ORIGINAL PAPER

ACID AND BILE TOLERANCE OF PROBIOTIC BACTERIA USED FOR LACTIC ACID FERMENTATION OF VEGETABLE JUICES

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Abstract. *The purpose of this study was to evaluate the probiotic Bifidobacterium animalis subsp. lactis BB-12 with regard to its resistance to simulated gastrointestinal stress. The tested strain was able to survive both in the presence of 0.3% bile salts for 5h and in the acidified MRS broth with pH 2.0 for 2h, the rate of viability being by 78% and 76% respectively. The presence of pepsin with a view to test the strain tolerance to gastric juice induced the decrease of the viable cells number with 1.4 log cycles after 6h. The behavior of Bifidobacterium lactis BB-12 was quite different when the exposure to gastric juice was combined with the exposure to bile salts, highest death rates being determined.*

Keywords: *cabbage juice, bifidobacteria, gastrointestinal stress, viability.*

1. INTRODUCTION

Brassica vegetables, including all cabbage-like ones, are consumed in enormous quantities throughout the world and are important in human nutrition [1]. Cabbage is rich in minerals, vitamin C, dietary fibers, and especially phytochemicals [2]. Many phytochemicals have antioxidant properties, being important to establish the contribution of ascorbic acid to this, and to assess how this information may translate into dietary intakes [3]. Lactic acid fermentation of cabbage juice with probiotic strains represents a way to obtain functional foods for lactose-intolerant persons, for vegetarians or for people interested to consume wholesome foods for their own well-being. As probiotic agents, bifidobacteria have been studied for their efficacy in the prevention and treatment of a broad spectrum of animal and/or human gastrointestinal disorders, such as colonic transit disorders, intestinal infections, and colonic adenomas and cancer [4]. Application of probiotic cultures in nondairy products represents a great challenge. Probiotic viability in the food matrix depends on factors, such as pH, storage temperature, oxygen levels, and presence of competing microorganisms and inhibitors. It is important that the formulation maintains the activity and viability of the probiotic for extended periods of time [5]. The minimum amounts of probiotics needed to obtain a clinical effect have not been established [6]. According the CODEX standard for fermented milks, the minimum counts of these microorganisms at the time of consumption should be 10^6 cfu g^{-1} [7]. In addition to their ability to survive in the product, many criteria have been suggested for the selection of probiotics, among them the tolerance of gastrointestinal conditions (acid and bile) and ability to adhere to intestinal mucosa [6]. The

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GIT survival of probiotic strains might not only depend on their number and physiological state but also food matrix and habits of food consumption which affect bile excretion [8].

2. MATERIALS AND METHODS

2.1. MATERIALS

Vegetables treatments. Fresh white cabbage (*Brassica oleracea L.*) was purchased from a retail market in Dambovita County (Romania) and specifically processed by removing the non-edible pieces. Using a home-made extractor, the vegetables were turned into juice. The heating treatment of the juice, applied at 80^0 C with a view to destroy the undesirable microorganisms under the limit of detection, was followed by cooling at 40^0 C.

Microorganisms and fermentation conditions. Bifidobacterium animalis subsp. lactis BB-12 was obtained from Christian Hansen (Romania). The lyophilized culture was aseptically inoculated into the cabbage juice (0.4 g/L) and vigorous homogenized for 15 min, according to the producer's specification. On the basis that the bifidobacteria are obligate anaerobes, growing better in static culture than in shake flasks, a series of fermentation experiments were performed in double in conical flasks by 50 mL, in static system. The lactic acid fermentation was performed in thermostat at 37 ± 0.2^0C. A 24-h or 48-h old culture was used as inoculum with a view to evaluate the resistance to simulated gastric juice, to bile salts and to simulated conditions of gastrointestinal tract respectively.

2.2. METHODS

The parameters for survival tests were adjusted according to literature and our previous results, in order to obtain noticeable loss in cell concentration during the tests.

Acid tolerance

Acid tolerance of the probiotic organism was studied according to Ding and Shah [9]. Briefly, MRS broth was adjusted to pH 2.0 with 5.0*M* HCl. After inoculation of the acidified MRS broth the incubation in anaerobiosis at 37^0C for 2 h was made. Samples were taken for plate counts at 30, 60, 90 and 120 min. intervals.

Bile salts tolerance

The strain of *Bifidobacterium* was assessed for rapidity of growth in MRS broth with and without bile salts. Over-two days culture was inoculated into broth containing 0%, 0.1%, 0.2% and 0.3% (w/v) bile salts respectively and incubated anaerobically at 37^0C for 3h and 5h.

Simulation of conditions in the gastrointestinal tract

Simulated gastric juice was prepared according to Shahidi et al. [10], adding 3 g pepsin to 1000 mL saline (0.5% w/w). After inoculation with over-night culture and incubation at 37^0C, the viability of *Bifidobacterium* was evaluated hourly until 6 h.

With a view to simulate the conditions prevailing in the stomach and the duodenum, MRS broth adjusted to pH 2.0 with 5.0*M* HCl and inoculated with 24-h old culture was incubated for 60 min. (under gastric conditions), then a solution of filter-sterilised 0.3% (w/v) bile salts was added. The samples were incubated for a further 120 min. To simulate a slower gastric transit followed by a tipical duodenum transit, the MRS broth inoculated with 24-h old culture was incubated for 120 min. under gastric conditions, then the bile salts were added and the samples were incubated for a further 120 min. The incubation temperature was by 37^0C.

The experimental described previously was made according to the method of Madureira et al. [11].

In all the cases, the count of *Bifidobacterium* sp. was determined by plate count method using Man–Rogosa-Sharpe agar, enriched with L-cysteine HCl, after serial tenfold dilutions in peptone water. The Petri plates were incubated for 48h at 37 °C in anaerobiosis (Anaerocult® A - anaerobiosis generator from Merck). The results were expressed as log colony forming units (CFU)/mL.

3. RESULTS AND DISCUSSION

The survival of the probiotic strain at pH 2.0 is shown in Fig. 1. The exposure to acid conditions induced loss in viability of *Bifidobacterium animalis* subsp. *lactis* BB-12 that ranged from 15.63% in the first 30 min. to 23.69% after 2 h. The decrease was significant in the first interval of time, probably due to accommodation of the strain to the new environment, until 2h the trend of the loss becoming steady.

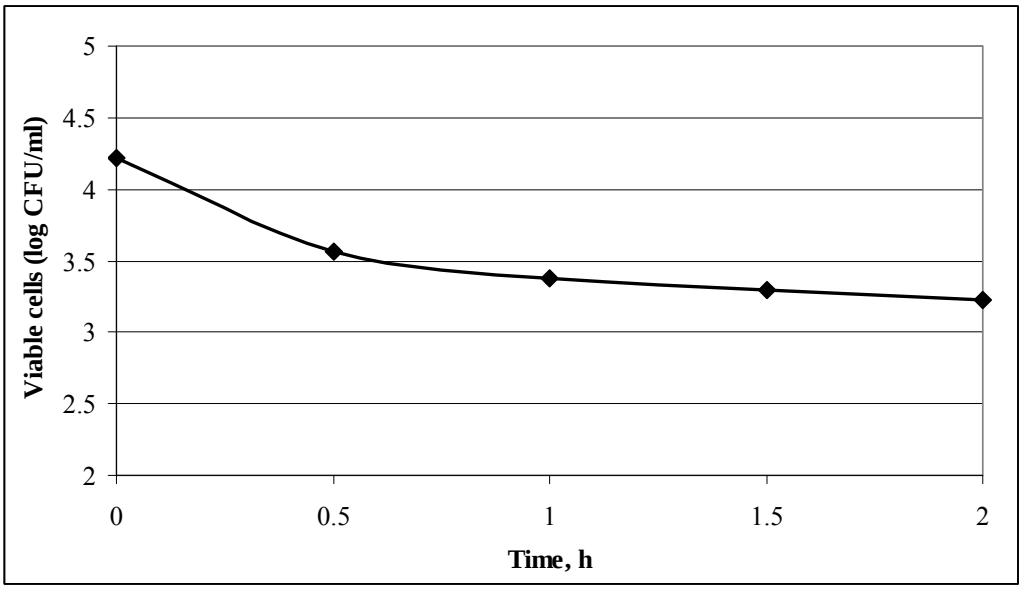

Fig. 1. Effect of pH 2.0 on viability of *Bifidobacterium sp*. BB-12.

According [9], the *Bifidobacterium* strains (including *B. longum, B. lactis* type Bl-O4 and *B. lactis* type Bi-07) were the most-acid sensitive compared with *Lactobacillus* strains. The effect of the bile salts on the viability of *Bifidobacterium* sp. is presented in Fig. 2. The mortality of the strain after 5h of exposure was by approximately 0.9 log cycles at the initial concentration of 0.1% bile salts, respectively by 1.4 log cycles at the initial concentration of 0.3% bile salts. Although after the first 3h of incubation the influence of the bile salts concentration is obviously, until 5h the differences between the samples with 0.1%, respectively 0.2% bile salts become relative unsignificant. These results are in agreement with other studies that have found that most probiotic bacteria can grow in MRS supplemented with up to 0.5% conjugated bile salts [9]. The highest mortality of the analyzed strain, by 22%, was observed in presence of 0.3% bile salts after 5h of incubation. According [12], a significant decrease of the viability of the bacterial strain *L. paracasei* IL2, of 25%, was determined after two hours of exposure at 3 mg/mL bile salts concentration.

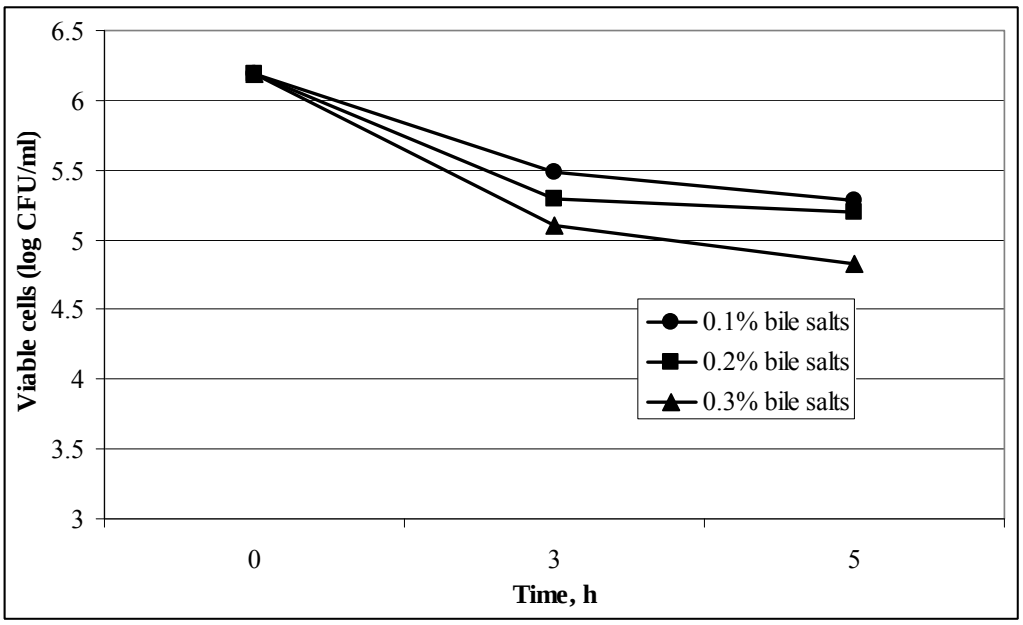

Fig. 2. Viability of *Bifidobacterium sp.* BB-12 in presence of bile salts.

Studying the bile tolerance of different probiotic strains, Ding and Shah [9] established that the strains most susceptible to oxgall bile salts were *B. lactis* type Bl-O4 and *B. lactis* type Bi-07 with 7.85 and 7.71 log CFU/mL reduction in viability at 8h of incubation, respectively. According Lukáčová et al. [13], *Bifidobacterium longum* strain CCM4990 (initial density approx. 10^9 KTJ.cm^{-3}) was able to survive in environments containing 0.3% and 0.5% bile salts respectively for 4h. The authors considered this time as sufficient for the microorganisms tested to survive in the digestive tract of the host, thus meeting the requirement regarding resistance to bile salts. The survival of the probiotic strain in simulated conditions prevailing in the stomach and the duodenum was by 46.55%, while when the slower gastric transit followed by a tipical duodenum transit was simulated, the above mentioned parameter was only by 28.09% (Fig. 3.). The highest death rates of the cells could be explained through the proposed theory, according to which the damage of bacterial cell envelope by low pH could make the cells more susceptible to bile action on cell membranes [6]. Referring to the viability profile throught the period of exposure to the combination of artificial gastric juice and bile salts, the results of Madureira et al. [11] are opposite to the current study. Thus, *Bifidobacterium animalis* BB-12 inoculated into whey cheese underwent a decrease of approximately 1.5 log cycles within 60 min. when pre-incubated for 60 min. in artificial gastric juice. This one it could be due to a lot of factors, including the different experimental design, the physiological state of the culture, and the initial number of viable cells. In Fig. 4. is shown the influence of pepsin on the survival of *Bifidobacterium animalis* subsp. *lactis* BB-12, the trend of the curve being quite similar with that one established in the case of the growth in MRS with pH 2.0.

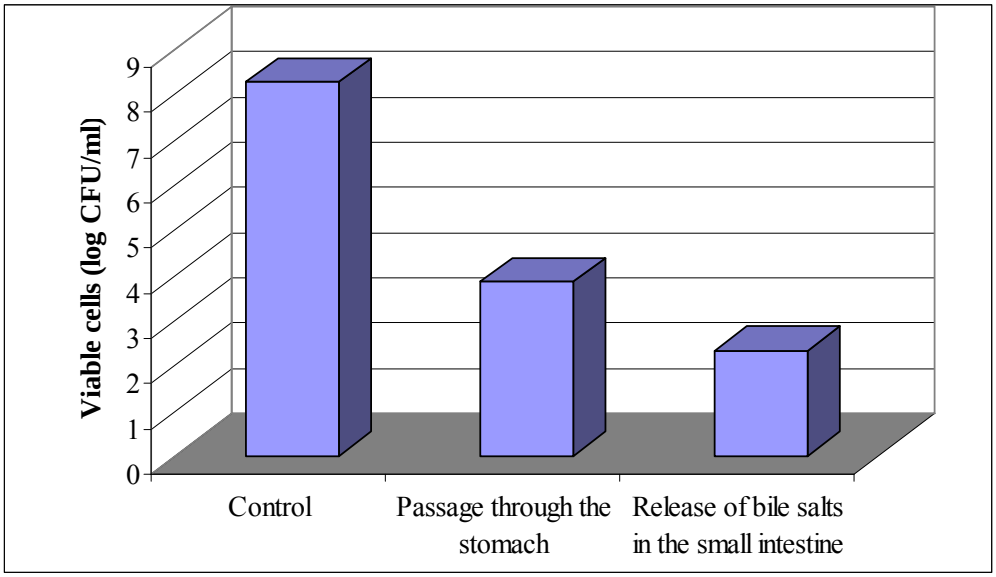

Fig. 3. The combined effect of exposure to gastric juice, followed by the effect of exposure to bile salts (simulated conditions) on the viability of *Bifidobacterium sp*. BB-12.

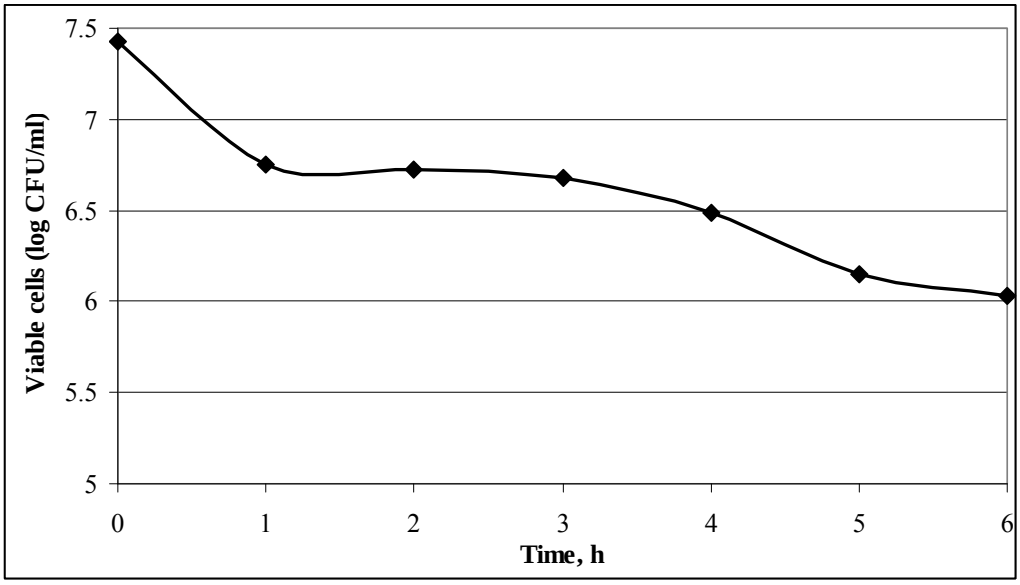

Fig. 4. Viability of *Bifidobacterium sp*. BB-12 in simulated gastric juice conditions.

The tested strain showed tolerance in simulated gastric juice conditions, the reduction of the viability being by approximately 1 log cycle after 4h. If the initial concentration of cells is about 7.5 log CFU/mL and the experimental conditions are those previously mentioned, after 6h of incubation at 37^0C the number of viable cells of *Bifidobacterium* sp. is by 10^6 CFU/mL, close to the value requested by the CODEX standard for fermented milks. Survival and stability of *Bifidobacterium animalis* strains BLC-1, BB-12 and Bo inoculated into a Portuguese whey cheese, when exposed to simulated gastrointestinal tract conditions, were assessed by Madureira et al. [11]. For *B. animalis* BB-12, the estimated death rates in viable counts exposed to artificial gastric juice and artificial gastric juice plus bile salts were 0.0122±0.0019 log ($CFUg^{-1}$) min^{-1}, respectively 0.0119±0.0004 log ($CFUg^{-1}$) min^{-1}. Our values are relative close for the analyzed parameters, the experimental conditions being somewhat different. Survival of microorganisms during processing and in the gastrointestinal tract depends on the physiological state of the culture, thus the production and maintenance of the bacteria in the same physiological state is crucial for comparative survival testing [8]. In

the same time, the protective effect of food as vector for probiotic strains is of crucial importance [11].

4. CONCLUSIONS

- The probiotic strain *Bifidobacterium animalis subsp. lactis* BB-12 exhibited, in the experimental conditions mentioned above, different viability profiles to various stress treatments.

- Thus, the 48-h old culture was resistant to artificial gastric juice and to bile salts, the decrease in viable cells number being by one log cycle after 4 h of incubation in the first case, respectively after 5h of exposure in the second.

- The simulation of the transit through the gastrointestinal tract (passage through the stomach, followed by release of bile salts in the small intestine) induced the survival diminution with 4.4 and 5.9 log cycles respectively. In *vivo* studies are however necessary to validate these results.

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