultivated in Fraser broth or LEB, and the capture profile were compared. Initial bacterial concentration were aprox. 10^6 CFU.ml^{-1}. Values are average of three separated experiments.

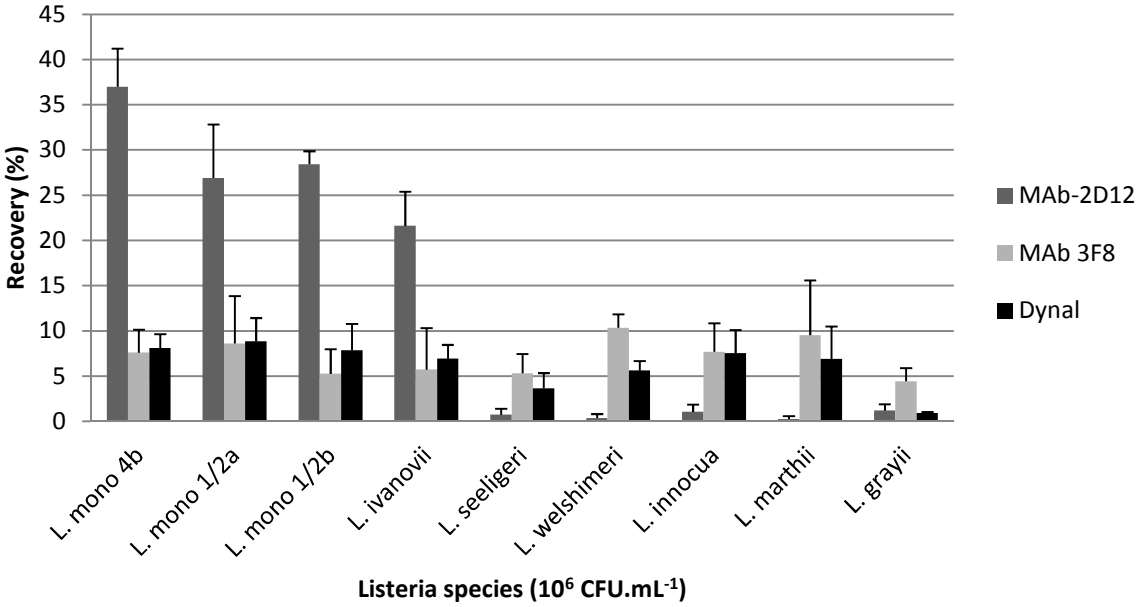

FIG. 12. Specificity of capture with PMBT1 coated with MAb-2d12 (InlA); MAb-3F8 (p30); and the control Dynabeads anti-*Listeria* (Dynal). Different *Listeria* spp. were cultivated in Fraser broth and the capture profile among the three bioreceptors were compared. Initial bacterial concentration were aprox. 10^6 CFU.ml^{-1}. Values are average of three separated experiments.

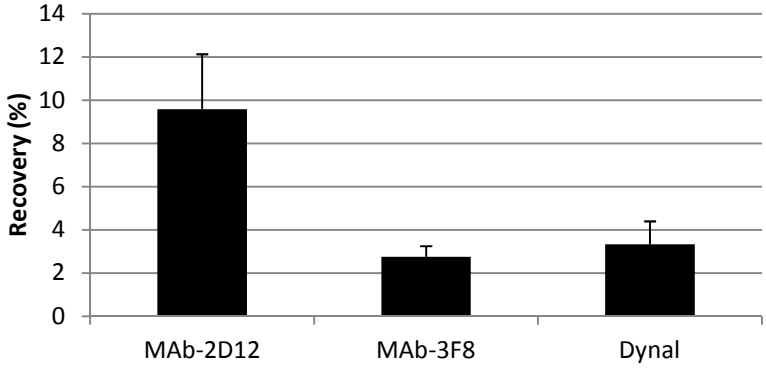

FIG. 13. Capture Efficiency of PMBT1 coated with MAb-2d12 (InlA); MAb-3F8 (p30) and Dynabeads anti-*Listeria* in co-culture of *L. monocytogenes* and *L. innocua* cultivated in Fraser broth. Bacterial concentration were aprox. 10^5 CFU.ml^{-1} of each. Mean average of three assays.

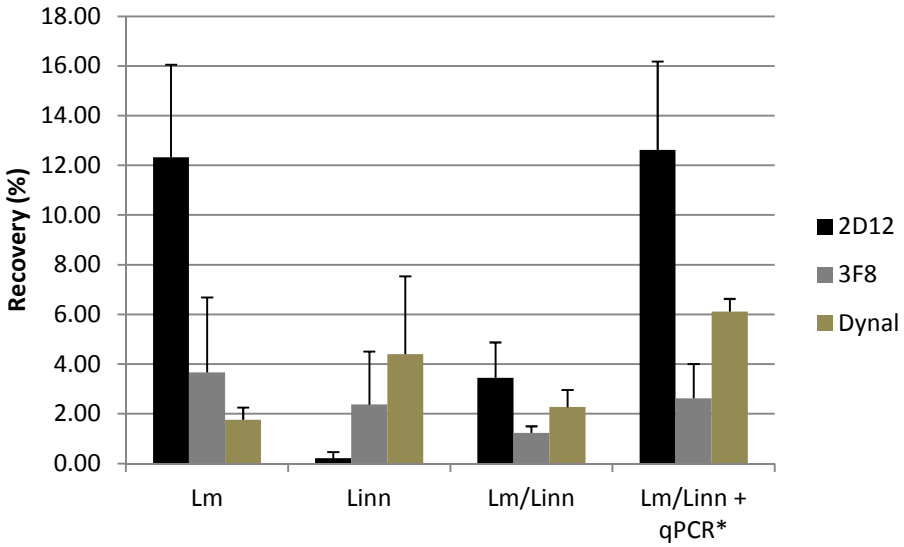

FIG. 14. Capture efficiency of PMBT1 coated with MAb-2D12 (InlA); MAB-3F8 and Dynabeads anti-*Listeria* from hotdog artificially contaminated with *L. monocytogenes* (Lm), *L. innocua* (Linn) and Lm/Linn; * Lm/Linn followed by real-time qPCR.

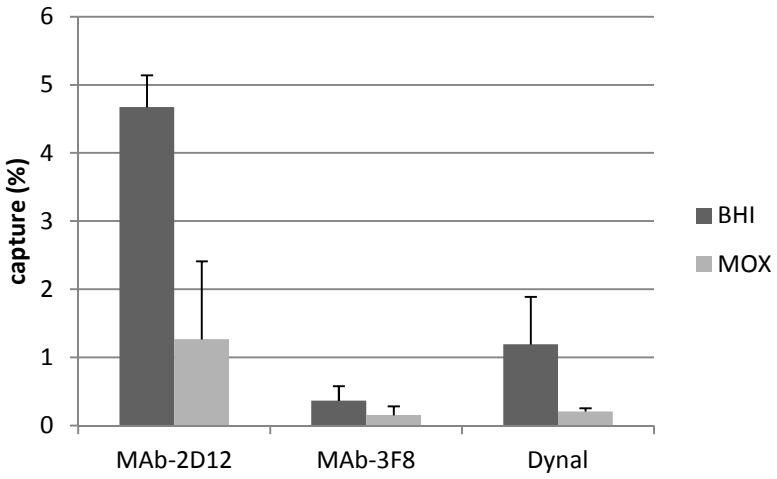

FIG. 15. Capture efficiency of PMBT1 coated with MAb-2D12 (InlA); MAb-3F8; Dynabeads anti-*Listeria* from goat cheese artificially contaminated with *L. monocytogenes* and *L. innocua*. After IMS, captured bacteria were plated onto BHI and MOX plates. Values are average of three independent assays.

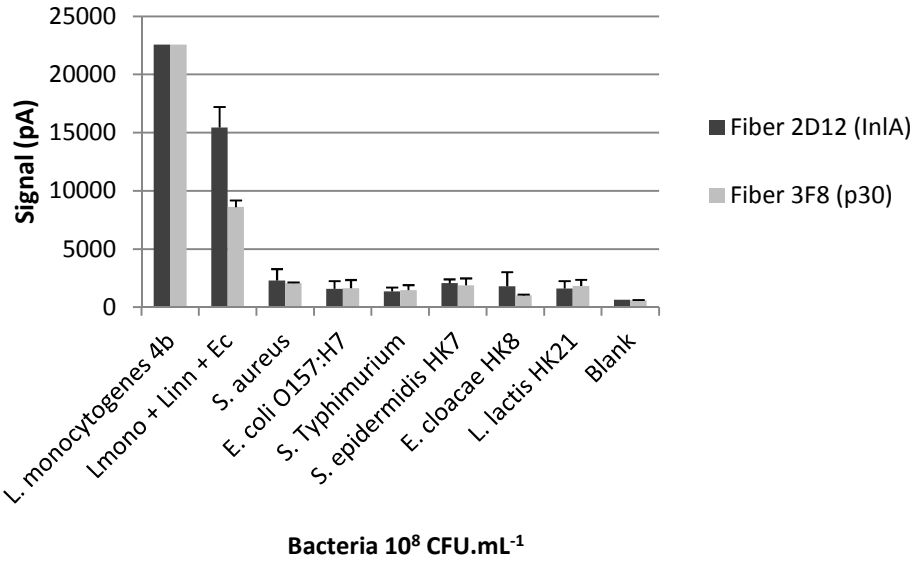

FIG. 16. Specificity of fiber-optic sensor using MAb-2D12 (InlA) and MAb-3F8 (p30) as capture antibody with different bacteria at concentration of 10^8 CFU.mL^{-1}. The Cy5 conjugated anti-InlA MAb-2D12 was used as antibody reporter for specific detection of *L. monocytogenes*. A mixed Lmono + Linn + Ec are respectively *L. monocytogenes* 4b, *L. innocua* and *E. coli* O157:H7 and were used at ~10^6 CFU.mL^{-1} of each. Signals are average of three fibers at 30 sec.

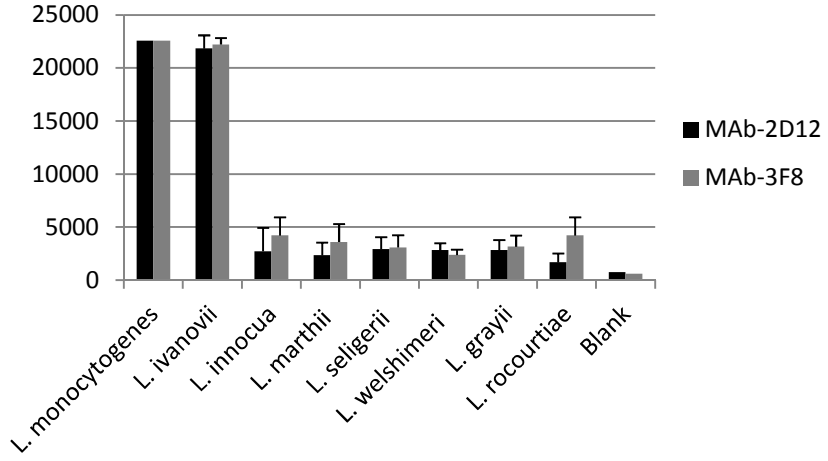

FIG.17. Specificity of fiber optic sensor using MAb-3F8 (p30) as capture antibody for all *Listeria spp.* The Cy5 conjugated anti-InlA MAb-2D12 as a reporter for specific detection of patogenic *Listeria*. Signals (pA) are average of three fibers at 30 sec.

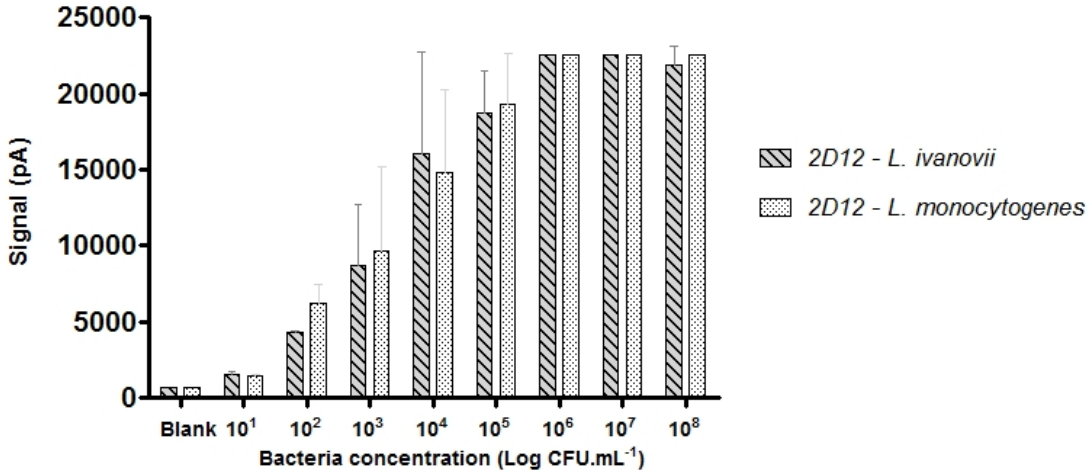

FIG. 18. Analysis of sensitivity of fiber-optic sensor with fibers coated with MAb-2D12 (InlA). Different concentrations of *L. monocytogenes* 4b (F4244) and *L. ivanovii* (ATCC 19119) were captured and signals were performed using Cy5 conjugated anti-InlA MAb-2D12. Values are average of three fibers with signals acquired at 30 sec.

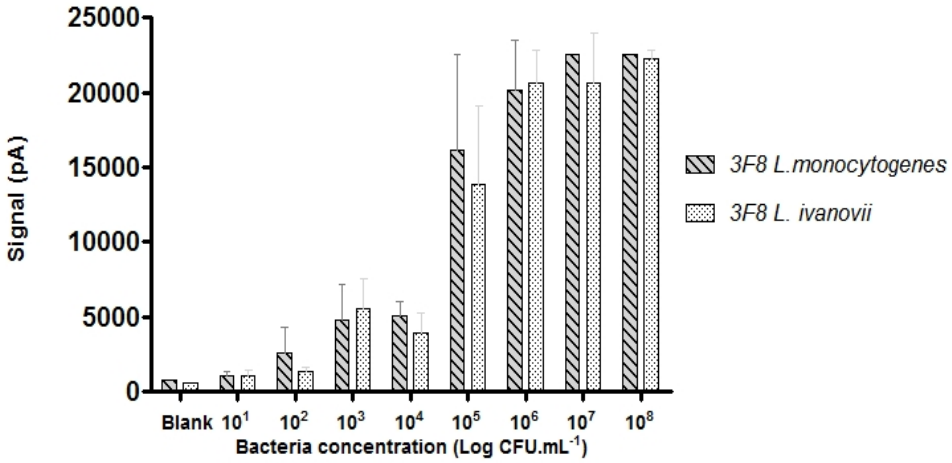

FIG. 19 – Analysis of sensitivity of fiber-optic sensor with fibers coated with MAb-3F8 (p30). Different concentrations of *L. monocytogenes* 4b (F4244) and *L. ivanovii* (ATCC 19119) were captured and signals were performed using Cy5 conjugated anti-InlA MAb-2D12. Values are average of three fibers with signals acquired at 30 sec.

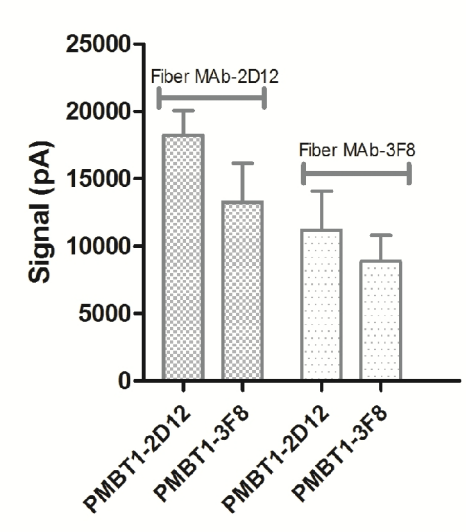

FIG. 20. Detection of *L. monocytogenes* captured with PMBT1 coated with MAb-2D12 or MAb-3F8 in buffer solution and directly tested with fibers coated with MAb-2D12 or MAb-3F8.

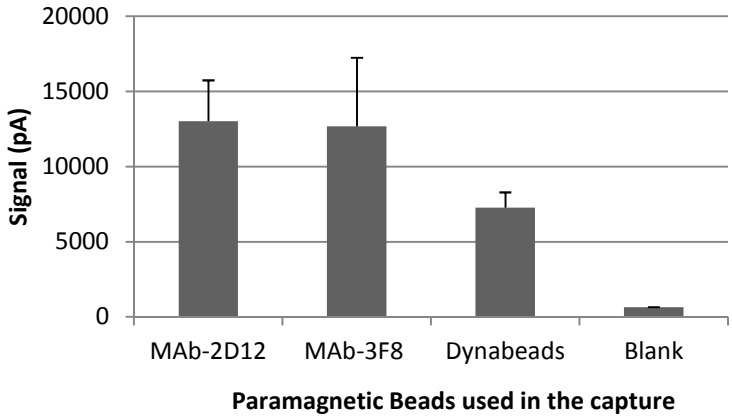

FIG. 21. Detection of *L. monocytogenes* from inoculated cheese using fiber-optic sensor after IMS. Goat cheese samples were inoculated with *L. monocytogenes* and *L. innocua*, and IMS using PMBT1-MAb-2D12 (InlA); PMBT1-MAb-3F8 (p30) and Dynabeads anti-*Listeria*. After released from PMBs, captured bacteria were allowed to react with fibers coated with MAb-2D12 (InlA), and signals were acquired with Cy5 conjugated MAb-2D12. Signals (pA) are average of three different assays. Blank, buffer only.

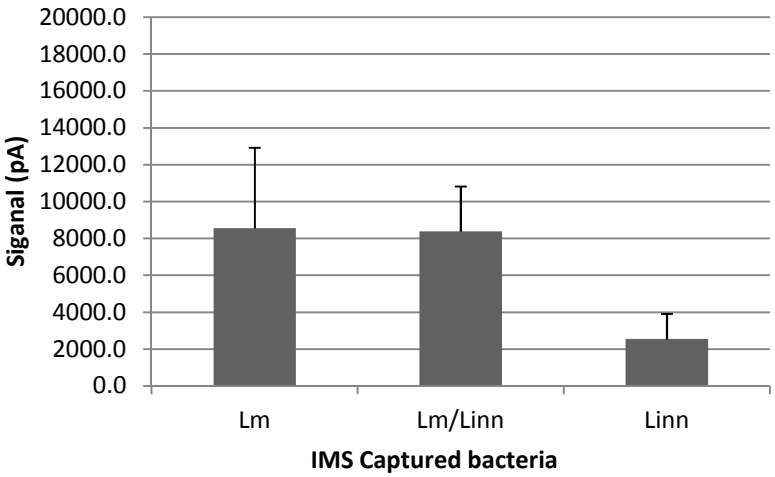

FIG. 22. Detection of *L. monocytogenes* from inoculated hotdogs using fiber-optic sensor after IMS. Hotdogs were inoculated with *L. monocytogenes, L. innocua* and both together. IMS was performed using PMB coated with MAb-2D12 (InlA). After released from PMB, captured bacteria were allowed to react with fiber coated with MAb-2D12, and signals were acquired with Cy5 conjugated MAb-2D12. Signals (pA) are average of three different assays.

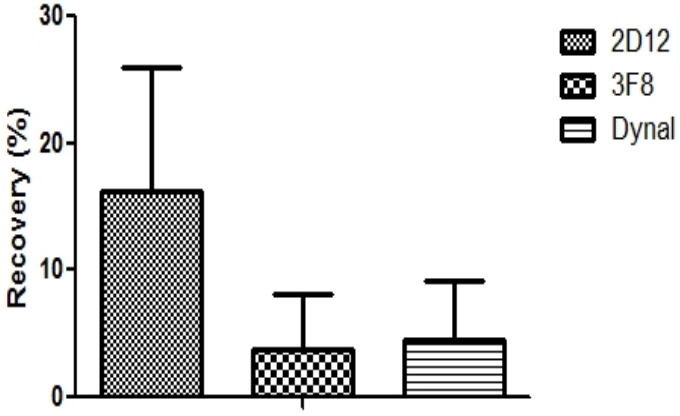

FIG. 23. Detection of *L. monocytogenes* from inoculated soft goat cheese using qPCR after IMS with PMBs coated MAbs. Real-time qPCR was performed with DNA samples from bacteria captured with PMB-MAb-2D12, PMB-MAb-3F8 and Dynabeads anti-*Listeria*. Values are average of three different assays.

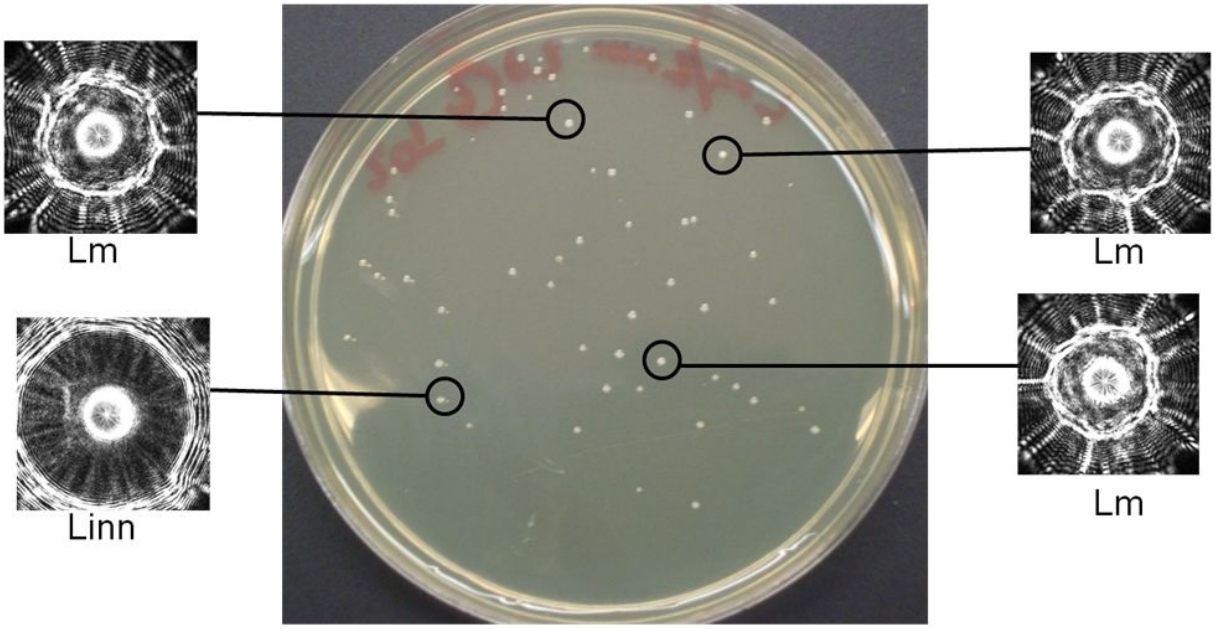

FIG. 24. Identification of *L. monocytogenes* (Lm) and *L. innocua* (Linn) from a mixed culture with light-scattering sensor (BARDOT). Bacteria were captured by PMBT1-MAb-2D12 from artificially contaminated soft goat cheese and plated onto BHI agar plates.

5 ARTIGO 2 - Characteristics of a novel monoclonal antibody with specific reactivity to the genus *Listeria*

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Characteristics of a novel monoclonal antibody with specific reactivity to the genus *Listeria*

ABSTRACT

Aims: This study aimed to characterize a monoclonal antibody (MAb) produced against the genus *Listeria* and demonstrated which protein target this MAb recognized.

Methods and Results: After used whole *L. monocytogenes* cells as immunogen, we selected the MAb-3F8 belonging to IgM serotype which had a commendable binding property for the genus *Listeria*. Western blot analysis demonstrated that this MAb reacted with an exquisite and single protein of about 30 kDa of all eight *Listeria* species, including the 13 serotypes of *L. monocytogenes*. Moreover, indirect ELISA and Dot blot assay shown that MAb-3F8 reacted with intact listerial cells. Furthermore, MAb-3F8 did not show any other cross-reactions with other non-*Listeria* bacteria tested in ELISA and Western blot. Aiming to identify the antigen target for this MAb, MALDI-TOF sequencing was performed and the protein identified as fructose 1,6-bisphosphate aldolase (FBA). In order to clarify whether or not this protein has any role in the attachment of *L. monocytogenes* to mammalian cells, we conducted an inhibition of adhesion assay, where did not show any involvement of this protein.

Conclusions: The MAb-3F8 obtained and characterized in this paper can be used as potential tool to study this protein in *Listeria*. Nevertheless, MAb-3F8 should be useful as a powerful biomolecule to detect *Listeria* spp. from food or environmental samples.

Significance and Impact of the Study: The FBA protein could be used for development of immunodiagnostic tools or used as novel drug and vaccine targets against *L. monocytogenes*.

Key words: *Listeria* spp., monoclonal antibody, fructose 1,6-bisphosphate aldolase, detection.

INTRODUCTION

Bacteria of the genus *Listeria* comprises a group of Gram-positive, facultative anaerobic, non-sporulating rods which are widely distributed in the environments (Hain et al. 2007). Due its ubiquitous nature, *Listeria* spp. can be introduced into food processing facilities, becoming persistent in the environment and thus contaminating food products (Carpentier and Cerf 2011). *L. monocytogenes* is a facultative intracellular foodborne pathogen which causes listeriosis, a serious systemic infection which primarily affects individuals with suppressed cellular immunity, such as elderly, cancer patients, AIDS patients, as well as pregnant women, their unborn fetuses and infants (Gasanov et al. 2005; Cossart 2007). Currently, the genus *Listeria* consist of eight species, *L. monocytogenes*, *L. ivanovii, L. innocua, L. seeligeri, L. welshimeri*, *L. grayi*, *L. marthii*, and *L. rocourtiae* (Mishra et al. 2011). However, only *L. monocytogenes* is pathogenic to humans and animals, and *L. ivanovii* has been implicated to cause listeriosis exclusively in animals, though it can also cause disease in humans as an opportunistic pathogen (Guillet et al. 2010).

Several outbreaks and sporadic cases of listeriosis have been attributed to contaminated foods (CDC 2011). Furthermore, *L. monocytogenes*-contaminated foodstuffs cause tons of recalled products annually, resulting in massive financial losses for food industries (Brougher and Greene 2011). The total elimination of this microorganism from the food processing plants is a difficult task. This is mainly due to the psychrotrophic nature of *Listeria* spp. that allows its survival in environments such as cold storage areas and floor drains (Farber and Peterkin 1991; Zhao et al. 2006). For this reason, *L. monocytogenes* can become a post-processing contaminant of ready-to-eat (RTE) foods, which carry a greater risk for foodborne illness than raw products because they are consumed without reheating (Rocourt et al. 2003). Hence, specific detection of *Listeria* spp. and *L. monocytogenes* is of utmost importance from the public health and food manufacturers' point of view.

Antibodies have been widely used as immunological test system for specific detection and identification of bacteria from different sources. The production of monoclonal antibodies (MAbs) against bacterial surface antigens allow not only the development of identification tests, but also can frequently provide a powerful tool for the study of bacterial proteins structures and functions (Nelson et al. 2000; Kumar et al. 2010). Since a MAb recognize an exclusive epitope of the antigen, it can be used

as an effective approach to identify new proteins that would be important in the bacterial pathogenesis, survival or adaptation in the environment (Sun et al. 2000). Moreover, MAbs offers a uniform reagent that can be produced in unlimited amounts, with high reproducibility of results after their application, providing improvement of immunoassays (Bhunia 2008).

In this paper we describe the production and characterization of a novel MAb that recognize specifically a 30 kDa protein of all eight *Listeria* species. In addition, we addressed the identification of this protein by MALDI-TOF mass spectrometry and investigated its role in adhesion to mammalian cells. To our knowledge, this is the first report of monoclonal antibody for a surface antigen that recognizes the whole genus *Listeria*.

MATERIALS AND METHODS

Cultures and growth conditions

Listeria species used in this study were grown at 37^{o}C for 16-18 h in Tryptic soy broth (TSB; BD, Beckton Dikinson, Sparks, MD, USA) containing 0.6% yeast extract (TSB-YE; Acumedia, Lansing, MI); *Listeria* enrichment broth (LEB, BD); or Fraser Broth (FB, BD). Other bacteria non-*Listeria* were grown in TSB-YE and lactic acid bacteria were grown in MRS broth at 37^{o}C for 16-18 h. All bacterial strains, including all *L. monocytogenes* used in this study are listed in Table 1.

Hybridoma Production

The immunization schedules and production of hybridoma cell line secreting MAb-3F8 was previously described Mendonça et al. (first paper pg. 35). Briefly, hybridomas producing antibodies reacting in an indirect ELISA with viable *L. monocytogenes* 4b cells, were cloned at least twice by the limiting-dilution technique. After established, the hybridoma lines were expanded to tissue culture flasks and supernatants re-tested by ELISA with *L. monocytogenes* (4b, 1/2a, 1/2b), *L. innocua*, *L. selligeri*. As negative control, *Salmonella enterica* serovar Enteritidis and *E. coli* O157:H7 were used. Those hybridomas clones that supernatants did not react with negative bacteria were stored in liquid nitrogen for future use and injected in mice to ascites production. Hybridoma supernatant was used to determine the MAb isotype

by ELISA isotyping kit (Sigma-Aldrich, USA). MAb-3F8 was purified by affinity chromatography using a protein A-Sepharose 4B column (GE Healthcare, Piscataway, NJ).

Characterization of MAb-3F8 by Indirect ELISA

Bacteria strains (table 1) were harvested by centrifugation and the concentrations adjusted to achieve OD_{600} of 1 (approx. 10^9 CFU/mL^{-1}) in 0.1 M sodium carbonate coating buffer (pH 9.6). Then, 96-well ELISA plates (Nunc, Maxisorp) were coated with 100 µL of each cell suspension for 16 h at 4°C. The plates were then washed three times with 200 µL PBS containing 0.5% Tween 20 (PBST), and blocked with a 5% solution of dry skimmed milk for 1 h at 37°C. Hybridoma ascites or purified antibody were diluted in PBST and 100 µL added to the wells and incubated for 1 h at 37°C. After new washing, a goat anti-mouse polyvalent antibody conjugated to horseradish peroxidase (HRP; Sigma) diluted in PBST (1:4000) was added and incubated at 37°C for 1 h. Lastly, wells were washed five times and the reaction developed by adding 100 µL of the substrate solution containing 10 mg of ortho-phenylenodiamine (OPD; Sigma) in 10 mL of 0.1 M citrate buffer (pH 5.0) and 10 µL of 30% H_2O_2.

Protein fractionation and immunoblotting

Protein fractionation was performed as described in Mishra et al. (2011), where secreted protein were isolated from broth supernatants and cell wall-associated proteins and intracellular proteins were isolated from the resulting bacterial pellets. Bacterial cultures were grown in 200 mL of broth at 37°C for 18 – 20h and centrifuged at 7000 g, 4°C for 10 min. The supernatant was filtered in 0.22 µm-pore membrane and precipitated overnight on ice with 10% trichloroacetic acid (w/v) followed by centrifugation (14000 g for 10 min at 4°C). The resulting pellet were resuspended in 1 mL of cold acetone and incubated for 10 min on ice and then centrifuged at 14000 g for 5 min at 4°C. After decanted the supernatant, pellets were air-dried and resuspended in 250 µl of alkaline rehydration buffer (3% SDS, 100mM Tris buffer, pH 11 and 3mM DTT). To isolate cell wall-associated protein, cell pellets from first centrifuge were resuspended in 0.5 ml of protein extraction buffer (0.5% SDS, 10mM Tris, pH 6.9) and incubated in water bath for 30 min at 37°C. After centrifugation (14000 g for 5 min, 4°C), the supernatant containing cell wall-

associated protein fraction were collected. Cell pellets were resuspended in 100 µl of sample solvent (4.6% SDS, 10% β-mercaptoethanol, 0.124 M Tris, 20% glycerol, pH 6.9), subjected to sonication (4 times) and centrifuged for 20 min at 14000 g at 4^{o}C, and then the supernatant containing the proteins were collected.

The proteins were separated in SDS-PAGE (Bio-Rad, Hercules, CA) and blotted onto Immobilon-P membranes (Millipore Bedford, MA, USA). The immunoblotting were performed after blocking the membranes with 5% skimmed milk for 1 h at room temperature (RT) and then washed with PBST. The MAb-3F8 was added and incubated for more 1 h at RT. After washing, the membranes were incubated with a HRP-conjugated goat anti-mouse polyvalent antibody. Antibody-reactive bands were developed with chemiluminescence substrate system (ECL kit: Thermo Fisher Scientific, Rockford, IL) or DAB (6 mg of 3.3-diaminobenzidine tetrahydrochloride; 10 µL of H_2O_2 30%; 9 mL of 50mM Tris-HCl pH 7.6; 1 mL of 0.3% sulphate nickel). In some experiments, MAb-2D12 against internalin A was used.

Dot blot

Five microliter of live or heat-killed *Listeria* spp., *Salmonella*, *E. coli* whole-cell suspension (~10^8 cells.ml^{-1}) was spotted on nitrocellulose membranes, allowed to air dry for 15 min and then blocked with 5% skimmed milk for 30 min. Immunoblotting was performed as described above and developed with DAB.

Protein Sequencing by MALDI-TOF-MS/MS

A matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF-MS) was performed to determine the identity of the 30 kDa protein (p30) recognized by the MAb-3F8. First, protein preparations from *L. monocytogenes*, *L. innocua*, *L. ivanovii* and *L. marthii* were separated in SDS-PAGE (10%-acrylamide) and visualized by Coommassie blue staining or electro-transferred onto PVDF membranes (Millipore, USA) and probed with MAb-3F8. The protein spots were then excised from the Coommassie-stained gel, rehydrated with 100 mM NH_4HCO_3 and analyzed by MALDI-TOF-MS/MS independently by two laboratories; Applied Biomics (Hayward, CA, USA) and Purdue University sequencing facility. The peptide mass was analyzed by using MASCOT protein identification software (Matrix Science, London, UK).

Inhibition of *L. monocytogenes* Adhesion to Epithelial Cells by MAbs

L. monocytogenes cells were cultured in TSB-YE at 37°C for 18 h, washed three times with PBS and resuspended in 1 ml of mammalian cell culture medium (DMEM-10F; Dulbecco's Modified Eagles Medium containing 10% fetal calf serum). Then, 0.5 mL of serially diluted cell suspensions containing aproximately 10^6 *L. monocytogenes* cells were incubated with 0.5 mL of each MAb (1 $mg.mL^{-1}$) at 37°C for 20 min with gentle agitation, washed, and resuspended in DMEM-10F. All experiments were carried out using as positive control MAb anti-internalin A (2D12) and negative MAb anti- N-acetylmuramidase (C11E9). To determine the rate of inhibition of the adhesion, a method described previously by Kim et al. (2006) was used with some modifications. First, HCT-8 cells (ileocecal cells; CCL 244; ATCC) were grown in 24-well plates, washed three times with Cell-PBS (137 mM NaCl, 5.4 mM KCl, 3.5 mM Na_2HPO_4, 4.4 mM NaH_2PO_4, 11 mM glucose, pH 7.2) and resuspended in DMEM-10F. Antibody-treated *L. monocytogenes* cells were then added to the cell monolayers at a multiplicity of infection (MOI) of 10:1 (bacteria : HCT-8 cell), and incubated at 37°C for 1 h. Non adherent bacterial cells were removed by washing thrice with Cell-PBS and treated with 0.1% Triton X-100 in Cell-PBS for 5 min at 37°C and bacterial adhesions were enumerated by plating onto BHI agar.

RESULTS

Production of MAb

A total of five cellular fusions were performed which yielded over 150 hybridomas. Those that upon initial screening reacted positively against *Listeria* species tested (*L. monocytogenes*, *L. innocua*, *L. seeligeri*), were chosen for cloning by limiting dilution. Of these, only four stable hybridomas secreting antibodies against bacteria of genus *Listeria* were selected (4E5; 4C1; 2A12 and 3F8). These MAbs were further characterized by indirect ELISA and three of them shown cross-reactions with other bacteria non-*Listeria*. Thus, after preliminary tests, just hybridoma 3F8 has shown secreting MAb with high specificity against only *Listeria* species. The MAb-3F8 was isotyped as IgM.

Characterization of MAb-3F8

MAb-3F8 had a significant reactivity by indirect ELISA with all *Listeria* species tested with this method, and displaying absorbance (OD_{450}) of over 1.5 for *L. monocytogenes*. Also, IgM MAb-3F8 has not shown any significant reaction above the background with other bacterial genus (Fig. 1). The Western blotting assay shows the specific reaction of MAb-3F8 with a single protein of molecular mass of about 30 kDa with all eight *Listeria* species, as well as with the 13 serotypes of *L. monocytogenes* (Fig. 2). The reactive band was visualized in the intracellular, cell wall, and as well as in secreted proteins evaluated in the supernatant fractions. In addition, this MAb did not show any cross-reactions with Gram-positive and negative bacteria, such as *S. aureus, B. subtilis, B. cereus, S.* Typhimurium*, S.* Enteritidis*, B. thuringiensis, E. coli O157:H7, L. lactis, E. aerogenes, L. paracasei, K. pneumonia* and *E. faecalis* (Fig. 3). Furthermore, the MAb-3F8 was able to react with live and heat-killed cells in a Dot blot assay (Fig. 4).

In order to assess and identify the antigen recognized by MAb-3F8, we also carried out tests against protein preparations of *sec*A2 deletion mutant of *L. monocytogenes* and *L. innocua* strains. The secA2 gene is responsible for the protein secretion and transport of some known virulence proteins across the bacterial cell wall (Mishra et al. 2011). As shown in the fig. 5, MAb-3F8 recognized intracellular proteins and cell wall proteins in both Δ*sec*A2 strains, thus revealed that the targeted protein of this MAb is not exported through SecA2 pathway.

Identification and characterization of the target antigen

Protein spots from SDS-PAGE where MAb-3F8 recognized the p30 protein in the Western blot were sliced, digested and subjected to sequencing by MALDI-TOF/MS-MS. Data from MS/MS based on at least two identified peptides with a MASCOT confidence score above 95% (C.I. %) were further analyzed. Three high scored proteins were selected, fructose 1,6-bisphosphate aldolase (FBA); pleiotropic transcriptional repressor (CodY); and enolase (Eno). In order to find out which of these three proteins would most likely the MAb-3F8 target, we used bioinformatics tools and protein databases such as Swiss-Prot and NCBI. Based on protein databases, CodY is 28 kDa weight, but that is not exported to cell surface, remaining exclusively in the cellular cytosol. Furthermore, *L. monocytogenes* enolase was identified as a SecA2-dependent (Lenz et al. 2003) and its molecular weight of about 46 kDa in *Listeria*. Thus, comparing with other known proteins deposited in protein sequence banks, and taking into account protein weight, location in the bacteria and functions, the results were consistent for the 30 kDa FBA protein.

Inhibition of *L. monocytogenes* Adhesion to Epithelial Cells by MAb

Ability of MAb-3F8 to block *L. monocytogenes* binding to mammalian cell line, HCT-8, was examined as an indirect way of confirming affinity of this antibody towards FBA protein and their role in adhesion. Internalin A is an adhesion and invasion factor (Lecuit et al. 1999), while the function of FBA is unknown. *L. monocytogenes* cells pretreated with anti-InlA MAb-2D12 significantly ($P < 0.05$) reduced adhesion of this bacterium to HCT-8 cells (Fig. 6) confirming specific interaction of antibody with InlA and subsequent interference with bacterial adhesion. On the other hand, MAb-3F8 did not show any significant reductions in adhesion of *L. monocytogenes* indicating that even though antibody reacted with FBA protein, it probably may not be involved in adhesion to mammalian cells. It was used as control antibody a MAb-C11E9 that reacts with N-acetylmuramidase and did not affect adhesion of *L. monocytogenes* (Bhunia et al. 1991).

DISCUSSION

L. monocytogenes is a food pathogen that causes one of the most widespread public health problems, creating not only social issues, but likewise economic burdens, making it a concern that all countries should address (McLauchlin et al. 2004; Gandhi and Chikindas 2007). Several attempts to produce monoclonal antibodies specific for *Listeria* species or that react only with *L. monocytogenes* have been described for many researchers (Bhunia et al. 1991; Kathariou et al. 1994; Kim et al. 2005; Hearty et al. 2006). Although the main goal is to raise MAbs against the pathogenic *L. monocytogenes*, most of these has failed in recognize just this specie, or has not shown specificity for an exclusive antigen of all *Listeria* species. Therefore, antibodies that are genus-specific or *L. monocytogenes*-specific are essential for assays development. In this study we have produced an IgM MAb by using whole cells of *L. monocytogenes* as immunogen, that was specific to fructose 1,6-biphosphate aldolase (FBA) of *Listeria* species.

Some MAbs are handy in many functional studies, for instance to characterize a surface antigen with respect to protein interactions or to determine conformational changes in response to activity effectors (Mancia et al. 2007). Furthermore, MAb-based immunoassays are considered highly specific for the identification and characterization of microorganisms from many different sources. However, the availability of an antigen-specific antibody is a crucial point to the success of immunoassays or antigen characterization (Bhunia 2008).

The FBA is a housekeeping glycolytic enzyme, which has also been found localized to the surface of several bacteria, where it can bind host molecules and exhibit non-glycolytic functions (Schaumburg et al. 2004; Tunio et al. 2010). Thus, FBA is considered as a “moonlighting” protein, which means that can perform more than one function, playing a potential role at the cell surface or in bacterial virulence (Schaumburg et al. 2004; Mujahid et al. 2007; Tunio et al. 2010). Although this protein is a common protein of many bacterial pathogens, it is very little known other exactly function of FBA in the genus *Listeria* (Schaumburg et al. 2004; Trost et al. 2005).

Tunio et al. (2010) demonstrated that FBA of *Neisseria meningitidis* besides of highly conserved, it participate in the adhesion of this bacterium to human cells. Nevertheless, MAb-3F8 was not capable to reduce the adhesion of *L. monocytogenes* to human colon HCT-8 cells (Fig. 6). Thus, indicating that this protein

may not have an important role in the attachment of *L. monocytogenes*, however, more studies should be done to confirm this assumption. Although a potential role of *Streptococcus pneumoniae* FBA in the infection has not been yet investigated, studies have been shown an antigenic immune response from human sera and a capacity of eliciting protective response in mice against Pneumococcal FBA (Ling et al. 2004). Besides, FBA of *M. tuberculosis* has been shown to bind human plasminogen (Santangelo et al. 2011).

Since FBA protein does not have a human ortholog, it has been suggested that it could provide a potential drug or vaccine for tuberculosis, streptococcosis and meningococcal infection (Ling et al. 2004; Pegan et al. 2009; Tunio et al. 2010; Santangelo et al. 2011). Moreover, FBA protein was defined to be the primary target site of nickel toxicity in *E. coli* (Macomber et al. 2011). The protein phosphorylation is often involved in the expression of virulence genes and in the formation of bacterial cell surface; or even has be implicated in the regulation of pathogenic functions by interfering biochemical host signaling cascades (Jers et al. 2008). Misra et al. (2011) have found that protein phosphorylation in *L. monocytogenes* is conserved in the FBA enzyme, and this fact can be linked with an unknown virulence factor through this protein. Like many other bacteria that can live ubiquitously in the nature, *Listeria* spp. has the ability of forming biofilms and become persistent in the environment. Hefford et al. (2005) have found a higher expression of FBA protein in *Listeria* biofilm-grown cells than planktonic cells, therefore speculating the participation of this protein in the biofilm formation.

Immunoblotting using our MAb-3F8, clearly showed reactivity with an unique epitope in the cell wall and intracellular fractions of all *Listeria* species. The presence of FBA protein was also verified in the cell wall of *S. pneumoniae* (Portnoi et al. 2006) and *M. tuberculosis* (Santangelo et al. 2011). Moreover, MAb-3F8 was capable to detect FBA in secreted proteins in the supernatant fraction (Fig. 7), which was also found in culture supernatant of *M. tuberculosis* (Rosenkrands et al. 2002; Santangelo et al. 2011). However, this protein was not visualized in supernatant fractionation of *N. meningitidis* (Tunio et al. 2010). Additionally, Ishihama et al. (2008) have demonstrated that FBA is one of most abundant soluble proteins of *E. coli* showing more than 47 thousand protein copies per cell. Our Western blot results suggest that this protein as well, is abundantly expressed in the genus *Listeria* (Fig. 7). The fig. 9 shows the reaction of MAb-3F8 and MAb-2D12 anti-InlA in the same blot, notice the

higher expression of FBA over InlA protein. It is worth to note that MAb-3F8 is the first reported antibody which recognizes the two recently discovered *L. marthii* and *L. rocourtiae*.

Altogether, however, there is a lacking of information regarding the properties of this enzyme in *Listeria* and that should be addressed to better understanding its functions. Once MAb-3F8 has shown an exclusive recognition of the fructose 1,6-bisphosphate aldolase of genus *Listeria*, it has a great potential for use as an analytical tool to study this protein in *Listeria* species. Furthermore, this MAb can be used to develop immunochemical methods for diagnostic of *Listeria* species and/or *L. monocytogenes*, when combined with genetic or immunological approaches for this pathogenic specie.

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TABLE

Table 1. Bacterial strains used this study

Bacteria / Serotype	Strain / Isolated	Source[a]
L. monocytogenes 1/2a	V7	FDA
L. monocytogenes 1/2b	F4260	CDC
L. monocytogenes 1/2c	7644	CDC
L. monocytogenes 4a	19114	ATCC
L. monocytogenes 4b	F4244	CDC
L. monocytogenes 4c	19116	ATCC
L. monocytogenes 4d	19117	ATCC
L. monocytogenes 4e	19118	ATCC
L. monocytogenes 4ab	Murray B	FDA
L. monocytogenes 3a	19113	ATCC
L. monocytogenes 3b	2540	ATCC
L. monocytogenes 3c	2479	SLCC
L. monocytogenes 7	2482	SLCC
L. innocua	F4248	CDC
L. innocua 6a	Li01	UFPel
L. welshimeri	35897	ATCC
L. seeligeri	3954	ATCC
L. seeligeri	Ls02	UFPel
L. ivanovii	SE98	USDA
L. grayii	19120	ATCC
L. marthii	BAA-1595	ATCC
L. rocourtiae	---	---
Bacillus subtilis	6633	ATCC
Bacillus thuringiensis	DUP-6044	MFM-Purdue
Escherichia coli O157:H7	EDL933	CDC
Lactococcus lactis	11454	ATCC
Enterococcus aerogenes	DUP-14591	MFM-Purdue
Lactobacillus paracasei	DUP-13076	MFM-Purdue
Klebsiella pneumonia	---	MFM-Purdue
Enterococcus faecalis	---	MFM-Purdue
Lactococcus lactis subsp. *lactis*	HK21	MFM-Purdue
Enterobacter cloacae	HK8	MFM-Purdue
Staphylococcus epidermidis	HK7	MFM-Purdue
Bacillus cereus	11778	ATCC
Staphylococcus aureus	13301	ATCC
Pseudomonas aeruginosa	10145	ATCC
Salmonella enterica ser. Typhimurium	DUP-1167	MFM-Purdue
Salmonella enterica ser. Enteritidis	13076	ATCC

[a] FDA: Food and Drug Administration, Washington, D.C.; CDC: Centers for Disease Control and Prevention, Atlanta, GA. ; ATCC: American Type Culture Collection, Rockville, MD.; SLCC: Special Listeria Culture Collection, Institute of Hygiene and Microbiology, Univ. of Würzburg, Germany; USDA: National Center for Agricultural Utilization Research, Peoria, Illinois, U.S.A.; MFM-Purdue: Molecular Food Microbiology Lab. Collection, Purdue.; UFPel: Laboratório de Microbiologia de Alimentos Collection, FAEM-UFPel.

FIGURES

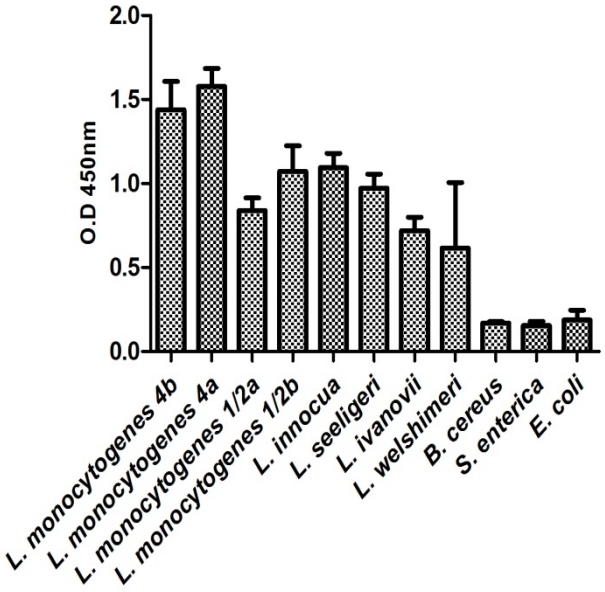

Fig. 1 – Binding of MAb-3F8 in indirect ELISA to different bacterial strains. Live bacteria were coated in the plates for 16 h at 4°C. Data are shown as mean ± SD.

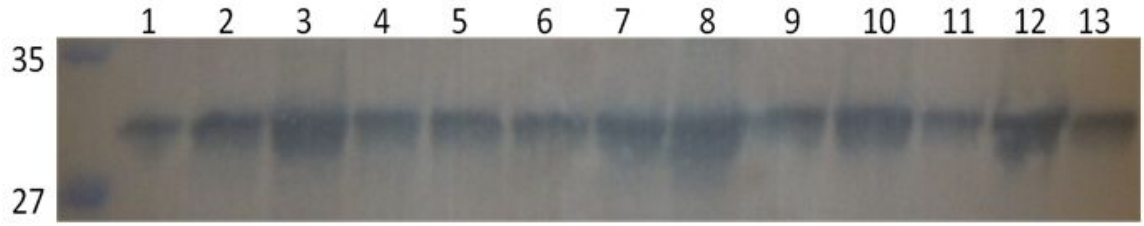

Fig. 2 – Immunoblotting using MAb-3F8 demonstrating the reaction with all 13 serotypes of *L. monocytogenes.* Cell wall proteins were resolved by 10% SDS-PAGE, transfer to PVDF membrane, and developed with DAB. 1- serotype 1/2a (V7); 2- serotype 1/2b (F4260); 3- serotype 1/2c (7644); 4- serotype 3a (19113); 5- serotype 3b (2540); 6- serotype 3c (2479); 7- serotype 4a (19114); 8- serotype 4b (F4244); 9- serotype 4c (19116); 10- serotype 4d (19117); 11- serotype 4e (19118); 12- serotype 4ab (Murray B); 13- serotype 7 (1323).

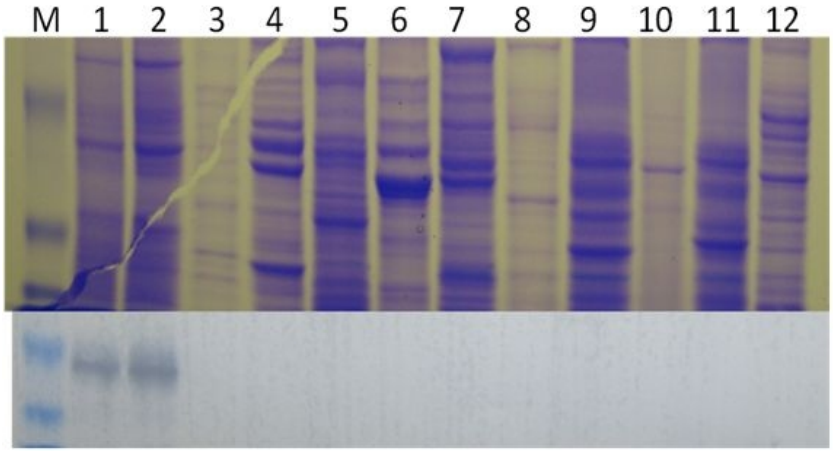

Fig. 3 – Immunoblotting using MAb-3F8 testing reaction with bacteria non-*Listeria*. Proteins from whole-cells lysates were resolved by 7.5% SDS-PAGE, transfer to PVDF membrane, and developed with DAB. M- Marker; 1- *L. monocytogenes* F4244; 2- *L. marthii*; 3- *Staphylococcus aureus*; 4- *Bacillus subtilis*; 5- *Salmonella* Typhimurium; 6- *Bacillus thuringiensis*; 7- *E. coli* O157:H7; 8- *Lactococcus lactis*; 9- *Enterococcus aerogenes*; 10- *Lactobacillus paracasei*; 11- *Klebsiella pneumonia*; 12- *Enterococcus faecalis*.

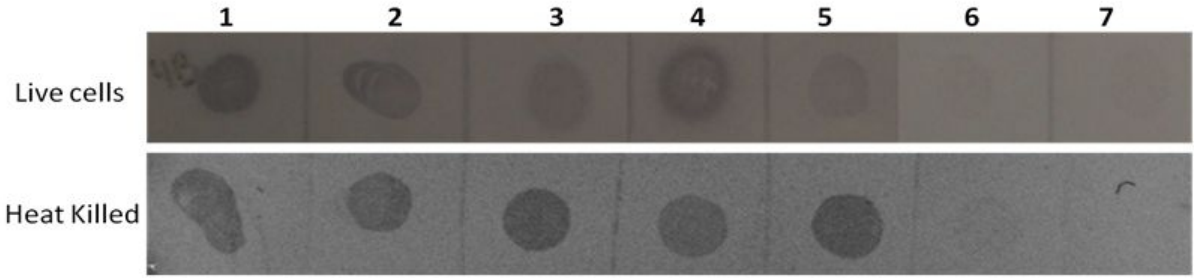

Fig. 4 – Dot blot assay of whole cells live and heat killed of different bacteria. 1- *L. monocytogenes* serotype 4b; 2- *L. monocytogenes* serotype 1/2a; 3- *L. monocytogenes* serotype 1/2b; 4- *L. monocytogenes* serotype 4d; 5- *L. innocua* 6a; 6- *Sal. enterica* Enteritidis; 7- *E*. coli O157:H7.

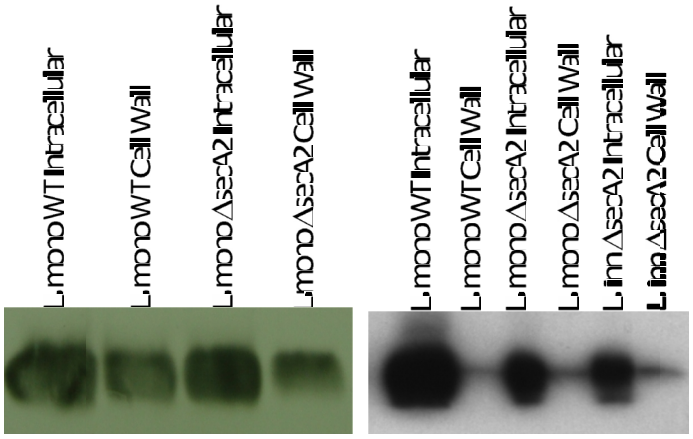

Fig. 5 – Immunoblotting of mutant SecA2 *L. monocytogenes* and *L. innocua* showing reaction of MAb-3F8 with intracellular and cell wall fractions. Proteins from intracellular and cell wall fractions were resolved by 7.5% SDS-PAGE, transfer to PVDF membrane, and developed with chemiluminescence.

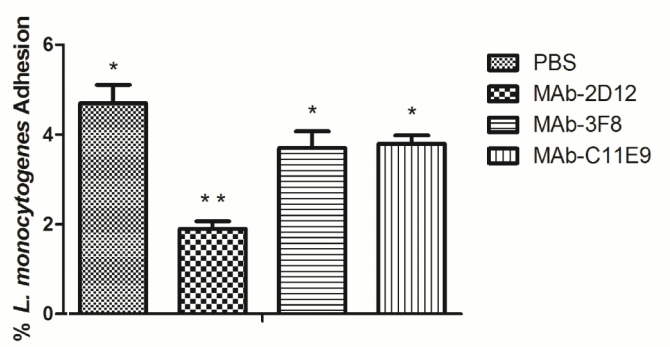

Fig. 6 – Inhibition of adhesion of *L. monocytogenes* to HCT-8 cell line followed pre-treatment with MAbs. Values are an average of three experiments. Marked asterisks indicate significant ($P < 0.05$) difference in adhesion.

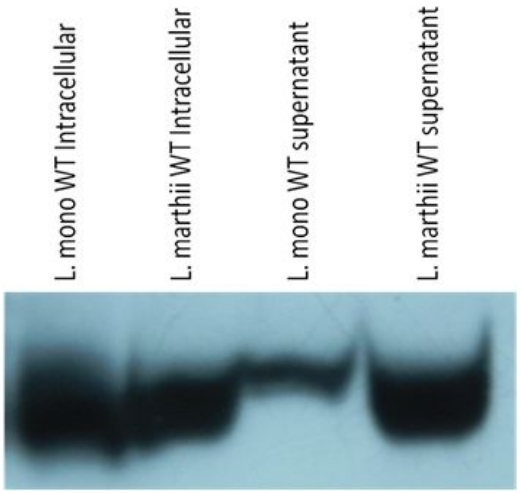

Fig. 7 – Immunoblotting using MAb-3F8 demonstrating the reaction with supernatant *L. monocytogenes* (F4244) and *L. marthii*. Proteins from intracellular and supernatant fractions were resolved by 7.5% SDS-PAGE, transfer to PVDF membrane, and developed with chemiluminescence.

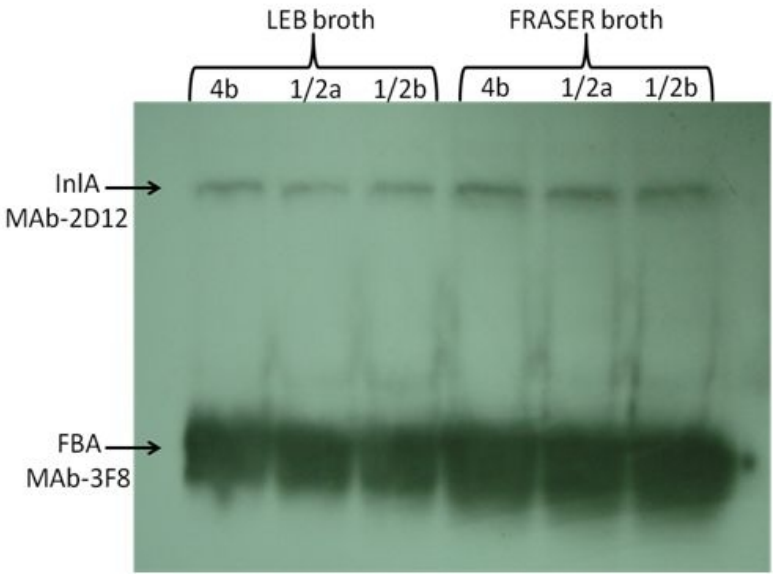

Fig. 8 – Immunoblotting using MAb-3F8 demonstrating the reaction with supernatant *L. monocytogenes* (F4244). Proteins from intracellular fraction were resolved by 10% SDS-PAGE, transfer to PVDF membrane, and developed with chemiluminescence.

CONCLUSÕES

1- Utilizando a proteína recombinante InlA produzida em *E. coli* e *L. monocytogenes* inativadas por fervura, foi possível estimular resposta imune humoral em camundongos BALB/c, gerando células secretoras de anticorpos anti-InlA e anti-*Listeria* spp.;

2- Foram obtidas três linhagens de hibridomas (2D12, 3B7, 4E4) estáveis secretores de anticorpos monoclonais (MAbs) contra a proteína InlA nativa de *L. monocytogenes*, e um hibridoma (3F8) que reconhece o gênero *Listeria*;

3- Os MAbs 2D12 e 3F8 demonstraram bons resultados na captura de *L. monocytogenes* por imunoseparação magnética (IMS), através de esferas paramagnéticas;

4- Pode ser demonstrado que, a utilização da plataforma sobre sensor de fibra óptica com o MAb anti-InlA 2D12 marcado com Cy5 foi eficaz na rápida detecção de *L. monocytogenes* após captura por ambos MAbs 2D12 e 3F8;

5- Foi possível detectar a presença de *L. monocytogenes* em alimentos artificialmente contaminados em menos de 22 horas usando o sistema de IMS, seguido do uso do biossensor de fibra óptica, para rápida captura e detecção de *L. monocytogenes*;

6- O antígeno que o MAb-3F8 reconhece foi caracterizado como fructose 1,6 bisphosphato aldalose, o qual possui potencial para ser utilizado no diagnóstico de *L. monocytogenes*.

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