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

Compaore, Clarisse S.; Jensen, Lars Bogø; Diawara, Brehima; Ouedraogo, Georges A.; Jakobsen, Mogens; Ouoba, Labia I. I.

Published in: African Journal of Food Science

Link to article, DOI: 10.5897/AJFS2013.1018

Publication date: 2013

Document Version
Publisher's PDF, also known as Version of record

Citation (APA): Compaore, C. S., Jensen, L. B., Diawara, B., Ouedraogo, G. A., Jakobsen, M., & Ouoba, L. I. I. (2013). Resistance to antimicrobials and acid and bile tolerance of Bacillus spp isolated from Bikalga, fermented seeds of Hibiscus sabdariffa. African Journal of Food Science, 7(11), 408-414. DOI: 10.5897/AJFS2013.1018
Full Length Research Paper

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

Clarisse S. Compaoré¹,²*, Lars B. Jensen³, Bréhima Diawara¹, Georges A. Ouédraogo², Mogens Jakobsen⁴ and Labia I. I. Ouoba⁵,⁶

¹Département Technologie Alimentaire (DTA/IRSAT/CNRST), Ouagadougou 03 BP 7047, Burkina Faso.
²Institut du Développement Rural/Université Polytechnique de Bobo, BAMSB, 01 BP 10 91 Bobo-Dioulasso, Burkina Faso.
³Technical University of Denmark (DTU), National Food Institute, Division of Food Microbiology, Mørkhøj Bygade 19, 2860 Søborg, Denmark.
⁴Department of Food Science, Faculty of Science, University of Copenhagen, Rolighedsvej 30, 1958 Frederiksberg C, Denmark.
⁵London Metropolitan University, FLSC/SHS, Microbiology Research Unit, 166-220 Holloway Road, London N7 8DB, United Kingdom.
⁶Independent Senior Research Scientist-Consultant, London, UK.

Accepted 24 September, 2013

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. licheniformis* 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 (catIP501, *erm(A)*, *erm(B)*, *erm(C)*, *aph(3")-I*, *aph(3")-III*, *ant(2")-I*, *blaZ*, *aadA*, *aadE*, *StrA*, *StrB*, *dftr(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,

*Corresponding author. E-mail: compaclara@yahoo.fr.*
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. sabdarif*a 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., 2013a, 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. sabdarif*a seeds.

**MATERIALS AND METHODS**

**Bacterial strains**

Thirteen (13) strains of *Bacillus* including six strains of *B. subtilis* spp. *subtilis* (F1, C3, C6, H4, G2, I7), four strains of *B. licheniformis* (E3, F9, J3, E5) and three strains of *B. amyloliquefaciens* spp. *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 (catIP501), erythromycin (erm(A), erm(B), erm(C)), kanamycin (aph(3′)-I, aph(3′)-III, ant(2′)-I), penicillin (bla2), 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 10⁶ 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...)
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, cefotifur, fluoroquinolones (ciprofloxacin), glycopeptides, florfenicol, gentamycin, linezolid, sulfamethoxazole (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. amyloylquefaciens 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.

| Antimicrobial         | Proposed breakpoints (µg/ml) | Source                  | Resistance genes investigated                     |
|-----------------------|-----------------------------|-------------------------|---------------------------------------------------|
| Avilamycin            | ≥ 16                        | CLSI (Enterococcus)     |                                                   |
| Bacitracin            | ≥ 16                        | Jensen et al. (2001)    |                                                   |
| Chloramphenicol       | > 8                         | EFSA (2008)             | catP501                                           |
| Cefotifur             | ≥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)   |                                                   |
| Gentamycin            | > 4                         | EFSA (2008)             |                                                   |
| Kanamycin             | > 8                         | EFSA (2008)             | aph(3')-I, aph(3')-III, ant(2')-I                 |
| linezolid             | ≥ 4                         | CLSI (Staphylococcus)   |                                                   |
| Penicillin            | ≥ 0.25                      | Luna et al. (2007)      | blAZ                                              |
| Salynamycin           | ≥ 16                        | CLSI (Enterococcus)     |                                                   |
| Spectinomycin         | ≥ 128                       | CLSI (Staphylococcus)   |                                                   |
| Streptomycin          | > 8                         | EFSA (2008)             | aadA, aadE, StrA, StrB,                           |
| Sulphamethoxazol      | ≥ 256                       | CLSI (Staphylococcus)   |                                                   |
| Synesicid             | > 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)             |                                                   |

Table 1. Antimicrobial breakpoints and resistance genes investigated.
subtilis Natto obtained from the Japanese soybean staple Natto (Hong et al., 2008) as well as the East European probiotic B. subtilis VKP M2335 (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 (catP501), erythromycin (erm(A), erm(B), erm(C)), kanamycin (aph(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. amylovorans 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 10⁴ CFU/ml) from 0 to 4 h.

---

**Table 2. Primers used.**

| Resistance gene | Primer | Annealing temperature (°C) |
|-----------------|--------|---------------------------|
| catP501         | 5'-GGATATGAAATTTATCCCTC-3' | 47 |
|                 | 5'-CAATCATCTACCTCATGAAAT-3' | 55 |
|                 | 5'-AAGCGGTAACCCCTCTGAG-3' | 52 |
| erm(A)          | 5'-TCAAAGCCTGTCGGAATTG-3' | 48 |
|                 | 5'-TTTACGACGAAACTGAGG-3' | 68 |
| erm(B)          | 5'-GAACATCTGTTGATGATCGG-3' | 52 |
|                 | 5'-GGGCAGCCGGATCCGAGGAT-3' | 67 |
| erm(C)          | 5'-CAATTTGAAATCCGCTACCAG-3' | 54 |
|                 | 5'-AACGCTCTTGGCTGAGGCCGG-3' | 50 |
| aph(3")-I      | 5'-GGCAAGATCGCTGTAGTCTGCG-3' | 56 |
|                 | 5'-GCCGATGTTGAATGAAA-3' | 50 |
| aph(3")-III    | 5'-GCTTGATCCCAAGTAAGTC-3' | 68 |
|                 | 5'-GGGCAGCCGGATCCGAGGAT-3' | 52 |
| ant(2")-I      | 5'-TATCGGCACCTGAAACGGGC-3' | 55 |
|                 | 5'-CAGTTCACATGGCACAAG-3' | 50 |
| blaZ            | 5'-TCACTCTTGGCGGTTTC-3' | 56 |
|                 | 5'-ATCCTTCCGCGGATTTG-3' | 50 |
|                 | 5'-GACGCAGCAATGACATTCTG-3' | 67 |
|                 | 5'-ATGGAAATTTCATCCACCTG-3' | 47 |
| aadA            | 5'-GCAATCGGAGATAGAAGGC-3' | 47 |
|                 | 5'-TCAAACCTCATTAAAGCC-3' | 52 |
| aadE            | 5'-CCGCGCAGATAGAAGGC-3' | 55 |
|                 | 5'-ATCCTTCCGCGGATTTG-3' | 50 |
| StrA            | 5'-CTTGGTGATAACCGGCATTC-3' | 56 |
|                 | 5'-CAATCGCAGATAGAAGGC-3' | 50 |
| StrB            | 5'-ATCCTTCCGCGGATTTG-3' | 55 |
|                 | 5'-GATCAGCAGCATATCCGCG-3' | 50 |
|                 | 5'-CTGTTGCACTTACAAATG-3' | 52 |
| dfr(A)          | 5'-CTGAAGATTCGACTTCC-3' | 50 |
Table 3. Minimal Inhibitory Concentrations (MIC) and antimicrobial susceptibility of *Bikalga Bacillus* spp.

| Antimicrobial | *B. amylo liquefaciens* 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 |
| 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 |
| Ceftidur       | 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 | 0.1 s | <0.12 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 | <0.12 s | <0.12 s | 0.25 s | <0.12 s | <0.12 s | <0.12 s | <0.12 s | 0.12 s | 0.25 s | >32 r | >32 r |
| 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 |
| Flornicol      | <1 s | <1 s | <1 s | <1 s | <1 s | <1 s | <1 s | <1 s | <1 s | <1 s | <1 s | <1 s |
| Gentamycin     | ≤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 | >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 |
| 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 | 0.5 s | 0.25 r | 0.25 r | 0.25 r |
| Salynnomicin   | <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 | 64 s | 64 s | 64 s | 64 s | 64 s | 64 s | 64 s |
| Streptomycin   | 8 s | 8 s | 4 s | 4 s | 4 s | 8 s | 4 s | 4 s | 4 s | 4 s | 4 s | 4 s |
| Sulphamethoxazol| 64 s | 128 s | 64 s | 64 s | 512 r | 512 r | 256 r | 16 s | >512 r | 256 r | 16 s | 256 r |
| Synercid       | 8 r | 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 |
| Tetracyclin    | 8 s | 8 s | 8 s | <0.5 s | <0.5 s | <0.5 s | 4 s | <0.5 s | 1 s | 2 s | <0.5 s | 8 s | <0.5 s |
| Tiamulin       | >32 r | >32 r | >32 r | >32 r | 32 r | <0.25 s | >32 r | >32 r | >32 r | <0.25 s | >32 r | >32 r |
| Tigecycline    | 0.06 s | 0.06 s | 0.06 s | 0.03 s | 0.03 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 | 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 |
| 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 |

* 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 10⁴ CFU/ml at 0 h up to 10⁷ 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
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 spp. plantarum A4, I8 and G3 and B. subtilis spp. subtilis H4 G2, C6 and I7 showed susceptibility to most antimicrobials tested and full resistance to simulated gastrointestinal 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.

**ACKNOWLEDGMENT**

This work was supported by a Danish International Development Agency (DANIDA) funded project, London Metropolitan University/FLSC/SHS/MRU, UK and the Technical University of Denmark/National Food Institute. They are gratefully acknowledged.
REFERENCES

Ammor MS, Belen Florez, Mayo B (2007). Antibiotic resistance in non-enterococcal lactic acid bacteria and bifidobacteria. Food Microbiol. 24:559-570.

Barbosa MT, Serra RC, La Ragione MR, Woodward JM, Henriches AO (2005). Screening for Bacillus isolates in the Broiler Gastrointestinal Tract. Appl. Environ. Microbiol. 71:968-978.

Bengaly MD (2001). Microbiological Study and nutritional value of traditional protein-rich condiment, obtained by natural fermentation of Hibiscus sabdariffa seeds. PhD Thesis, University of Ouagadougou, p. 116.

Bengaly MD, Béré A, Traoré A (2006). The chemical composition of bikalga, a traditional fermented roselle (Hibiscus sabdariffla L.) seeds condiment. Part II: evaluation of mineral, total polyphenols and phytic acid content, predicting the iron bioavailability. Elec. J. Food Plant Chem. 1:7-11.

Chateau N, Deschamps AM, Sassi AH (1994). Heterogeneity of bile salts resistance in the Lactobacillus isolates of a probiotic consortium. Lett. Appl. Microbiol. 18:42-44.

Compaoré CS, Nielsen DS, Ouoba LII, Berner TS, Nielsen KF, Sawadogo-Lingani H, Diawara B, Ouédraogo GA, Jakobsen M, Thorsen L (2013a). Co-production of surfactin and a novel bacteriocin by Bacillus subtilis subsp. subtilis H4 isolated from Bikalga, an African alkaline Hibiscus sabdariffa seeds fermented condiment. Int. J. Food Microbiol. 162:297-307.

Compaoré CS, Nielsen DS, Sawadogo-Lingani H, Berner TS, Nielsen KF, Adimpong DB, Diawara B, Ouédraogo GA, Jakobsen M, Thorsen L (2013b). Bacillus amyloliquefaciens ssp. plantarum strains as potential protective starter cultures for the production of Bikalga, an alkaline fermented food. J. Appl. Microbiol. 115:133-146.

Danielsen M, Wind A (2003). Susceptibility of Lactobacillus spp. to antimicrobial agents. Int. J. Food Microbiol. 82:1-11.

Duc LH, Hong HA, Barbosa TM, Henriches AO, Cutting SM (2004). Characterization of Bacillus probiotics available for human use. Appl. Environ. Microbiol. 70:2161-2171.

EFSA (2008). Technical guidance prepared by the Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) on the update of criteria used in the assessment of bacterial resistance to antibiotics of human or veterinary importance. J. EFSA. 732:1-15.

European Commission (2001). Opinion of the Scientific Committee on Animal Nutrition on the Criteria for Assessing the Safety of Microorganisms Resistant to Antibiotics of Human and Veterinary Importance. [http://www.europa.eu.int/comm/food/fs/sc/scan/out64_en.pdf].

Goldin B, Gorbach S (1992). Probiotics for humans. In R. Fuller (Ed.), Probiotics: the scientific basis (pp. 355-376). London: Chapman and Hall.

Guo X, Li D, Lu W, Piao X, Chen X (2006). Screening of Bacillus strains as potential probiotics and subsequent confirmation of the in vivo effectiveness of Bacillus subtilis MA139 in pigs. Antonie van Leeuwenhoek. 90:139-146.

Holzapfel WH, Haberer P, Snel J, Schillinger U, Huis in't Veld JHJ (1998). Overview of gut flora and probiotics. Int. J. Food Microbiol. 41:1-101.

Hong HA, Huang JM, Khaneja R, Hiep LV, Urdaic MC, Cutting SM (2008). The safety of Bacillus subtilis and Bacillus indicus as food probiotics. J. Appl. Microbiol. 105:510-520.

Huang Y, Adams MC (2004). In vitro assessment of the upper gastrointestinal tolerance of potential probiotic dairy propionibacteria. Int. J. Food Microbiol. 91:253-260.

International Organization for Standardization: ISO 7218 (2007). Food Microbiology-General Requirements and Recommendations. pp. 65.

Jensen BL, Baloda S, Boye M, Aarestrup MF (2001). Antibiotic resistance among Pseudomonas spp. and the Bacillus cereus group isolated from Danish agricultural soil. Environ. Int. 26:581-587.

Jensen BL, Frimodt-Møller N, Aarestrup FM (1999). Presence of erm gene classes in Gram-positive bacteria of animal and human origin in Denmark. FEMS Microbiol. Lett. 170:151–158.

Klingberg TD, Axelsson L, Naterstad K, Elsser D, Budde BB (2005). Identification of potential probiotic starter cultures for Scandinavian-type fermented sausages. Int. J. Food Microbiol. 105:419-431.

Luna VA, King DS, Gulledge J, Cannons AC, Amuso PT, Cattani J (2007). Susceptibility of Bacillus anthracis, Bacillus cereus, Bacillus mycoides, Bacillus pseudomycoides and Bacillus thuringiensis to 24 antimicrobials using Sensititre® automated microbroth dilution and Etest® agar gradient diffusion methods. J. Ant. Chem. 60:555-567.

Mhamadou BA, Mbolung CM, Thouvenot D (2010). Characterization of some atypical lactic acid bacteria associated with the fermentation of Hibiscus sabdariffa seeds. Afr. J. Microbiol. Res. 4(24):2655-2660.

Ouoba LII, Parkouda C, Diawara B, Scotti C, Varnam AH (2008). Identification of Bacillus spp from bikalga, fermented seeds of Hibiscus sabdariffa: phenotypic and genotypic characterization. J. Appl. Microbiol. 104:122-131.

Papamanol E, Tzanetakis N, Litopoulou-Tzanetaki E, Kotzekidou P (2003). Characterization of lactic acid bacteria isolated from a Greek dry-fermented sausage in respect of their technological and probiotic properties. Meat Sci. 65:859-867.

Parkouda C, Diawara B, Ouoba LII (2008). Technology and physico-chemical characteristics of bikalga, alkaline fermented seeds of Hibiscus sabdariffa. Afr. J. Biotechnol. 7:916-922.

Pennachia C, Ercolini D, Blaiotta G, Pepe O, Mauriello G, Villani F (2004). Selection of Lactobacillus strains from fermented sausages for their potential use as probiotics. Meat Sci. 67:309-317.

Sorokulova IB, Pinchuk IV, Denayrolles M, Osipova IG, Huang JM, Luna VA, Pinchuk IV, Denayrolles M, Osipova IG, Huang JM, King DS, Gulledge J, Cannons AC, Amuso PT, Cattani J (2007). Susceptibility of Bacillus anthracis, Bacillus cereus, Bacillus mycoides, Bacillus pseudomycoides and Bacillus thuringiensis to 24 antimicrobials using Sensititre® automated microbroth dilution and Etest® agar gradient diffusion methods. J. Ant. Chem. 60:555-567.

Yagoub AEGA, Mohamed BE, Ahmed AHR, El Tinay AH (2004). Study and characterization of probiotic Bacillus strains from two fermented sausages: Bifidobacterium longum and a novel bacteriocin-producing Bacillus subtilis as food probiotics. J. Appl. Microbiol. 105:122-131.