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Performance evaluation of a low conductive growth medium (LCGM) for growth of healthy and stressed *Listeria monocytogenes* and other common bacterial species

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Abstract

The performance of a low conductive growth medium (LCGM) (conductivity of <1300 μS) was evaluated for its ability to support growth of food borne bacterial pathogens including *Listeria monocytogenes* and to determine the expression of the two key virulence proteins in *L. monocytogenes* for possible applications in an impedance-based microfluidic biochip detection platform. Growth of *Listeria* was monitored spectrophotometrically and the lag phase, generation time, growth rate and maximum population density were determined using the Gompertz equation. LCGM had a lag phase of 2.3 h and showed a higher cell density compared to Luria Bertini (LB) broth. Length of lag phase was highly dependent on initial inoculum concentrations. The changes in conductivity with respect to growth in the low conductive medium were monitored using a conductivity probe. *L. monocytogenes* growth could be detected within 2 h (0.1 mS) in LCGM and within 6 h in LB. The performance of the media was also evaluated for the recovery of *Listeria* cells exposed to various stresses as 42 °C for 1, 2 or 6 h, an osmotic stress in 10.5% NaCl, an acidic stress at pH 2, 3 or 5 and a combined stress of 10.5% NaCl, pH 5 and 1 h exposure at 42 °C. The recovery rate was comparable with that of Tryptic soy broth containing yeast extract (TSBYE). *L. monocytogenes* in LCGM supported the expression of two key virulence markers, actin polymerization protein (ActA) and internalin B (InlB), which could be detected using specific antibodies. In general LCGM also supported the growth of several other bacterial species suggesting its implication in microbial quality monitoring of products. In conclusion, LCGM is a sensitive low conductive medium that supports the growth as well as the expression of virulence markers for potential applications in sensitive detection of *L. monocytogenes* or other food borne pathogens in impedance-based sensor platform.

Keywords: Low conductive growth medium; Bacteria; *Listeria monocytogenes*; Conductivity; Internalin B; ActA

1. Introduction

The presence of bacteria in water, foods, biological or environmental samples could be monitored by measuring the physicochemical changes caused by bacterial growth or metabolism. The following techniques are commonly employed which are based on conductance or impedance measurements (Wawerla et al., 1999), bioluminescence assay to detect bacterial ATP (adenolsine triphosphate) (Griffiths, 1993; Lee and Deininger, 2004; VanderZee and Huisin'tVeld, 1997), fluorescence to detect viable bacteria by direct epifluorescent filter technique (DEFT) (McLauchlin and Pini, 1989), analysis of cellular fatty acid compositions of bacteria by using gas chromatography as in microbiological identification system (MIS) of Microbial ID (Newark, DE) (Dziezak, 1987) and by metabolic fingerprinting by Biolog system (Hayward, CA).

Although the concept of impedance microbiology is more than a century old, it gained its popularity only in the mid-seventies. Impedance has been defined as the resistance to flow of an alternating current as it passes through a conducting material. Microbial metabolism results in an increase in both conductance and capacitance causing a decrease in impedance and a consequent increase in admittance; it is the admittance, which is plotted

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against time by the Rapid Automated Bacterial Impedance Technique (RABIT) system (Bolton, 1990). The final electrical signal is frequency and temperature dependent and has a conductive and a capacitative component (Eden and Eden, 1984). The commercially available impedance-based systems include Malthus AT analyzer (Malthus Instruments, Bury, UK), Vitec-Bactometer system (BioMerieux, Marcy l'Etoile, France), BacTrac™ and μ-Trac microorganism growth analyzer (Sy-Lab, Purkersdorf-Vienna, Austria).

Bacterial contamination in milk has been analyzed using the Bactometer for quality assessment purposes (Biggadike et al., 2000; Garro et al., 2001). For specific detection of *Listeria*, this system uses a high conductive *Listeria* electrical detection (LED) medium with a conductivity of >2 mS and *Listeria* selective supplements, and it takes about 30 h to induce a 30% change in capacitance (Capell et al., 1995). This medium was applied for the detection of *Listeria* in cheese samples, which was shown to be better than the Fraser broth (Rodrigues et al., 1995; Duarte et al., 1999). Sy-Lab (Purkersdorf-Vienna, Austria) also markets general impedance broth, which has a conductivity value of about 2.8 mS.

Sensitivity of the impedance-based detection was substantially improved in a microfluidic biochip using Luria Bertani (LB) broth, which uses nanoliter volumes of the sample (Gomez et al., 2001, 2002a,b). Because of the decreased threshold for conductivity of the biochip, the existing commercial media cannot be used. A newly formulated commercial low conductive growth medium (LCGM; BioVittesse, Inc., San Jose, CA, USA) was evaluated for its performance with a battery of bacterial cultures including *Listeria* for use with our biochip or any other applications where low conductive media is employed for growth monitoring by impedance microbiological methods. In addition, growth patterns of previously stress-exposed cells were determined in order to check the efficiency of this media in recovering injured or stressed cells. Ability of this medium to support the expression of surface associated virulence factors including internalin B (InlB) and actin polymerization protein (ActA) was examined. This was important since specific antibody or nucleic acid probes for target could be introduced into the biochip platform for selectivity.

Table 1
List of cultures used for growth analysis in low conductive growth medium

Cultures (serotypes)	Growth by 24 h (OD_{595})	Growth grouping
Listeria monocytogenes V7 (1/2 a)	0.590	A
Listeria monocytogenes F4233 (1/2 b)	0.500	A
Listeria monocytogenes ATCC 2540 (3b)	0.268	B
Listeria monocytogenes SLCC 2373 (3a)	0.434	B
Listeria monocytogenes SLCC 2479 (3c)	0.188	C
Listeria monocytogenes SLCC 2482 (7)	0.147	C
Listeria monocytogenes Murray B (4ab)	0.408	B
Listeria monocytogenes ATCC 19116 (4c)	0.496	A
Listeria monocytogenes F4244 (4b)	0.511	A
Listeria monocytogenes Scott A (4b)	0.468	B
Listeria monocytogenes ATCC 7644 (1/2c)	0.318	B
Listeria monocytogenes ATCC 19114 (4a)	0.289	B
Listeria innocua ATCC 33090	0.466	B
Listeria seeligeri SE31	0.426	B
Listeria grayi ATCC 19120	0.520	A
Listeria welshimeri ATCC 35897	0.281	C
Listeria ivanovii V12 (5)	0.355	B
Enterobacter aerogenes	0.394	B
Enterococcus faecalis ATCC 344	0.794	A+
Enterococcus faecalis CG110	0.320	B
Staphylococcus aureus β-lysin+	0.357	B
Bacillus subtilis	0.413	B
Bacillus cereus ATCC 14579	0.229	C
Bacillus cereus MS1-9	0.287	B
Bacillus cereus A926	0.188	C
Bacillus cereus HS23-11	0.272	B
Bacillus cereus AS41-2	0.364	B
Bacillus polymyxa	0.252	B
Salmonella enterica subsp. Enteritidis ATCC 13096	0.460	B
Salmonella enterica subsp. Typhimurium #25	0.310	B
Escherichia coli EDL 933 (O157:H7)	0.672	A
Escherichia coli K12	0.294	B
Serratia marcescens	0.354	B
Pseudomonas aeruginosa ATCC 10145	0.762	A+
Escherichia coli ATCC 43890 (O157:H7)	0.191	C
Escherichia coli ATCC 35150	0.202	C
Salmonella enterica subsp. Typhimurium	0.131	C
Lactobacillus plantarum	0.192	C

Growth was arbitrarily grouped into A+, excellent growth; A, good growth; B, medium growth; C, fair growth and was in no comparison of growth in any other media.

2. Materials and methods

2.1. Bacterial cultures

Listeria monocytogenes V7 serotype 1/2a (Ribotype DUP-1039) was used as standard culture for this study. Brain heart infusion (BHI) broth (Difco, Sparks, MD) was used for inoculum preparation. The performance of the LCGM was tested using the cultures listed in Table 1.

2.2. Media

Ready to use sterile LCGM broth, containing proprietary formulations of tryptone, yeast extract and bovine serum albumin, was procured from BioVittesse, Inc. (San Jose, CA). Luria Bertani (LB) broth was prepared as per Difco formulations (Difco Manual, Sparks, MD) with some modifications which contained tryptone (5 g/l) and yeast extract (3 g/l), pH 7.2±.2 (Gomez et al., 2002a). Antibiotic supplements [modified oxford agar supplement (Difco), containing moxalactam (0.15%) and colistin sulphate (0.1%)] were added to LB (LBs) and LCGM (LCGMs) to provide selectivity for *L. monocytogenes*. Other media such as *Listeria* repair broth (LRB) (Busch and Donnelly, 1992), buffered *Listeria* enrichment broth (BLEB) (Difco, Sparks, MD) and a general impedance broth or total viable count (TVC) media (SYlab, Purkersdorf-Vienna, Austria) were used as comparative media for evaluation purposes only.

The initial conductivity of each medium was recorded at room temperature using a conductivity meter (Accumet AP85, Fisher Scientific International, Inc., Hampton, NH). Tryptic soy broth with 0.5% yeast extract (TSBYE) was used as a comparative medium for recovery of stressed cells.

2.3. Growth kinetics

2.3.1. Inoculum preparation

Initial inoculums of the cultures were prepared by growing the bacteria in brain heart infusion (BHI) broth at 37 °C for 16–18 h. One milliliter of each culture was centrifuged (8000 ×*g*, 5 min), cell pellets were washed three times with sterile distilled water to remove any residual media components, and finally resuspended in 1 ml of the sterile distilled water. Similar fresh cultures were prepared for each experiment.

2.3.2. Growth of L. monocytogenes

One percent of the inoculum was added into 3 ml of various media and was incubated at 37 °C in DU-640 spectrophotometer (Beckman Instruments, Inc., Fullerton, CA) configured for data acquisition at every 20 min intervals. The growth was measured at OD_{595} for up to 16–24 h. The growth was also monitored spectrophotometrically in a plate reader (Bio-Rad, Hercules, CA). The 96-well sterile untreated tissue culture plates (Evergreen scientific, Los Angeles, CA) with 250 μl of each medium per well and inoculated with 1% of each culture are listed in Table 1. Growth was measured periodically, by reading the absorbance at 595 nm in a microplate reader (BioRad). Prior to the readings, the plate contents were mixed for 15 s using the preset mixing program to ensure uniform dispersion of cells in the wells. All the experiments were done in triplicates, unless otherwise indicated.

2.3.3. Effect of inoculum size on the growth of L. monocytogenes in LCGM

To determine the lag time for the varying inoculum concentrations, the cells were inoculated into LCGM with 10^3–10^7 CFU/ml and was incubated at 37 °C in DU-640 spectrophotometer (Beckman). Viable plate counts on BHI agar were also carried out for cell inoculum levels of 10^5 and 10^6 CFU/ml over a period of 24 h with every 2 h intervals.

2.4. Validation of growth rate by real-time conductivity measurement

L. monocytogenes was inoculated at 1% concentration into LB and LCGM and the conductivity probe (Jenco Instruments, San Diego, CA) was immersed into the tube containing the inoculated media and the tube was sealed with UV sterilized parafilm to avoid contamination. The changes in conductivity were recorded digitally at every 10 min interval for up to 24 h. Control experiments without the inoculation were run in parallel to confirm the sterility. Separate experiments were run for each media in triplicates.

2.5. Growth analysis of stressed cells in LCGM

2.5.1. Heat stress (45 °C)

L. monocytogenes was grown in BHI broth for 16–18 h at 37 °C, in an orbital shaker incubator (New Brunswick Scientific Co, Edison, NJ) set at 140 rpm. An aliquot was taken for initial viable plate count. The culture tubes were transferred to 45 °C and were incubated for 1, 2 and 6 h. At each time point, the cells were washed twice in sterile water by centrifuging at 8000 ×*g* for 5 min. Finally, the cell pellets were suspended in sterile water and 1% of this was used as inoculum for recovery studies using LCGM and an aliquot was used for viable plate count.

2.5.2. Osmotic stress (10.5% NaCl), acid stress (HCl: pH 2, 3 and 5) and combined stress (10.5% NaCl, pH 5 and 45 °C for 1 h)

L. monocytogenes was grown in BHI broth as above and an aliquot was drawn for viable plate count and the rest of the culture was centrifuged (8000 ×*g* for 5 min), the supernatant was discarded and an equal volume of BHI broth with a final concentration of 10.5% NaCl was added (for osmotic stress) or BHI broth adjusted to pH 2, pH 3 or pH 5 (for the acid stress) or BHI broth with a final concentration of 10.5% NaCl, adjusted to pH 5, for combined stress, was mixed with the cell pellets. Cultures receiving osmotic and acid stress were incubated at 37 °C for 1 h and 3 h, respectively. Culture receiving combined stress was incubated at 45 °C for 1 h. An aliquot was taken for viable plate count and the remaining culture was centrifuged and resuspended in sterile water. One percent of this preparation was used as an inoculum for recovery studies using LCGM and was compared with TSBYE.

2.6. Expression of surface proteins, internalin B (InlB) and actin polymerization protein (ActA) in LCGM by Western blotting

Crude surface proteins were extracted from *L. monocytogenes* cells following growth in LB and LCGM for 12 h under static conditions at 37 °C. The cell concentrations were adjusted to 0.5 at OD_{595}, washed and treated with 150 μl of SDS-PAGE sample solvent (Bhunia et al., 1991). The cell pellets were mixed well by repeated pipetting and were incubated for 1 h at 37 °C, centrifuged (10,000 ×*g*, 10 min), and 50 μl of each preparation was loaded per well and electrophoresed in 10% pre-cast SDS-PAGE (Bio-Rad). Proteins were transferred onto Immobilon-P membranes (Millipore, Bedford, MA) and immunoprobed with either MAb C11E9 (1:500 of 1 mg/ml) (Bhunia et al., 1991) or anti-ActA or anti-InlB polyclonal antibodies (1:100 of 1 mg/ml) (Lathrop et al., 2004).

2.7. Statistical analysis

Growth of *L. monocytogenes* was modeled and the growth parameters were calculated using the following Gompertz equation (Zwietering et al., 1990; Silk et al., 2002):

$$y = a.\exp[-\exp(b-ct)]$$

where: y = absorbance value (OD_{595}); t = time at which each absorbance was read; a, b, c = Gompertz growth parameters.

The curves were fitted with least square error minimization method using MATLAB (Version 6.5.1.199709 Release 13, ITAP, Purdue University). Gompertz equation parameters were

Table 2
Comparison of *Listeria monocytogenes* growth in various low and high conductive media

Media[a]	Initial conductivity	Exponential growth rate	Generation time [h]	Lag phase duration [h]	Maximum population density
LCGM	1280 μS*	0.08 ± 0.017^{B}	12.9 ± 2.77^{B}	2.3 ± 0.45^{AB}	0.41 ± 0.06^{C}
LB	1200 μS	0.07 ± 0.004^{B}	12.7 ± 0.21^{B}	1.7 ± 0.25^{B}	0.2 ± 0.009^{D}
LCGMs	1500 μS	0.04 ± 0.021^{B}	28.9 ± 2.72^{A}	3.44 ± 0.52^{AB}	0.18 ± 0.02^{E}
LBs	1500 μS	0.04 ± 0.007^{B}	26.2 ± 2.86^{A}	4.05 ± 0.57^{A}	0.16 ± 0.01^{E}
TVC	2800 μS	0.1 ± 0.02^{AB}	10.3 ± 2.45^{C}	3.02 ± 1.66^{AB}	0.46 ± 0.01^{C}
LRB	15.08 mS†	0.15 ± 0.01^{A}	6.85 ± 0.58^{C}	3.44 ± 0.98^{B}	1.73 ± 0.19^{A}
BLEB	16.00 mS	0.16 ± 0.05^{A}	9.24 ± 0.18^{C}	4.29 ± 0.06^{A}	0.88 ± 0.02^{B}

[a]Media designations are; LCGM, low conductive growth medium; LB, Luria Bertani; LCGMs, LCGM with antimicrobial selective agents (Moxalactam (0.15%) and Colistin sulphate (0.1%) — see Materials and methods section); LBs, LB with antimicrobial selective agents; TVC, total viable count media (Sy-lab, Austria); LRB, *Listeria* repair broth (Busch and Donnelly, 1992); BLEB, buffered *Listeria* enrichment broth (DIFCO, MD). Values labeled with different capital letters (A, B, C, D, E) in each column are significantly different at $P<0.05$.
*μS — microsiemens.
†mS — millisiemens.

used to calculate the kinetic parameters: generation time (GT)=e/ac; lag phase duration (LPD)=$(b-1)/c$; maximum population density (MPD)=a (Silk et al., 2002).

Statistical comparisons were carried out using the ANOVA (SAS, Cary, NC) and the groupings were based on the Tukey's multiple comparison of means at $P<0.05$.

3. Results

3.1. Growth of L. monocytogenes and other bacteria in LCGM

A total of 38 different bacteria were tested for their ability to grow in LCGM. Growth of individual culture was assessed by monitoring final absorbance reading (OD_{595}) taken after 24 h. Twelve different strains of *L. monocytogenes* and five strains of other *Listeria* spp. and 21 other bacteria were tested and the growth was arbitrarily divided as excellent (A+), good (A), medium (B) or poor growth (C). This grouping is based only on the growth of each bacterium in LCGM and no comparison with other media was made. Of these, *Enterococcus faecalis* ATCC 344 and *Pseudomonas aeruginosa* ATCC 10145 were grouped under A+ since they showed excellent growth reaching an OD_{595} of up to 0.76, followed by the strains of *L. monocytogenes* V7, F4233, F4244 and ATCC 19116, *L. grayi* ATCC 19120, *E. coli* O157:H7 EDL 933, grouped under A, which showed a good growth with an average growth OD_{595} of ca. 0.5. All the other stains reaching an absorbance value of between ca. 0.25 and 0.5 were grouped under B, which included *L. monocytogenes* strains ATCC 2540, SLCC 2373, Murray B, Scott A, ATCC 7644 and ATCC 19114, *L. innocua* ATCC 33090, *L. seeligeri* SE31, *L. ivanovii* V12, *L. welshimeri* ATCC 35897 and other bacterial cultures including *Enterobacter aerogenes*, *E. faecalis, Staphylococcus aureus*, *Bacillus subtilis*, *Salmonella* Enteritidis ATCC 13096, *S.* Typhimurium #25, *E. coli* K 12 and *Serratia marcescens*. The rest of the cultures showing an absorbance value below 0.25 were grouped under C, which included two strains of *L. monocytogenes* SLCC 2479 and SLCC 2482 and six of the other bacterial strains. Nevertheless, all the bacteria reached a minimum OD_{595} of ca. 0.13 when grown in LCGM.

3.2. Growth kinetics for L. monocytogenes in LCGM and comparison with other media

Growth parameters such as exponential growth rate (EGR), generation time (GT), lag phase and maximum population density (MPD) for *L. monocytogenes* in each medium were calculated using Gompertz equation (Table 2). A representative growth curve and the model fitted curves for *L. monocytogenes* grown in LB and LCGM are presented in Fig. 1. Similar model fittings were obtained for other media used in the study. Comparison of the values between the media was carried out using ANOVA and the Tukey's grouping. The commonly used *Listeria* selective media, LRB (15.08 mS) and BLEB (16 mS) were used as controls with high conductive values. These media had the best exponential growth rate of 0.15 and 0.16 OD_{595}/h and high population density (OD_{595}) of 1.73 and 0.88, respectively. The generation time was 6.85 h in LRB and was about 9.2 h in BLEB. The lag time was 3.4 h and 4.29 h in LRB and BLEB, respectively. A commercially available low conductive media TVC (2800 μS) and LB (1200 μS) were used as controls with low conductive values. TVC showed an intermediate growth rate of 0.1 OD_{595}/h and the generation time was statistically similar ($P<0.05$) to that of LRB or BLEB; however, the lag time was about 3 h and the maximum population density was 0.46, which were different from that of LRB and BLEB. In LCGM, the exponential growth rate (0.08 OD_{595}/h), lag time (2.3 h) and maximum population density (0.4) values were

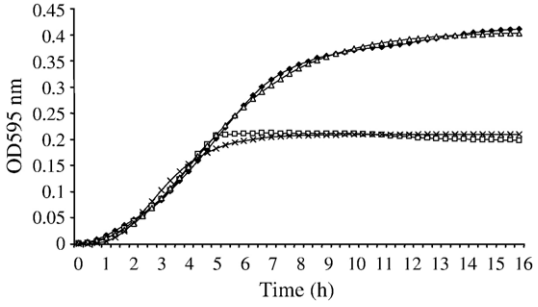

Fig. 1. Comparison of growth of *L. monocytogenes* in Luria Bertani (LB) (□) and in low conductive growth medium (LCGM) (♦) along with the Gompertz fitted curves for LB (×) and LCGM (ρ).

statistically similar to that of TVC. Only significant difference ($P<0.5$) was observed in the generation time; LCGM had 12.9 and TVC had 10.3 h. Most importantly, the conductivity value for LCGM was 1280 μS, while TVC has 2800 μS. Thus LCGM appeared to be a better low conductive medium than TVC. Although LB gave a similar growth rate (0.07 OD_{595}/h), generation time (12.7 h) as that of LCGM, the maximum population density value was decreased by 2-fold. LB had the lowest lag phase value (1.7 h) in comparison to all the media used in the study but the maximum cell density was also the poorest. Addition of selective agents in either LB or LCGM, resulted in poor growth rate (0.04 OD_{595}/h), longer generation time (26–28 h) and lower cell density (0.16 to 0.18). However, the lag phase duration was similar to that of BLEB ($P<0.05$) with about 3.4 or 4 h. In summary, LCGM had statistically similar lag phase, growth rate and generation time, but a better maximum population density and conductivity values compared to two other low conductive media, LB or TVC. However, the presence of selective agents suppresses the growth of *L. monocytogenes* in LCGM.

3.3. Validation of growth rate by real-time conductivity measurement

The absolute changes in conductivity were measured in relation to time. There was a considerable difference when the growth rate in LCGM was compared with LB (Fig 2). To obtain an increase of 0.1 mS in the absolute conductivity, it took about 6 h when *L. monocytogenes* was grown in LB and only about 2 h in LCGM (Fig. 2). Thus the detection time was 4 h less in LCGM compared to LB.

3.4. Growth and recovery of stressed Listeria in LCGM

Efficiency of recovery of stressed *Listeria* in LCGM without any selective agents was determined by comparing the duration of lag phase and maximum population density with the commonly used recovery media TSBYE. *L. monocytogenes* cells were subjected to various stress conditions such as, heat stress at 45 °C for 1 h, 2 h or 6 h. The viable plate count showed the initial cell number before stress to be about 2.4×10^9 CFU/ml which was decreased to about 2×10^9 CFU/ml in 1 h, 1×10^6 CFU/ml in 2 h and was $<10^2$ CFU/ml by 6 h. The recovery of

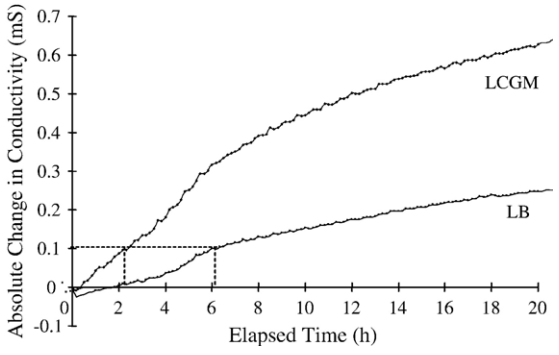

Fig. 2. Online conductivity measurements to determine the growth rate of *L. monocytogenes* V7 in low conductive growth medium (LCGM) and comparison with Luria Bertani (LB) broth.

Table 3
Recovery of stressed *Listeria monocytogenes* cells using low conductive growth medium (LCGM) and comparison with the Tryptic soy broth with yeast extract (TSBYE)

Stress condition	Cell number (CFU/ml)	Media			
		LCGM		TSBYE	
		Lag phase[a]	MPD[b]	Lag phase	MPD[b]
Unstressed cells	$2.4\pm0.14\times10^9$	3 h	0.56±0.02	3 h	0.94±0.01
Heat stress:					
45 °C–1 h	$1.96\pm0.3\times10^9$	3 h	0.53±0.01	3 h	0.93±0.01
45 °C–2 h	$1.0\pm0.04\times10^6$	10.7 h	0.53±0.008	ND	ND
45 °C–6 h	$<10^2$	14.7 h	0.57±0.04	ND	ND
Unstressed cells	$2.4\pm0.15\times10^9$	3 h	0.56±0.02	3 h	0.94±0.01
Osmotic stress:					
10.5% NaCl for 1 h	$1.4\pm0.17\times10^9$	3.6 h	0.43±0.09	3.6 h	0.9±0.01
Unstressed cells	$2.1\pm0.7\times10^9$	2.5 h	0.56±0.02	2.5 h	0.94±0.01
Acid stress: for 3 h					
pH 5	$2.1\pm0.21\times10^9$	3.3 h	0.51±0.001	2.6 h	0.94±0.02
pH 3	$2.3\pm0.28\times10^7$	9 h	0.56±0.05	8.6 h	0.96±0.005
pH 2	$<10^5$	19 h	0.21±0.11*	18.3 h	0.38±0.075†
Unstressed cells	$2.4\pm0.15\times10^9$	3.6 h	0.43±0.09	3.6 h	0.9±0.01
Combined stress:					
10.5% NaCl, pH 5 and 45 °C for 1 h	$4.97\pm0.03\times10^8$	4 h	0.51±0.04	3.6 h	0.95±0.001

[a]Lag phase as measured on the graph when the cell density reached 0.1 OD_{595}.
[b]Maximum population density (OD_{595}) at 20 h.
*Reached OD_{595} of 0.52±0.06 by 24 h.
†Reached an OD_{595} of 0.9±0.02 by 24 h.
ND, not determined.

one hour stressed cells were comparable with that of TSBYE which had the same lag time of about 3 h but the maximum population density values were higher in TSBYE (0.94) compared to LCGM (0.56). Highly injured cells took a longer lag time of 10 to 14 h in LCGM (Table 3). Nevertheless, it is quite promising that even the highly injured cells could be recovered using LCGM.

The recovery of osmotic (10.5% NaCl) stressed cells in LCGM was again comparable to that of TSBYE (Table 3). The decrease in the cell numbers from 2.7×10^9 to 1.4×10^9 CFU/ml due to osmotic stress does not appear to be large; however the lag time duration was prolonged by 0.6 h when recovered in either LCGM or TSBYE. The final cell density was also slightly decreased in both the media (Table 3).

The recovery of acid stressed cells (at pH 2, 3 or 5 for 3 h) revealed the effect of pH 3 and 2 to be more severe on the cells compared to pH 5 (Table 3). The viable cell count before stress was $2.1\pm0.7\times10^9$ CFU/ml which remained the same after exposure to pH 5 but decreased by 2 and 4 logs, respectively, when exposed to pH 3 and 2 for 3 h. From the growth curves,

the lag phase for the normal cells and pH 5 stressed cells grown in TSBYE or LCGM was found to be ~2–3 h, for pH 3 it was 7–8 h in either of the media and it took about 18–19 h of lag time for the pH 2 stressed cells. The recovery of these stressed cells using LCGM was also comparable to that of TSBYE.

Recovery of *Listeria* cells exposed to combined stress of 10.5% NaCl, pH 5 and 45 °C for 1 h in LCGM was conducted to simulate the natural stress conditions, likely to be encountered by bacteria in a food product. Stress resulted in about one-log reduction in cell counts. The lag phase in LCGM was 4 h compared to 3.6 h in TSBYE; however the considerable differences were seen in maximum population density values of LCGM with 0.51 and TSBYE with 0.95 (Table 3). Overall, the performance of LCGM appeared to be comparable to that of the TSBYE medium in recovering stressed cells.

3.5. Effect of inoculum size on the lag phase of L. monocytogenes grown in LCGM

LCGM was inoculated with variable concentrations of cells ranging from 10^3 to 10^7 CFU/ml. It had about 11 h of lag phase when inoculated with 10^3 CFU/ml, 9 h with 10^4 CFU/ml, 7.5 h with 10^5 CFU/ml, 5.5 h with 10^6 CFU/ml and about 3.3 h with 10^7 CFU/ml. The growth rate was similar in all and reached a maximum population density at OD_{595} of ca. 0.4 to 0.5 in about 16 h (Fig 3a). The viable plate counts for two of the inoculations (5.5 and 6.5 $\log_{10}$ CFU/ml) supported the OD data. There was a change in lag time, after 2 h, in the two inoculum sizes of 5.5 and 6.5 $\log_{10}$ CFU/ml (Fig 3b). However, both reached to about

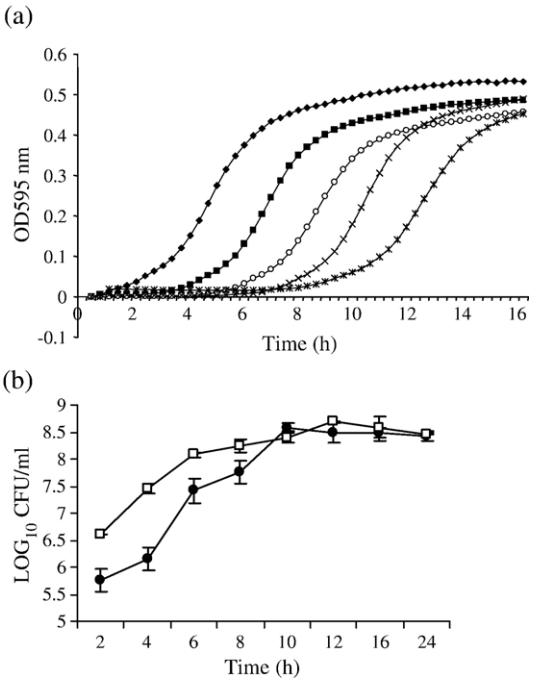

Fig. 3. Effect of inoculum size on the lag phase of *L. monocytogenes* growth in LCGM as monitored by (a) measuring absorbance at 595 nm and (b) by viable plate count methods. Legends for panel (a): (♦) 3×10^7 CFU/ml, (■) 3×10^6 CFU/ml, (○) 3×10^5 CFU/ml, (×) 3×10^4 CFU/ml, (*) 3×10^3 CFU/ml. Legends for panel (b): (□) 6×10^6 CFU/ml, (✺) 3×10^5 CFU/ml.

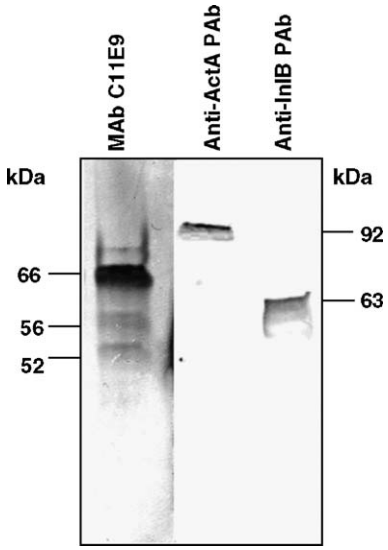

Fig 4. Western blot analysis of protein extracts from *L. monocytogenes* V7 with MAb-C11E9 and anti-ActA and anti-InlB polyclonal antibodies.

8.5 ± 0.2 $\log_{10}$ CFU/ml and 8.3 ± 0.08 $\log_{10}$ CFU/ml, respectively, within 10 h and 8 h.

3.6. Expression analysis of surface antigens, ActA and InlB by Western blotting

Western immunoblot revealed the expression of 92 kDa ActA and 63 kDa InlB proteins by *L. monocytogenes* V7 grown in LCGM (Fig. 4). Conversely, there was no apparent expression of the ActA protein in LB broth (data not shown). Surface antigenic expression was examined for a monoclonal antibody, C11E9 that reacts with several protein bands. The expression of all the three proteins in the molecular range of 66, 56 and 52 kDa was seen in *L. monocytogenes* as reported earlier (Bhunia et al., 1991).

4. Discussions

The major focus of the present study was to evaluate the performance of a proprietary low conductive growth medium (LCGM), marketed by Biovittesse Inc, for use in a microfluidic biochip for bacterial growth monitoring by impedance measurements (Gomez et al., 2001) or any other applications where this media will allow sensitive monitoring of bacterial growth by impedance microbiology for quality assurance purposes. A low conductive, Tris-Gly-Dext buffer (33.5 μS) was used earlier in the biochip to detect low number of cells but the detection was limited to about 10^7 cells/ml to observe any considerable change (Gomez et al., 2001). Substituting the buffer with the LB medium (2 mS) did not improve impedance measurements. However, LB broth is far better than Tris-Gly-Dext buffer, since it does not impart stress to the cells. Lack of nutrients and low osmolarity of the buffer possibly caused reduced growth in Tris-Gly-Dext buffer (Gomez et al., 2002a). Therefore, a better low conductive media which could support the growth and sensitive detection of *L. monocytogenes* by impedemetric methods was required. The LCGM media with an initial conductivity of

1200 μS were, thus evaluated for use in biochip-based detection, for: (i) growth of different bacteria, (ii) growth of *L. monocytogenes* in LCGM in the presence or absence of selective agents, (iii) recovery of stressed bacterial cells, and (iv) expression of the diagnostically important proteins.

4.1. LCGM for the growth of Listeria and other bacteria

The LCGM in the absence of selective agents could be used for the growth of most of the bacteria tested, as evidenced by the high OD_{595} values. About 55% of the tested isolates showed medium to good growth. Fifteen percent of the isolates had good growth and 5% showed excellent growth, which included the strains of *L. monocytogenes*, *E. faecalis* and *P. aeruginosa*. About 21% strains showed a rather poor growth, which could be either due to the susceptibility of certain strains to the low ionic environment or inability of these strains to utilize bovine serum albumen (BSA), one of the major components in the medium. BSA has been used earlier by investigators to grow fastidious organisms like *Helicobacter pylori* (Albertson et al., 1998). Although the growth between the organisms looks random and seems to be strain dependent, most or all of the *Listeria* grew well in this medium. With the exception of *Lactobacillus* and some strains of *E. coli*, the media could be used as a universal low conductive growth medium for major food related bacteria.

4.2. Growth of L. monocytogenes in various media

Most of our studies were concentrated on the growth of *L. monocytogenes*, because of the obvious interest on the rapid detection of this bacterium on biochip (Gomez et al., 2001, 2002a,b; Huang et al., 2003) using the LCGM. When compared with LB, *L. monocytogenes* grown in LCGM had a shorter generation time, longer lag phase and two-fold higher cell density. The other selective media, LRB and BLEB yielded a better growth rate, shorter generation time and lag phase, which were statistically different from the other low conductive media. LCGM showed a good growth with no statistically significant difference ($P<0.5$) with TVC or LB medium. But the higher maximum population density and lower initial conductivity values make the LCGM medium more attractive.

The presence of selective agents in LCGM or LB medium reduced the growth by half, showing a statistically different ($P<0.05$) cell density and generation time. Although the lag phase duration was similar to BLEB, the reduction in the cell mass was very evident. The reduction in growth could be due to the stress effect exerted by the presence of antibiotics on bacterial cells, thus prolonging the lag phase (Golden et al., 1988; Silk et al., 2002).

Growth of *L. monocytogenes* in LCGM was further validated as a function of changes in conductivity by an online conductivity measurement setup. Measurable differences could be achieved within 60 min of incubation. It is possible that the complex organic molecule like BSA catabolizes to simpler inorganic ions during the growth of *L. monocytogenes*, which was detected by the conductivity probe. Listerial growth has been measured earlier using a bactometer broth or LED broth based on the changes in capacitance of the media, which took up to 30 h to induce about 30% changes in signal (Capell et al., 1995; Rodrigues et al., 1995). LCGM however could detect the growth of *Listeria* within 2 h compared to LB. Thus, it is shown to be a reliable and better medium than LB for the application in any conductivity-based rapid detection of *L. monocytogenes*.

4.3. Recovery of stressed Listeria cells in LCGM

An efficient growth media should be able to support the recovery of stressed or injured bacterial cells. *L. monocytogenes* cells when exposed to 45 °C for a period of 2 or 6 h, considerably reduced cell counts were obtained and the lag phase was prolonged by 9–10 and 13–14 h, respectively. Silk et al. (2002) compared eight enrichment broths for the recovery of heat-injured (20 min heating) cells of *L. monocytogenes* and found that LRB gave the best recovery, within 5.5 h. TSB repaired the cells in about 5.68 h and Fraser broth took the longest of about 18 h. In another study, a recovery period of about 18 h was observed when heat-injured (55 °C for 20 min) cells were grown in BHIEM (BHI supplemented with magnesium chloride and egg yolk) and it took about 22 h and 48 h, respectively, in UVM I and UVMII (Jacobsen, 1999). The effect of salt stress (10.5% NaCl for 1 h) on *Listeria* was not considerably high, as observed in our study. Acid stress caused a very high decrease in the cell number at lower pH values (pH 2 and pH 3), yielding longer lag phases. A combined stress of NaCl and pH 5 at 45 °C caused a one-$\log_{10}$ reduction in the cell number. LCGM again showed a recovery rate comparable to that of TSBYE. Studies have shown that the presence of glucose in the media could influence the recovery of the osmotic stressed *L. monocytogenes* and an increased incubation temperature to 41.5 °C can repair the acid-injured cells, exposed for longer stress periods (Besse et al., 2000). We did not observe a very high influence of these elements under shorter stress conditions.

4.4. Effect of inoculum size on the lag phase of L. monocytogenes in LCGM

The effect of the inoculum size on the growth has been shown to be an important factor in order to understand the growth patterns in bacteria (Gay et al., 1996; Augustin et al., 2000a,b; Pascual et al., 2001; Robinson et al., 2001). The physiological state and temperature history of the cells have significant effect on the lag phase of growth (Buchanan and Klawitter, 1991; Robinson et al., 2001). The lag phase variation in LCGM at different inoculum size was studied. Under normal conditions, the healthy cells of *L. monocytogenes*, required ca. 11 h of lag time at lower cell concentrations (10^3 CFU/ml) and ca. 3.5 h at the highest initial cell number (10^7 CFU/ml). Thus, it requires a minimum of 11–12 h of detection time for *L. monocytogenes* (10^2 to 10^3 CFU/ml), grown in LCGM by absorbance measurements. Similar observations were made in a complementary study, by our group using pH and/or conductivity measurements, where it was shown that the detection time is inversely proportional to the initial cell number (Yang et al., 2005). Ruan et al. (2003) also found similar results,

when an electrochemical voltammetry method was used for the detection of *L. monocytogenes* by monitoring the total oxygen consumption in the medium.

4.5. Expression of virulence proteins in LCGM

The performance of LCGM media in its capacity to support the expression of two selected virulence factors was examined. ActA responsible for actin-based motility and InlB, required for cell invasion, were targeted because of the availability of the specific antibodies in our laboratory. Another antibody C11E9 was used which reacts with a 66 kDa protein identified as murien hydrolase in *L. monocytogenes* (Carroll et al., 2003). The data showed that the expression of internalin B and C11E9 reactive proteins were good in both LB and LCGM. However, ActA expression was variable. The LB broth has shown to be a poor medium for ActA expression (Moors et al., 1999). It could be postulated that the possible expression of ActA in the presence of BSA could be due to the induction of expression by suppressing the catabolite repression, similar to the effect of charcoal (Ermolaeva et al., 2004). Reaction with MAb C11E9 antibody was very important since, this antibody has been used in a number of immunosensor assays for *L. monocytogenes* (Lathrop et al., 2003; Geng et al., 2004; Gray and Bhunia, 2005) and on the protein biochip (Huang et al., 2003). These results demonstrate the ability of LCGM to support the expression of some selected proteins and it would be useful for specific capture and detection in a sandwich configuration on a biochip or any other immunosensor applications.

In conclusion, although several media have been developed to enrich and isolate stressed or injured *L. monocytogenes* from foods (Busch and Donnelly, 1992), no single medium has been shown to fulfill all the requirements of an ideal medium. There have been some which recovers the fewest cells from the foods, like the LEB medium and there are some which yields higher cell densities, like the LRB medium (Osborne and Bremer, 2002; Silk et al., 2002). The efficiency of a media is rather dependent on the type of stress the cells have been exposed to and also varies with the strains (Purwati et al., 2001; Osborne and Bremer, 2002; Silk et al., 2002). Fraser broth is a USDA/FSIS recommended media for the selective isolation of *L. monocytogenes* from foods, and it was able to recover the injured cells but required longer repair time (Silk et al., 2002). Also this media inhibit expression of certain proteins (Geng et al., 2004). Although LCGM is a low conductive medium it could recover the heat injured cells in about 14 h and could also support the expression of some selected virulence proteins. Thus, it could be successfully used in any impedance-based methods, for sensitive detection of *Listeria* or other bacteria. Furthermore, specific protein expression at early stages of growth would facilitate antibody-based capture and detection on the biochip or similar sensor platforms.

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