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ORIGINAL ARTICLE

Combined antibodies against internalins A and B proteins have potential application in immunoassay for detection of *Listeria monocytogenes*

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Abstract *Listeria monocytogenes* is a food-borne bacterium that causes listeriosis upon the ingestion of contaminated food. Traditional methods to detect *L. monocytogenes* require pre-enrichment broths to increase its concentration. To improve the screening of contaminated food and prevent listeriosis outbreaks, rapid, specific and sensitive assays are needed to detect *L. monocytogenes.* This study developed a prototype lateral flow immunochromatographic assay (LFIA) employing antibodies against *L. monocytogenes* Internalin A (InlA) and Internalin B (InlB) proteins, that are involved in non-phagocytic cell invasion. The following antibodies were used to capture *L. monocytogenes* antigenic targets: mouse anti-Internalin A monoclonal antibody (MAb-2D12) conjugated to colloidal gold nanoparticles and a mouse anti-Internalin B polyclonal antibody. This test was able to detect pure *L. monocytogenes* from culture with a limit of detection (LOD) ranging from 5.9×10^3 to 1.5×10^4 CFU/mL. In milk artificially contaminated with *L. monocytogenes*, the LOD was 1×10^5 CFU/mL. This prototype test discriminated *L. monocytogenes* from other bacterial species (*Listeria innocua*, *Enterobacter cloacae*, *Bacillus cereus*). Results indicate that this LFIA developed using antibodies against *L. monocytogenes* InlA and InlB proteins is a sensitive and specific tool that can be potentially useful to rapidly detect *L. monocytogenes* in contaminated food.

Keywords *Listeria monocytogenes* · Listeriosis · Lateral flow immunoassay · Rapid test · Anti-Internalin A monoclonal antibody · Anti-Internalin B polyclonal antibodies

Leonardo Lopes-Luz and Ernandes Silva-Filho should be considered joint first author.

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Abbreviations

AuNP	Colloidal Gold Nanoparticle
BHI	Brain Heart Infusion
InlA	Internalin A
InlB	Internalin B
LFIA	Lateral Flow Immunochromatographic Assay
LM	*Listeria Monocytogenes*
LOD	Limit of Detection
MAb	Monoclonal Antibody
NC	Nitrocellulose Membrane
OD	Optical Density
PAb	Polyclonal Antibody
TSB	Tryptic Soy Broth

Introduction

Listeria monocytogenes is a Gram-positive invasive food-borne bacterial pathogen that upon ingestion of highly

contaminated food (up to ~ 10^9 bacteria) by healthy adults causes listeriosis, a mild to severe gastroenteritis characterized by diarrhea, abdominal cramps and flu-like symptoms (Schlech 2019). However in children, elderly, immunocompromised individuals and pregnant women, even low levels of food contamination (~ 10^2–10^4 bacteria) can be potentially lethal (Mclauchlin 1990). Following the ingestion of contaminated food, *L. monocytogenes* cross the intestinal barrier, spread into the bloodstream through the lymph nodes and disseminate to target tissues, such as the liver and the spleen manifesting a plethora of effects on the cells due to the activity of potent virulence factors (Drolia and Bhunia 2019). In immunocompromised individuals, *L. monocytogenes* can cross the blood–brain barrier or the fetoplacental barrier and cause potentially fatal meningitis, sepsis, premature birth or abortion. Despite its low incidence, the mortality rate of listeriosis ranges from 20 to 30% (Schlech 2019).

Listeria genus comprises 26 species and two of them, *L. monocytogenes* and *Listeria ivanovii* are pathogenic to humans and ruminants, respectively (Carlin et al. 2021). *L. ivanovii* has also been described as an opportunistic pathogen associated with gastroenteritis and bacteremia in humans (Snapir et al. 2006). *L. innocua* represents the most prevalent bacteria in listeria-contaminated foods and this poses a difficulty for the specific capture and detection of the pathogenic listeria (Oravcová et al. 2008).

Traditional methods for *L. monocytogenes* detection in food include long time bacterial culture with selective pre-enrichment steps (Bhunia 2018). Sensitive and specific molecular methods based on the detection of bacterial nucleic acid such as the polymerase chain reaction (PCR) have been proposed, but they are still considered expensive and complex for routine use (Wu 2019). In contrast, lateral flow immunochromatographic assay (LFIA) represents a simple, affordable, user- and field-friendly diagnostic tool that can provide quick visual results for *L. monocytogenes* detection (Cho et al. 2015; Li et al. 2017; Liu et al. 2017). Commercially available rapid tests for *L. monocytogenes* have low detection capacity, are time consuming and costly due to prior culture required to increase the amount of bacteria to detectable levels (Ueda et al. 2013).

The advances in the understanding of *L. monocytogenes* biology including the steps involved in host cell invasion revealed new potential targets for immunodiagnosis such as the internalin A (InlA) and InlB, two members of a family of 25 proteins known as internalins, which bind to eukaryotic cell membrane receptors (Radoshevich and Cossart 2018). *L. monocytogenes* can be uptaken by phagocytic cells or internalized into non-phagocytic cells, which is considered one of its hallmarks. In non-phagocytic cells, such as goblet cells, InlA binds the E-cadherin while in trophoblasts, InlB binds the hepatocyte growth factor receptor, inducing bacterial uptake through receptor-mediated endocytosis (Drolia and Bhunia 2019). InlA and InlB proteins have been shown to be immunogenic representing important targets for antibody recognition (Banada and Bhunia 2008; Drolia and Bhunia 2019). The production of anti-InlA monoclonal antibodies (MAb) and of anti-InlB polyclonal antibodies (PAb) and their application for the detection of *L. monocytogenes* in food samples has been reported (Banada and Bhunia 2008; Tully et al. 2008; Mendonça et al. 2012; Lathrop et al. 2014). InlA and InlB in the *Listeria* genus have highly immunogenic and non-conserved amino acid sequences (Bierne and Cossart 2007).

The goal of this study was to develop a prototype lateral flow diagnostic tool for the detection of *L. monocytogenes* based on the use of a combination of antibodies against InlA and InlB proteins. As a proof of concept, the performance of this prototype was evaluated in pure culture of *L. monocytogenes* and in artificially contaminated milk samples. The specificity of this test was also evaluated.

Materials and methods

Antibodies and bacteria

The prototype LFIA employed a mouse anti-internalin A monoclonal antibody (MAb-2D12) previously described (Mendonça et al. 2012) and a mouse anti-internalin B polyclonal antibodies (PAb-InlB), which were kindly provided by the Laboratory of Applied Immunology (Federal University of Pelotas, Pelotas, RS, Brazil). The strains of *L. monocytogenes* (ATCC 7644 and 19,117), *Listeria innocua* (CLIP 12,612), *Enterobacter cloacae* (ATCC 13,047) and *Bacillus cereus* (ATCC 11,778) were provided by the Laboratory of Food Microbiology (Federal University of Pelotas, Pelotas, RS, Brazil).

Bacterial cultures

In order to obtain bacterial activation prior to use, *L. monocytogenes* and *L. innocua* strains were cultivated at 37 ºC for 16- 18 h in tryptic soy broth (TSB, 22,092, Sigma-Aldrich, St. Louis, USA) supplemented with 0.6% (w/v) yeast extract (Y1,625, Sigma-Aldrich). The strains of *E. cloacae* and *B. cereus* were cultivated in brain heart infusion broth (BHI, 53,286, Sigma-Aldrich) at 37 °C for 16- 18 h. The bacterial cultures were diluted ten-fold (ratio factor 1:5), and each dilution was plated onto TSB agar plates to count colony forming units (CFU). The optical density (OD_{600}) of each dilution was recorded (Metter Toledo, Columbus, USA) and the OD values were plotted on graphs to generate standard curves and linear regression equations from bacteria growth (Fig. 1).

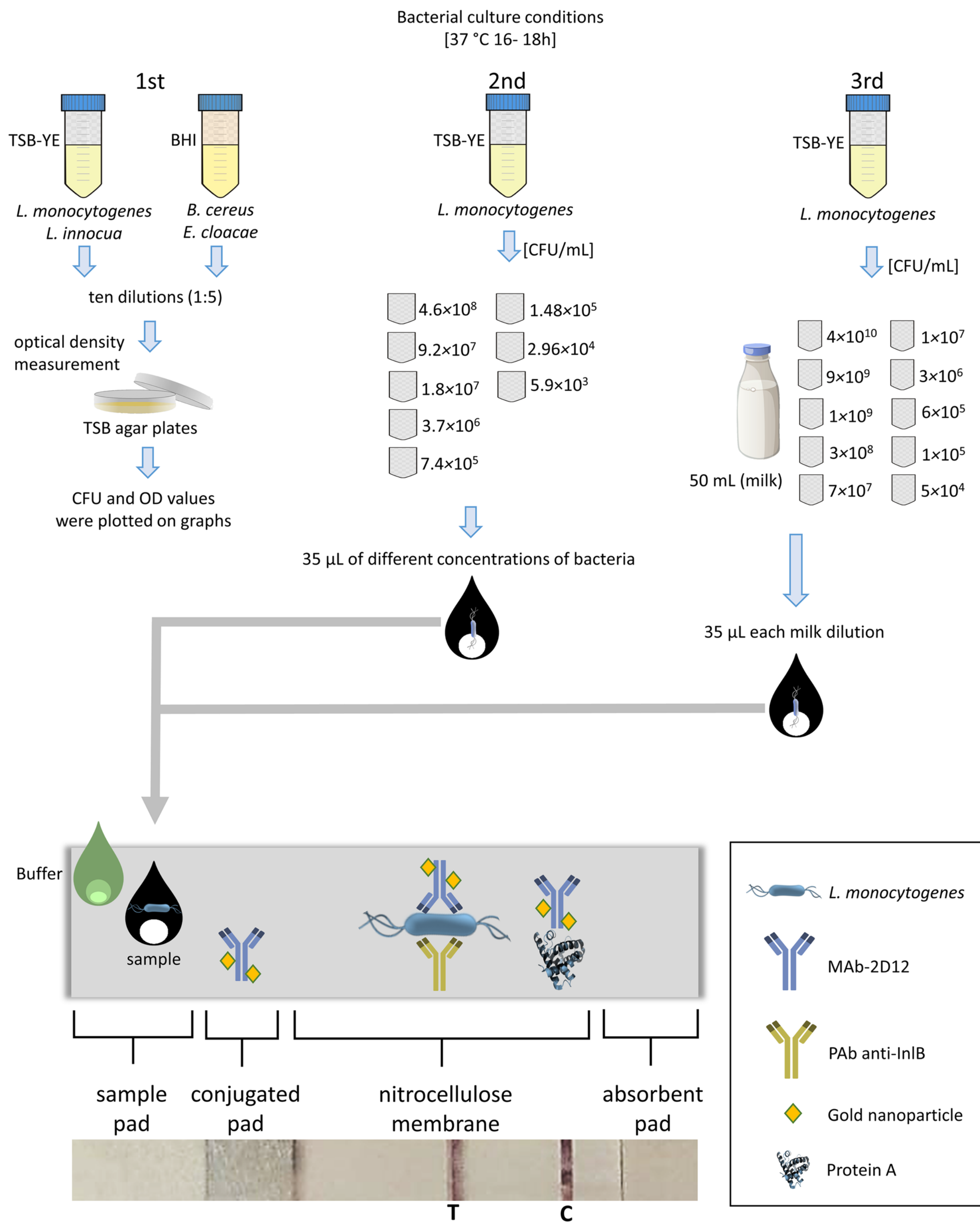

Fig. 1 Methodology employed for the development of the prototype lateral flow immunochromatography assay (LFIA) for the detection of *Listeria monocytogenes* by using a combination of antibodies: anti-Internalin A (MAb-2D12) conjugated to colloidal gold nanoparticles and anti-Internalin B (PAb anti-InlB). Pure cultures of *L. monocytogenes* and artificially contaminated milk (2nd and 3rd schemes) were used as test samples while cultures of *Listeria innocua*, *Bacillus cereus* and *Enterobacter cloacae* (1st scheme) were used as specificity controls (1st scheme). Bacterial cultures were plated onto TSB agar plates and colony forming units (CFU) were counted (1st scheme). The optical density (OD_{600}) of each dilution was recorded, and values were plotted on standard curves and linear regression equations were based on the concentrations (CFU/mL) and respective ODs. Next, *L. monocytogenes* was cultivated in TSB-YE and eight different dilutions (2nd scheme) were tested with the prototype LFIA. Lastly, ten different dilutions of *L. monocytogenes* were artificially inoculated into pasteurized milk samples (3rd scheme) and these samples were tested in lateral flow prototypes. TSB-YE: Tryptic Soy Broth with Yeast Extract; BHI: Brain Heart Infusion; OD: optical density; T: test line; C: control line; CFU: colony formed units

Preparation of the *L. monocytogenes* Internalin lateral flow immunochromatographic assay (LM Internalin LFIA) prototype

As depicted in Fig. 1, the LFIA prototype developed in this study was composed of a sample pad, a conjugated pad containing 33 µg/mL of MAb-2D12 conjugated to colloidal gold nanoparticles (AuNP, 520,918, Sigma-Aldrich), a nitrocellulose (NC) membrane containing the test and the control lines and an absorbent pad. As previously described, the MAb-2D12 is an IgG2a antibody, highly specific to the *L. monocytogenes* Internalin A epitope that recognizes all *L. monocytogenes* serotypes (Mendonça et al. 2012). The conjugation of gold nanoparticles (AuNP) to the MAb-2D12 was performed as described elsewhere (Snowden and Hommel 1991; Gussenhoven et al. 1997). Anti-InlB polyclonal antibodies at 1 mg/mL and protein A were dropped on the NC membrane (Test/T and Control/C lines, respectively). A XYZ Airjet Dispenser System from BioDot Inc (Irvine, CA, USA) was used to deposit the capture agent in NC cards and to dispense the conjugate in the fiber-fleece. Lastly, the assembled card was cut into strips (5 mm width) using CM4000 BioDot Paper Cutter (Irvine, CA, USA) and stored at 4 °C until the strips were built into a proper plastic housing.

Determination of the limit of detection (LOD) of the LM Internalin LFIA prototype test

To determine the LOD of the test, pure culture of *L. monocytogenes* (TSB medium, 37 °C for 16–18 h) was used in different concentrations that were calculated by linear regression equation: 5.9×10^3, 2.96×10^4, 1.48×10^5, 7.42×10^5, 3.71×10^6, 1.85×10^7, 9.28×10^7, 4.64×10^8, 1×10^9 CFU/mL. The *L. monocytogenes* dilutions were prepared in TSB medium and PBS pH 7.2, and 35 µL of each dilution was tested with the LFIA prototype. Test results were visually read after 15 min and the test was considered valid when the control line was clearly visible. The tests were considered positive whenever a distinct purple staining of the test line was observed. To assess the prototype reproducibility, a second test batch and a second curve were prepared and tested by an independent researcher (data not shown) using a different lot and the respective concentrations of *L. monocytogenes* culture, prepared as previously described. To assess the test specificity, *L. innocua, E. cloacae* and *B. cereus* strains were cultivated in BHI at 37 °C for 24 h and different dilutions were tested as described for *L. monocytogenes* strain.

Preparation of *L. monocytogenes* contaminated sample food and detection

A food sample, of artificially contaminated pasteurized milk, purchased from a local market was tested with the prototype. For this, *L. monocytogenes* at 5.0×10^9 CFU/mL (TSB medium) was artificially inoculated into 50 mL of the milk sample and homogenized for 1 min. Nine serial dilutions of the artificially contaminated milk were prepared and each milk dilution was used as an individual sample for testing.

Results

Evaluation of the performance of the LM Internalin Prototype LFIA to detect *L. monocytogenes* and of its sensitivity

The prototype LM Internalin LFIA was evaluated for its capacity to detect different concentrations of *L. monocytogenes* obtained from pure cultures. For the quantitative determination of *L. monocytogenes* in culture samples, the linear relationship between the optical density (OD_{600}) and the log concentrations was developed which ranged from 1×10^1 to 1×10^{10} (linear regression equation $y = 4.754 \times 10^9\, x - 2.570 \times 10^8$; $R^2 = 0.9975$) (Fig. 2 and Fig. S1). When the two batches of cultures were tested, the LOD of the LM Internalin LFIA prototype was 5.9×10^3 CFU/mL (Fig. 3a) and 1.5×10^4 CFU/mL (Fig. 3b) of *L. monocytogenes*, respectively.

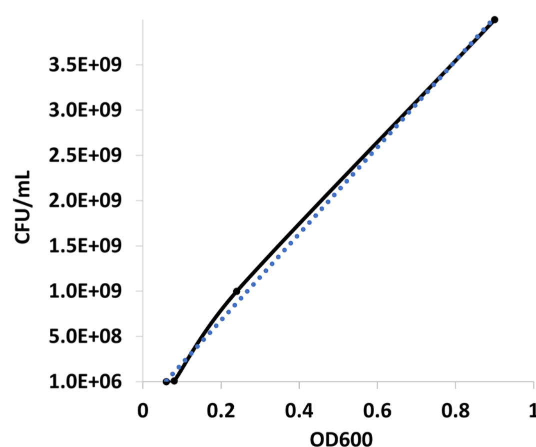

Fig. 2 Correlation between optical density of *L. monocytogenes* cultures and bacterial concentration. *L. monocytogenes* grown in tryptic soy broth were serially diluted and plated to determine CFU. A graph correlating the ODs and CFU/mL was plotted and the linear regression equation was determined. CFU: colony forming units

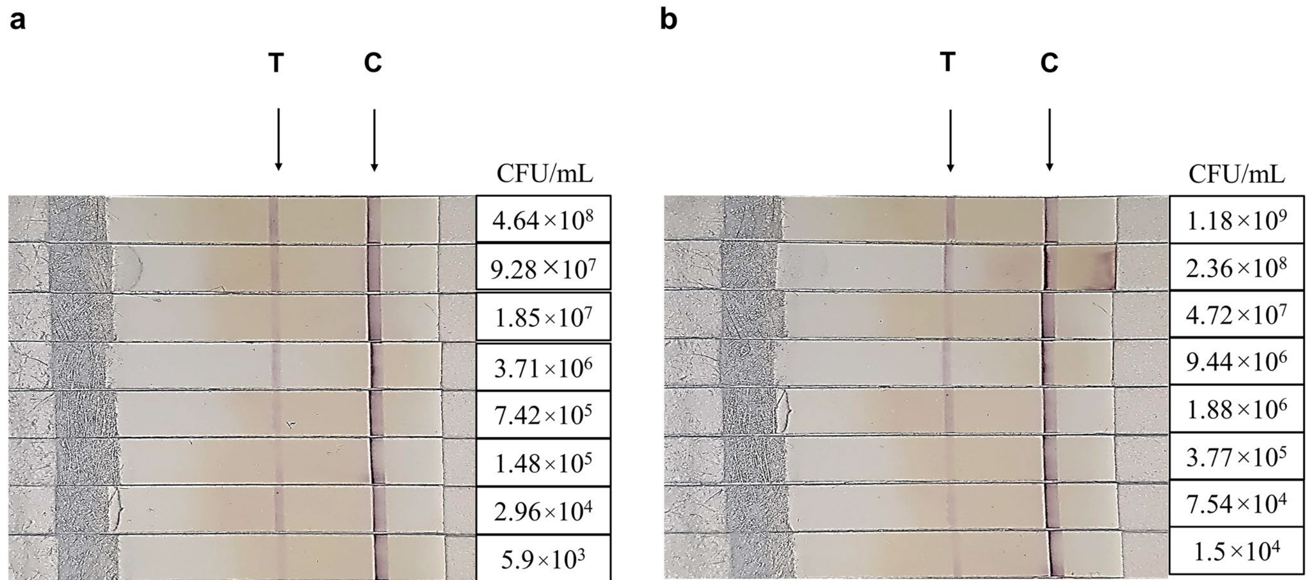

Fig. 3 Evaluation of the limit of detection (LOD) of the LM Internalin LFIA prototype for *L. monocytogenes* detection employing different culture concentrations from batches 1 (A, LOD 5.9×10^3) and 2 (B, LOD 1.5×10^4). T: test line; C: control line; CFU: colony forming units

LM internalin LFIA and the detection of *L. monocytogenes* in artificially contaminated milk

Commercially available pasteurized milk contaminated in the laboratory with different concentrations of *L. monocytogenes,* obtained from cultures, were tested with the LM Internalin LFIA. *L. monocytogenes* concentrations in milk samples ranged from 5×10^4 CFU/mL to 4×10^{10} CFU/mL and as depicted in Fig. 4 *L. monocytogenes* in milk was detected in the range of 1×10^5 to 4×10^{10} CFU/mL concentrations.

Evaluation of the specificity of the LM Internalin LFIA prototype

L. innocua, E. cloacae and *B. cereus* strains at concentrations of 5×10^9 CFU/mL tested negative with the LM Internalin LFIA (Fig. 5).

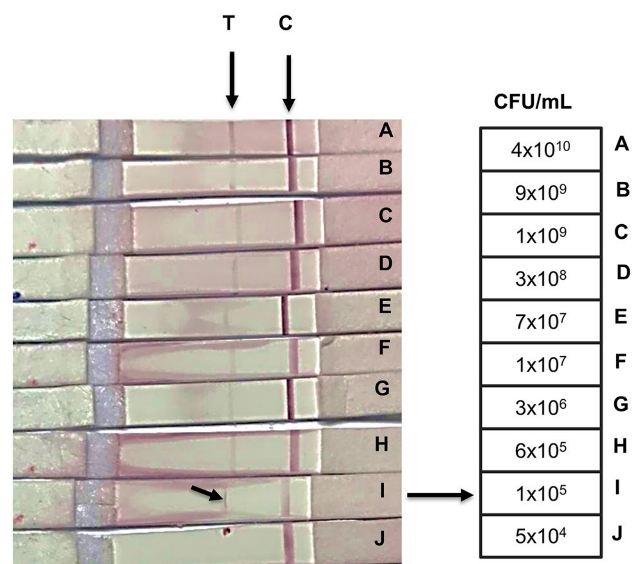

Fig. 4 Evaluation of the limit of detection (LOD) of the LM Internalin LFIA prototype for *L. monocytogenes* in artificially contaminated pasteurized milk. The following concentrations were used: 4×10^{10}, 9×10^9, 1×10^9, 3×10^8, 7×10^7, 1×10^7, 3×10^6, 6×10^5, 1×10^5, 5×10^4 CFU/mL)\; CFU: colony forming units

Discussion

This study presents the results of a sensitive and specific prototype rapid lateral flow immunoassay for *L monocytogenes* detection (LM Internalin LFIA) which was developed based on the use of anti-Internalin A and B antibodies. Highly specific and sensitive antibodies produced against immunogenic virulence/adhesion factors of *L. monocytogenes,* such as the Internalin A and B, are considered key reagents for the development of immunodetection tools (Li et al. 2017). *L. monocytogenes* is a ubiquitous opportunistic pathogen found in food-processing environments and food products (Bhunia 2018) representing both a constant threat to the food industry and also a challenge to detect and eliminate this pathogen from final products and from the processing environments (Phraephaisarn et al. 2017). The advantage of a qualitative rapid test for *L. monocytogenes* detection, as the one

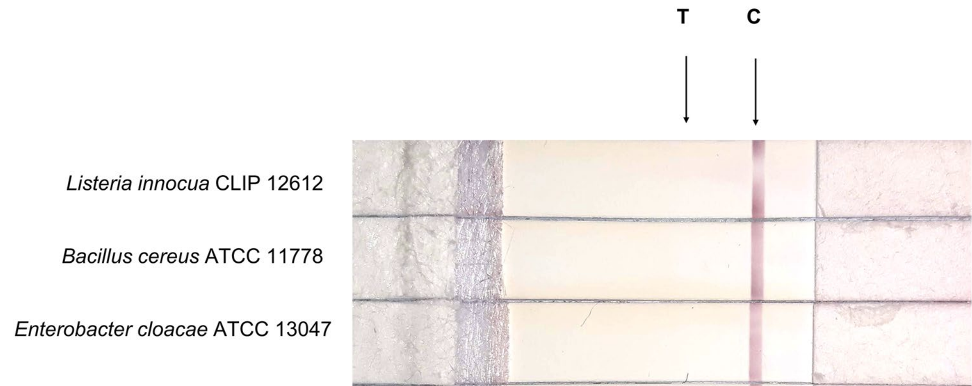

Fig. 5 Evaluation of the specificity of LM Internalin LFIA prototype using culture samples of *L. innocua* (CLIP 12,612 at 5×10^9 CFU/mL), *B. cereus* (ATCC 11,778 at 5×10^9 CFU/mL) and *E. cloacae* (ATCC 13,047 at 5×10^9 CFU/mL). T: test line; C: control line

described here in, lies on the fact that immunochromatography represents the simplest and quickest method for pathogen detection in a sample requiring around 15 min for final results (Liu et al. 2017; Wu 2019). Also, the use of colloidal gold nanoparticles as a detection label, allows the easy monitoring of the color reaction which can be scored by naked eye, without the use of any costly equipment. Although outbreaks are relatively rare, consumption of *L. monocytogenes* tainted food products can cause overwhelming health and economic consequences (Hoffmann et al. 2014). Therefore, a high-throughput, rapid, specific and sensitive method to detect *L. monocytogenes* is always highly needed to guarantee the safety and quality of food, especially meat and dairy products. Considering the features of the LM Internalin LFIA reported in this study, it represents a potential point-of-care (POC) diagnostic tool for *L. monocytogenes* detection.

Another potential advantage of the use of a sensitive, rapid lateral flow tests is the direct detection without the need of previous conventional culture methods, that although simple, of low cost and reliable, may take 5– 7 days for its detection and confirmation in food products (Bhunia 2014 2018). The prototype LM Internalin LFIA developed using a combination of Internalin A and B antibodies was able to directly detect *L. monocytogenes* both from culture and in artificially contaminated milk with good sensitivity. Although other food products were not tested, it is possible that it may be able to identify the pathogen in processed food products without the need of previous culture. Usually, due to the low number of contaminating bacteria in food samples, pre-enrichment cultures are needed to expand bacterial growth to detectable numbers, however, besides the long time needed, during the culture time, the suspected sample can be contaminated by sample residues and by other bacteria (Ueda et al. 2013; Bhunia 2014; Li et al. 2017). An ideal diagnostic tool for the detection of *L. monocytogenes* and the prevention of listeriosis outbreaks should be simple, specific, sensitive, affordable and provide rapid results after testing as the lateral flow test provides.

We consider that the use of highly specific and sensitive anti-Internalin antibodies employed in this LFIA was crucial for achieving good sensitivity, especially the use of the monoclonal antibody Mab-2D12 which was conjugated to colloidal gold nanoparticles and employed as a label signal. The anti-InlA MAb-2D12 was previously described to be specific for *L. monocytogenes* and *L. ivanovii* and when used in immunomagnetic separation assay provided highly specific capture efficiency for both bacteria enabling their detection at low levels from buffer or food using fiber-optic sensor (Mendonça et al. 2012). In immunodiagnostic tests, antibodies have been employed as affinity ligands to separate and concentrate the target analyte from sample matrices when coupled to paramagnetic beads (Banada and Bhunia 2008; Mendonça et al. 2012; Bhunia 2014) or as recognition or reporter molecules (Tully et al. 2008; Dwivedi and Jaykus 2011). Different immunologic detection methods including ELISA (Mendonça et al. 2012; Lv et al. 2019), colloidal gold immunochromatography (Ueda et al. 2013; Cho et al. 2015; Liu et al. 2017; Wu 2019) and immunomagnetic separation method (Uusitalo et al. 2016) have been described for the detection of pathogens and toxins. However, one the greatest pitfalls of immunologic methods to detect *L. monocytogenes* is their low sensitivity characterized by a high bacteria concentration for their LOD. The prototype LM Internalin LFIA presented in this study showed good sensitivity in two batches of bacterial cultures (5.9×10^3 and 1.5×10^4 CFU/mL) and from these concentrations, the color signals produced by gold nanoparticles at the test line on the nitrocellulose immunostrip increased. The LOD of commercially available lateral flow rapid tests (Dupont Qualicon, Neogen Corp., and Oxoid Ltd) ranged from 10^5 to 10^6 cells/mL (Ueda et al. 2013). Other studies have reported tests with lower sensitivity for *L. monocytogenes* detection: Ueda et al. (2013) (LOD 6.9×10^6 CFU/mL), Li et al. (2017) (LOD 4.0×10^5 CFU/mL). A previous study reported that the use of immunomagnetic beads as bacterial pre-treatment increased the LOD by one log (4.0×10^4 CFU/mL) however, this increase in sensitivity required an additional step of

pre-enrichment, making it more complex and costly (Li et al. 2017). Therefore, the good sensitivity of the LM Internalin LFIA without pre-enrichment step increases the chances of its applicability in the field as a POC.

When the LM Internalin LFIA was performed with artificially contaminated milk, the obtained LOD (1×10^5 CFU/mL) was lower than the sensitivity of a previously described lateral flow enzyme concentration assay using artificially contaminated milk samples (LOD of 1×10^2 CFU/mL) (Cho and Irudayaraj 2013). However, this test required a special equipment because of the additional step for bacterial separation and concentration by magnetic nanoparticles. A fluorescent lateral flow using immunomagnetic separation of bacteria in artificially contaminated milk reported a LOD of 1×10^4 CFU/mL (Li et al. 2017), nevertheless with the drawback of additional time and cost. The sensitivity of the tests mentioned above was improved by combining other non-immunological techniques which added complex reading steps, while the LOD of our prototype defined by visual readings was established by the use of highly specific antibodies against InlA and InlB proteins that are present on the bacterial surface (Radoshevich and Cossart 2018; Drolia and Bhunia 2019).

Another important feature of a diagnostic tool refers to its specificity. No cross reaction was observed when pure culture of *B. cereus*, *E. cloacae* and *L. innocua* at concentration of 5×10^9 CFU/mL were tested indicating that the LM Internalin LFIA can discriminate *L. monocytogenes* from other *Listeria* species and from other non-*Listeria* gastroenteric bacteria. The absence of cross-reaction with *L. innocua* is also an advantage of the prototype described, as *L. innocua* is the most frequently found bacteria in *Listeria*-contaminated food, representing a difficulty for the specific capture and detection of the pathogenic Listeria (Oravcová et al. 2008). Also, food products tainted with *L. monocytogenes* may often be contaminated with other *Listeria* spp. both pathogenic and non-pathogenic as well as other background microbiota and, as *L monocytogenes* grows slowly, other bacteria may outcompete, so lower concentrations of *L. monocytogenes* may be expected and lead to false-negative results (Gnanou Besse et al. 2010; O'Connor et al. 2010). As our prototype did not require pre-enrichment step, the possibility of false negative results due to overgrowth of other species is reduced. As previously mentioned, the Mab used in the LM Internalin LFIA is specific for both human pathogenic bacteria, *L. monocytogenes* and *L. ivanovii* (Mendonça et al. 2012), therefore although not tested, this prototype has also the potential to detect the pathogenic *L. ivanovii* that is an opportunistic pathogen that is associated with gastroenteritis and bacteremia in humans (Snapir et al. 2006).

Although promising, we acknowledge that this study has limitations such as the need to test if the LM Internalin LFIA can detect *L. monocytogenes* in other types of food besides milk. However, a previous study demonstrated that the MAb-2D12 was used as a capture agent and reporter antibody in a fiber-optic sensor test that was able to detect *L. monocytogenes* in hot dogs and soft cheese (Mendonça et al. 2012). It is known that the recovery of low numbers of pathogens from complex food matrices can interfere with their rapid and sensitive detection (Bhunia 2008). Moreover, the use of polyclonal antibodies limits the detection of low populations of *L. monocytogenes* because antigen recognition is less specific, and antibodies can cross-react with the food matrix (Bhunia 2008). There is no availability of anti-InlB monoclonal antibodies on the market, limiting our current capacity to improve the LOD of our prototype. We also acknowledge that the capacity of the LM Internalin LFIA to discriminate other pathogens should be tested.

Conclusions

We have developed a sensitive and specific rapid lateral flow immunochromatographic assay to detect *L. monocytogenes* based on the use of anti-Internalin A monoclonal antibodies and anti-Internalin B polyclonal antibodies. Our prototype test detected *L. monocytogenes* from cultures and in artificially contaminated milk with good sensitivity compared to commercially available tests. No cross reaction was observed with the non-pathogenic *L. innocua* and other gastro-enteric bacteria (*B. cereus*, *E. cloacae)*. Preliminary data on the sensitivity and specificity of this prototype test, which provides visual results within 15 min, indicate its potential use as a point-of-care test, for the rapid detection of *L. monocytogenes* in contaminated food or industry environment.

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Authors' contributions Conception and design: RSM, ESF, MM, FRC, MMAS, SBS; Acquisition of data: RSM, ESF, AV, DRS, ANM, TGS; Analysis and interpretation of data: LLL, ESF, RSM, AK, MMAS, SBS; Drafting the manuscript: LLL, ESF, SBS, MMAS; Revising the manuscript critically: LLL, RSM, AK, MM, FRC, MMAS, SBS; Approval of the final manuscript: LLL, ESF, MM, ANM, AV, DRS, TGS, RSM, MMAS, FRC, AK, SBS.

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Code Availability Not Applicable.

Declarations

Conflicts of interest The authors declare that they have no conflict of interest.

Consent to participate Not Applicable.

Consent for publication Not Applicable.

Ethics approval Not Applicable.

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