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Full Length Research Paper

Resistance to antimicrobials and acid and bile tolerance of *Bacillus* spp isolated from *Bikalga*, fermented seeds of *Hibiscus sabdariffa*

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In the aim of selecting starter cultures, thirteen species of *Bacillus* spp. including six *Bacillus subtilis* ssp. *subtilis*, four *Bacillus licheniformis* and three *Bacillus amyloliquefaciens* ssp. *plantarum* isolated from traditional *Bikalga* were investigated. The study included, for all isolates, genes, determination of minimal inhibitory concentration (MIC) for 24 antimicrobials and detection of resistance by PCR using specific primers. The isolates were also examined for their resistance to pH 2.5 and their tolerance to 0.3% bile over 4 h. Results showed that most studied isolates, in particular *B. subtilis* ssp. *subtilis* G2, H4, C6, I7 and *B. amyloliquefaciens* ssp. *plantarum* A4, I8, G3 were susceptible to most antimicrobials tested while all *B. licheniformis* isolates showed high resistance level. The resistance observed towards the antimicrobials (chloramphenicol, erythromycin, kanamycin, penicillin, streptomycin and trimethoprim) in this study may be intrinsic as no positive amplicon was observed for the most prevalent resistance genes investigated (*catp*IP501, *erm*(A), *erm*(B), *erm*(C), *aph*(3")-I, *aph*(3")-III, *ant*(2")-I, *blaZ*, *aadA*, *aadE*, *StrA*, *StrB*, *dfr*(A)). Furthermore, based on their good survival in pH 2.5 and in 0.3% bile all the tested isolates may be able to resist passage through the gastro-intestinal tract conditions. Regarding these results, isolates G2, C6, I7, H4, A4, I8 and G3 may be useful as starter cultures to optimize *Hibiscus sabdariffa* seeds fermentation into *Bikalga*.

Key words: *Bikalga, Bacillus*, antimicrobial resistance, acid resistance, bile tolerance, starter cultures.

INTRODUCTION

Bikalga is a traditional food condiment obtained by an alkaline fermentation of *Hibiscus sabdariffa* seeds. It is generally used as a seasoning condiment in most staples in Burkina Faso and is also known in other African countries under different names such as *Dawadawa bosto* in Niger, *Datou* in Mali, *Furundu* in Sudan and *Mbuja* in Cameroun (Yagoub et al., 2004). The main steps of *Bikalga* production process include cleaning of the seeds,

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cooking and a two-stage spontaneous fermentation (Parkouda et al., 2008). The nutritional value of *Bikalga* has been studied showing that it is a rich source of proteins, lipids, carbohydrates, essential amino acids, fatty acids and vitamins (Bengaly et al., 2006; Parkouda et al., 2008; Yagoub et al., 2004). Members of the *B. subtilis* group were shown to be the main microorganisms involved in the fermentation of *H. sabdarifa* seeds into *Bikalga* (Ouoba et al., 2008). The nutritional value of *Bikalga* as well as other similar African traditional fermented foods is believed to be due to the fermentation process and metabolic activities of the associated microorganisms (Bengaly 2001; Mohamadou et al., 2010). However, the production of such products relies on spontaneous fermentation with uncontrolled processes and hazardous starters, leading to a product of varying hygienic, nutritional and organoleptic quality. Therefore, more emphasis has since been put on the mastering of starter cultures for use in a more standardized process to produce African fermented products. In this aim, in previous studies, we have pre-selected potential starter cultures from *Bikalga* predominant *Bacillus* spp. based on their capacity to inhibit pathogens and spoilage microorganisms via bacteriocin and lipopeptide antibiotics production (Compaoré et al., 2013 a, b).

However, in addition to antimicrobial properties, another required property of starter cultures is that they are safe for human consumption (Ammor et al., 2007). Such safety includes that they do not harbor acquired and transferable antimicrobial resistance elements. Indeed, antimicrobial resistance is a worldwide public health problem that continue to grow and bacteria used as starter cultures for the production of foods could be a source of spread of antimicrobial resistances, which might be transferred to commensal or pathogenic bacteria (Danielsen and Wind, 2003; Ammor et al., 2007). Furthermore, in order to survive and establish within the human Gastro Intestinal Tract (GIT), some of the desirable properties of starter cultures/probiotics include their ability to resist the acidity (pH 2.5-pH 3.5) of the stomach and the exposure to bile in the upper part of the intestine (Holzapfel et al., 1998; Huang and Adams, 2004).

The aim of the present study was to determine the antimicrobial resistance profile of the predominant *Bacillus* spp. isolated from *Bikalga* and to explore their capacity to survive in acidic pH and to tolerate bile salts, in order to select suitable starter cultures for a controlled fermentation of *H. sabdariffa* seeds.

MATERIALS AND METHODS

Bacterial strains

Thirteen (13) strains of *Bacillus* including six strains of *B. subtilis* ssp. *subtilis* (F1, C3, C6, H4, G2, I7), four strains of *B. licheniformis* (E3, F9, J3, E5) and three strains of *B. amyloliquefaciens* ssp. *plantarum* (A4, I8, G3) isolated from different productions of *Bikalga* were investigated. The *Bacillus* isolates were maintained as stock cultures at -80°C in Brain Heart Infusion (BHI, broth, Oxoid CM1135 Basingstoke, Hampshire, England) supplemented with 20% (v/v) glycerol. The strains were sub-cultured in 10 ml BHI broth at 37°C for 24 h before the cells were used.

Resistance of bacteria to antimicrobials

Determination of the susceptibility of the studied bacteria to antimicrobials: determination of Minimal Inhibitory Concentrations (MIC)

For 24 antimicrobials (Table 1), MICs were determined by the microwell and agar dilution methods using BHI broth (Oxoid CM1135) and BHI agar (Oxoid CM1136). This was done according to standards set by Clinical and Laboratory Standard Institute (CLSI), Wayne, USA. Breakpoint values towards antimicrobials for *Bacillus* spp, were used as described by European Food Safety Agency (EFSA, 2008). For antimicrobials not included in the EFSA (2008) list, breakpoints for closely related Gram-positive species were used tentatively (Table 1).

Detection of resistance genes by PCR

For some antimicrobials to which the tested bacteria showed reduced susceptibility, PCR were conducted to screen the isolates for the presence of resistance genes. Amplification of genes associated with resistance to chloramphenicol (*catp*IP501), erythromycin (*erm*(A), *erm*(B), *erm*(C)), kanamycin (a*ph*(3")-I, *aph*(3")-III, *ant*(2")-I), penicillin (*blaZ*), streptomycin (*aadA, aadE, StrA, StrB*), and trimethoprim (*dfr(A)*) was done accordingly to Jensen et al. (1999). All PCR were performed using the following temperature program: 94°C for 3 min, 25 or 35 cycles of 94°C for 1 min, 45–65°C, according to annealing temperature for the individual primers (Table 2) and extension at 72°C for 1 min. A final extension step at 72°C for 10 min ended the PCR protocol. The PCR products (10 µl) were subjected to electrophoresis on 1.5% agarose gels (120 V, 2 h) and the products were visualized by staining with ethidium bromide.

Screening of the *Bacillus* isolates for acid resistance and bile tolerance

Preparation of inocula

From BHI agar plates incubated for 24 h at 37°C, the *Bacillus* strains were sub-cultured under agitation for 18 h at 37°C in 10 ml BHI broth, pH 7. The cultures were centrifuged at 5000 g, 4°C for 10 min and the pellet re-suspended in 5 ml of sterile saline solution (Becton 211677; Sparks, MD, USA), pH 7.0. The number of cells was estimated by microscopy using a counting chamber (Neubauer, Wertheim, Germany) and dilutions were made in sterile saline to obtain an inoculum concentration of approximately 10^6 CFU/ml.

Acid resistance

The method described by Klingberg et al. (2005) was used. The survival of the bacteria in acidic pH was examined in BHI broth adjusted with hydrochloric acid (HCl) 1 N to obtain a final pH of 2.5. For each *Bacillus* isolate, 100 µl of inoculum were added into 10 ml of BHI broth, pH 2.5 and incubated at 37°C in a rotary shaker at 120 cycles per min. Samples were taken at various times (0, 1, 2, 3

Table 1. Antimicrobial breakpoints and resistance genes investigated.

Antimicrobial	Proposed breakpoints (µg/ml)	Source	Resistance genes investigated
Avilamycin	≥ 16	CLSI (*Enterococcus)*	
Bacitracin	≥ 16	Jensen et al. (2001)	
Chloramphenicol	> 8	EFSA (2008)	*catp*IP501
Ceftiofur	≥8	CLSI (*Staphylococcus*)	
Ciprofloxacin	≥ 4	Jensen et al. (2001)	
Daptomycin	≥ 8	CLSI (*Enterococcus)*	
Erythromycin	> 4	EFSA (2008)	*erm*(A), *erm*(B), *erm*(C)
Flavomycin	≥ 16	CLSI (*Enterococcus*)	
Florfenicol	≥ 32	CLSI (*Staphylococcus*)	
Gentamicin	> 4	EFSA (2008)	
Kanamycin	> 8	EFSA (2008)	a*ph*(3")-I, *aph*(3")-III, *ant*(2")-I
linezolid	≥ 4	CLSI (*Staphylococcus*)	
Penicillin	≥ 0.25	Luna et al. (2007)	*blaZ*
Salynomycin	≥ 16	CLSI (*Enterococcus*)	
Spectinomycin	≥128	CLSI (*Staphylococcus*)	
Streptomycin	> 8	EFSA (2008)	*aadA, aadE, StrA, StrB,*
Sulphamethoxazol	≥ 256	CLSI (*Staphylococcus*)	
synercid	> 4	EFSA (2008)	
Tetracyclin	> 8	EFSA (2008)	
Tiamulin	≥ 32	CLSI (*Staphylococcus*)	
Tigecycline	> 0.5	Luna et al. (2007)	
Trimethoprim	≥ 16	CLSI (*Staphylococcus*)	*dfr(A)*
TMP+SMX	≥4	Luna et al. (2007)	
Vancomycin	> 4	EFSA (2008)	

and 4 h), serially 10-fold diluted and plated in duplicate onto BHI agar. The plates were incubated at 37°C for 24 h and bacterial colonies were counted. The number of bacteria was calculated according to the standard of ISO 7218 (2007).

Tolerance to bile salts

The bile tolerance was examined using the method described by Klingberg et al. (2005). For each *Bacillus* isolate, 100 µl of inoculum were added into 10 ml of BHI broth, pH 7 containing 0.3% (w/v) oxgall bile (Sigma-Aldrich 30209037, Steinheim, Germany) and incubated at 37°C in a rotary shaker at 120 cycles per min. Samples were taken at various times (0, 1, 2, 3 and 4 h), serially 10-fold diluted and plated in duplicate onto BHI agar. The plates were incubated at 37°C for 24 h and bacterial colonies were counted. The number of *Bacillus* was calculated according to the standard of ISO 7218 (2007).

To determine bile tolerance of the *Bacillus* isolates after pre-exposure to low pH, 100 µl of each *Bacillus* inoculum were first added into 10 ml of BHI broth at pH 2.5. After incubation for 3 h at 37°C, cells were harvested by centrifugation (5000 g, 10 min),re-suspended into 10 ml of BHI broth containing 0.3% (w/v) oxgall bile and incubated at 37°C in a rotary shaker. Bacterial growth was checked after 24 h of incubation at 37 °C (from the beginning of the first incubation) by plate counting on BHI agar.

For the experiments described above, cells growth for each isolate in 10 ml BHI broth, pH 7 was monitored simultaneously as a positive control. The experiments were conducted in duplicate on two separate occasions.

RESULTS AND DISCUSSION

Antimicrobial resistance

The antimicrobial susceptibility of the studied *Bacillus* isolates was variable according to the *Bacillus* isolate and the antimicrobial tested (Table 3). For all tested bacteria, no resistance to avilamycin, ceftiofur, fluoroquinolones (ciprofloxacin), glycopeptides, florfenicol, gentamycin, linezolid, salynomycin, spectinomycin, kanamycin (except *B. subtilis* ssp. *subtilis* F1), tetracyclin, trimethoprim (except *B. subtilis* ssp. *subtilis* C6), trimetoprim + sulfametoxazole (except *B. subtilis* ssp. *subtilis* C3), and tigecycline was observed. However, reduced susceptibility towards flavomycin was found for all isolates with the exception of *B. subtilis* ssp. *subtilis* C3. All *B. licheniformis* showed reduced susceptibility towards chloramphenicol, daptomycin, β-lactams (penicillin) and streptomycin while all *B. amyloliquefaciens* ssp. *plantarum* were susceptible to the same antimicrobials (Table 3). The reduced susceptibility of all *B. licheniformis* toward chloramphenicol correlate with the findings of Sorokulova et al. (2008) who reported that the East European probiotic strain *B. licheniformis* 31 (BL31) was resistant to chloramphenicol. Similar to *B. subtilis* PY79, a laboratory strain derived from the 168 type strain and *B.*

Table 2. Primers used.

Resistance gene	Primer	Annealing temperature (°C)
*catp*IP501	5'-GGATATGAAATTTATCCCTC-3'	47
	5'-CAATCATCTACCCTATGAAT-3'	
erm(A)	5'-AAGCGGTAAAACCCCTCTGAG-3'	55
	5'-TCAAAGCCTGTCGGAATTGG-3'	
erm(B)	5'-CATTTAACGACGAAACTGGC-3'	52
	5'-GGAACATCTGTGGTATGGCG-3'	
erm(C)	5'-CAAACCCGTATTCCACGAGG-3'	48
	5'-ATCTTTGAAATCGGCTCAGG-3'	
a*ph*(3")-I	5'-AACGTCTTGCTCGAGGCCGCG-3'	68
	5'-GGCAAGATCCTGGTATCGGTCTGCG-3'	
aph(3")-III	5'-GCCGATGTGGATTGCGAAAA-3'	52
	5'-GCTTGATCCCCAGTAAGTCA-3'	
ant(2")-I	5'-GGGCGCGTCATGGAGGAGTT-3'	67
	5'-TATCGCGACCTGAAAGCGGC-3'	
blaZ	5'-CAGTTCACATGCCAAAGAG-3'	54
	5'-TACACTCTTGGCGGTTTC-3'	
aadA	5'-ATCCTTCGGCGCGATTTTG-3'	56
	5'-GCAGCGCAATGACATTCTTG-3'	
aadE	5'-ATGGAATTATTCCCACCTGA-3'	50
	5'-TCAAAACCCCTATTAAAGCC-3'	
StrA	5'-CTTGGTGATAACGGCAATTC-3'	55
	5'-CCAATCGCAGATAGAAGGC-3'	
StrB	5'-ATCGTCAAGGGATTGAAACC-3'	56
	5'-GGATCGTAGAACATATTGGC-3'	
dfr(A)	5'-CCTTGGCACTTACCAAATG-3'	50
	5'-CTGAAGATTCGACTTCCC-3'	

subtilis Natto obtained from the Japanese soybean staple Natto (Hong et al., 2008) as well as the East European probiotic *B. subtilis* VKPM B2335 (Sorokulova et al., 2008), most *B. subtilis* ssp. *subtilis* (G2, H4, C6 and I7) investigated in the present study were susceptible to most antimicrobials tested including those highlighted by EFSA (2008). Knowledge on the antimicrobial resistance of *B. subtilis* group species is limited and therefore little is known on the population distributions of susceptibility for these compounds.

In the present study, no positive amplicons were obtained when the isolates were screened for the presence of the most prevalent genes associated with resistance to chloramphenicol (*catp*IP501), erythromycin (*erm*(A), *erm*(B), *erm*(C)), kanamycin (a*ph*(3")-I, *aph*(3")-III, *ant*(2")-I), penicillin (*blaZ*), streptomycin (*aadA, aadE, StrA, StrB*), and trimethoprim (*dfr(A)*). This result suggests that the reduced susceptibility towards chloramphenicol, erythromycin, kanamycin, penicillin, streptomycin and trimethoprim may be intrinsic or natural. This finding is very important, since antimicrobial resistance plasmids are of special interest from the safety point of view, because they may be transferred to other strains including pathogens. Indeed, intrinsic resistance is considered to present a minimal risk for spread whereas acquired resistance mediated by mobile genetic elements like plasmids and transposons is considered to have a high risk for spread (European Commission, 2001). Given that the studied *B. subtilis* ssp. *subtilis* and *B. amyloliquefaciens* ssp. *plantarum* are for the most part susceptible to the antimicrobials tested and that they do not carry the most prevalent resistance genes for chloramphenicol, erythromycin, kanamycin, penicillin, streptomycin and trimethoprim, they can be considered as potentially safe for use as starter cultures. However, additional studies will be required to determine the exact nature of the antimicrobial resistance observed in this study.

Acid resistance and bile tolerance of the *Bacillus* isolates

As seen in Table 4, all 13 *Bacillus* isolates studied were able to survive in BHI broth (pH 2.5) following 4 h of incubation at 37°C. There was no sensitive variation in viable cell numbers (about 10^4 CFU/ml) from 0 to 4 h.

Table 3. Minimal Inhibitory Concentrations (MIC) and antimicrobial susceptibility of *Bikalga Bacillus* spp.

Antimicrobial	MIC (µg/ml)*												
	B. amyloliquefaciens* ssp. *plantarum			***B. subtilis* ssp. *subtilis***						***B. licheniformis***			
	A4	**I8**	**G3**	**G2**	**H4**	**F1**	**C3**	**C6**	**I7**	**J3**	**E5**	**E3**	**F9**
Avilamycin	<2 s	<2 s	<2 s	<2 s	4 s	<2 s	<2 s	<2 s	<2 s	<2 s	<2 s	<2 s	<2 s
Bacitracin	>64 r	>64 r	>64 r	>64 r	8 s	>64 r	4 s	4 s	>64 r	>64 r	>64 r	>64 r	>64 r
Chloramphenicol	4 s	4 s	<2 s	8 s	4 s	8 s	16 r	8 s	4 s	>64 r	32 r	32 r	16 r
Ceftiofur	0.25 s	0.25 s	0.25 s	1 s	0.5 s	2 s	<0.12 s	0.5 s	0.5 s	0.5 s	1 s	1 s	1 s
Ciprofloxacin	<0.12 s	<0.12 s	<0.12 s	<0.12 s	0.5 s	1 s	<0.12 s	<0.12 s	<0.12 s	<0.12 s	<0.12 s	<0.12 s	<0.12 s
Daptomycin	4 s	4 s	2 s	2 s	2 s	4 s	2 s	4 s	4 s	16 r	>16 r	16 r	>16 r
Erythromycin	<0.12 s	<0.12 s	<0.12s	<0.12 s	0.25s	<0.12 s	<0.12 s	<0.12 s	<0.12s	0.25 s	>32 r	>32 r	0.5 s
Flavomycin	>32 r	>32 r	>32 r	>32 r	>32 r	>32 r	8 s	>32 r	>32 r	>32 r	>32 r	>32 r	>32 r
Florfenicol	<1 s	<1 s	<1 s	<1 s	<1 s	<1 s	<1 s	2 s	<1 s	2 s	2 s	2 s	1 s
Gentamycin	≤2 s	≤2 s	≤2 s	≤2 s	≤2 s	4 s	≤2 s	≤2 s	≤2 s	≤2 s	≤2 s	≤2 s	≤2 s
Kanamycin	≤2 s	≤2 s	≤2 s	≤2 s	≤2 s	>64 r	≤2 s	≤2 s	≤2 s	4 s	4 s	4 s	4 s
Linezolid	<1 s	<1 s	<1 s	<1 s	<1 s	<1 s	<1 s	<1 s	<1 s	<1 s	<1 s	<1 s	<1 s
Penicillin	<0.06 s	<0.06 s	<0.06 s	<0.06 s	<0.06 s	<0.06 s	<0.06 s	<0.06 s	<0.06 s	0.5 r	0.25 r	0.25 r	0.25 r
Salynomycin	<2 s	<2 s	<2 s	<2 s	<2 s	<2 s	<2 s	<2 s	<2 s	<2 s	<2 s	<2 s	<2 s
spectinomycin	64 s	64 s	64 s	64 s	64 s	32 s	32 s	32 s	32 s	32 s	64 s	32 s	64 s
Streptomycin	8 s	8 s	4 s	4 s	4 s	8 s	4 s	4 s	4 s	64 r	16 r	16 r	32 r
Sulphamethoxazol	64 s	128 s	64 s	512 r	512 r	256 r	16 s	>512 r	256 r	16 s	256 r	32 s	64 s
Synercid	8 r	8 r	8 r	2 s	<0.5 s	>16 r	2 s	<0.5 s	<0.5 s	4 s	4 s	4 s	2 s
Tetracyclin	8 s	8 s	8 s	<0.5 s	<0.5 s	4 s	<0.5 s	1 s	2 s	<0.5 s	8 s	<0.5 s	2 s
Tiamulin	>32 r	>32 r	>32 r	32 r	<0.25 s	>32 r	>32 r	<0.25 s	0.5 s	32 r	>32 r	32 r	0.5 s
Tigecycline	0.06 s	0.06 s	0.06 s	0.03 s	0.03 s	0.06 s	0.03 s	0.03 s	0.03 s	<0.015 s	0.03 s	<0.015 s	<0.015 s
Trimethoprim	<1 s	<1 s	<1 s	<1 s	<1 s	<1 s	<1 s	32 r	<1 s	<1 s	<1 s	<1 s	<1 s
TMP+SMX	<0.25 s	<0.25 s	<0.25 s	0.5 s	<0.25 s	<0.25 s	8 r	2 s	<0.25 s	<0.25 s	<0.25 s	<0.25 s	<0.25 s
Vancomycin	<2 s	<2 s	<2 s	<2 s	<2 s	<2 s	<2 s	<2 s	<2 s	<2 s	<2 s	<2 s	<2 s

* s, sensitive; i, intermediary; r, resistant; according to the proposed breakpoints mentioned in Table 1.

Viable counts from 1, 2 and 3 h were identical to viable counts at 4 h (data not shown). This indicates that these strains may be able to survive the acidic conditions of the stomach. The pH value (2.5) used in the present study for the selection of potential starter cultures has been shown to be very selective and even though it is not the most common pH value of the human stomach it assures the isolation of very acid-tolerant strains (Pennachia et al., 2004). Interestingly, the vegetative cells of all *Bacillus* spp. showed excellent resistance to 0.3% bile. A growth was even observed from 10^4 CFU/ml at 0 h up to 10^6 CFU/ml after 4 h of incubation at 37°C (Table 4). After pre-exposure to BHI broth pH 2.5 for 3 h, all *Bacillus* isolates were

Table 4. Survival of *Bacillus* spp. in low pH and tolerance to bile.

Strain		Viable count (CFU/ml)								
		pH 7		pH 2.5		Bile		Bile/ pre-exposure to low pH		
		0 h	4 h	0 h	4 h	0 h	4 h	0 h	3 h/pH2.5	24 h/Bile
B. amyloliquefaciens ssp. *plantarum*	A4	1.3×10^{5}	1.2×10^{7}	2.3×10^{4}	1.8×10^{4}	2.2×10^{4}	3.0×10^{6}	5.0×10^{4}	4.2×10^{4}	7.8×10^{7}
	I8	8.3×10^{4}	1.7×10^{7}	8.6×10^{4}	7.4×10^{4}	1.9×10^{4}	4.2×10^{6}	6.2×10^{4}	2.5×10^{4}	1.5×10^{8}
	G3	9.4×10^{4}	2.1×10^{7}	1.2×10^{4}	1.0×10^{4}	6.5×10^{4}	6.2×10^{6}	5.4×10^{4}	1.6×10^{4}	6.5×10^{7}
B. subtilis ssp. *subtilis*	H4	2.0×10^{4}	8.9×10^{6}	2.6×10^{4}	2.3×10^{4}	3.6×10^{4}	2.3×10^{6}	1.1×10^{4}	6.5×10^{3}	1.4×10^{7}
	F1	5.5×10^{4}	9.6×10^{6}	6.0×10^{4}	4.4×10^{4}	5.4×10^{4}	5.6×10^{6}	5.3×10^{4}	3.4×10^{4}	1.3×10^{8}
	C3	4.2×10^{4}	7.7×10^{6}	2.4×10^{4}	1.8×10^{4}	1.2×10^{4}	8.0×10^{5}	1.5×10^{4}	4.0×10^{3}	5.4×10^{6}
	C6	4.5×10^{4}	1.1×10^{6}	1.4×10^{4}	1.3×10^{4}	2.1×10^{4}	4.5×10^{5}	5.5×10^{4}	3.4×10^{4}	1.0×10^{6}
	G2	5.1×10^{4}	4.9×10^{6}	8.6×10^{4}	7.7×10^{4}	5.7×10^{4}	5.0×10^{5}	3.7×10^{4}	2.0×10^{4}	7.5×10^{7}
	I7	4.3×10^{4}	4.7×10^{6}	1.4×10^{4}	1.2×10^{4}	3.0×10^{4}	3.1×10^{6}	2.0×10^{4}	1.9×10^{4}	8.2×10^{7}
B. licheniformis	E3	2.7×10^{5}	1.8×10^{7}	4.0×10^{4}	1.5×10^{4}	3.5×10^{4}	7.5×10^{5}	4.5×10^{4}	1.2×10^{4}	9.2×10^{7}
	F9	8.0×10^{4}	5.9×10^{6}	1.9×10^{4}	7.7×10^{3}	5.1×10^{4}	4.2×10^{5}	1.6×10^{4}	1.1×10^{4}	5.1×10^{7}
	J3	9.0×10^{4}	3.2×10^{6}	4.0×10^{4}	2.8×10^{4}	4.9×10^{4}	1.7×10^{5}	3.8×10^{4}	1.0×10^{4}	2.1×10^{8}
	E5	6.8×10^{4}	3.7×10^{6}	5.1×10^{4}	3.4×10^{4}	5.4×10^{4}	1.6×10^{6}	3.2×10^{4}	2.5×10^{4}	1.1×10^{8}

able to grow in BHI broth containing 0.3% oxgall bile with viable cell counts reaching up to 10^{8} CFU/ml after 24 h incubation at 37°C (Table 4). The concentration of bile (0.3%) used has been recommended to be suitable for the selection of probiotics (Goldin and Gorbach, 1992) while other authors reported that this concentration is discriminatory (Chateau et al., 1994; Papamanoli et al., 2003). The fact that our isolates showed full resistance to acidic pH and bile is not in agreement with the findings of Barbosa et al. (2005). Indeed, these authors reported that vegetative cells of different *Bacillus* species (including *B. subtilis* and *B. licheniformis*) isolated from chicken fecal materials were unable to survive the simulated gastro-intestinal tract conditions. However, they observed that spores of the same *Bacillus* species showed excellent tolerance to bile salts and simulated gastric conditions. In contrast, some *Bacillus* spp. spores were reported to be susceptible to these conditions (Duc et al., 2004; Guo et al., 2006). *B. amyloliquefaciens* ssp. *plantarum* A4, I8 and G3 and *B. subtilis* ssp. *subtilis* H4 G2, C6 and I7 showed susceptibility to most antimicrobials tested and full resistance to simulated gastro-intestinal tract conditions making them promising starter cultures candidates for *H. sabdariffa* fermentation into *Bikalga*. However, to complete the selection of the starter cultures, further studies including for example proteolytic, lipolytic and organoleptic properties need to be addressed. It will also be interesting to investigate the probiotic potential of these isolates.

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