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ORIGINAL ARTICLE

Tolerance to challenges miming gastrointestinal transit by spores and vegetative cells of *Bacillus clausii*

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Bacillus clausii, bile tolerance, gut microbial ecology, pH tolerance, probiotics.

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Abstract

Aims: To study *Bacillus clausii* from a pharmaceutical product (Enterogermina O/C, N/R, SIN, T) and reference strains (*B. clausii* and *Bacillus subtilis*) for eco-physiological aspects regarding the gut environment.
Methods and Results: Spores and vegetative cells were challenged *in vitro* miming the injury of gastrointestinal transit: pH variations, exposure to conjugated and free bile salts, microaerophilic and anaerobic growth. No relevant differences were found studying the growth at pH 8 and 10, whereas at pH 7 the yields obtained for O/C and SIN were higher than those obtained for N/R and T strains. The spores were able to germinate and grow in the presence of conjugated bile salts (up to 1%, w/v) or free bile salts (0·2%) and also exhibited tolerance for the combined acid-bile challenge. As evidenced by lag-time, growth rate and cell yield the tolerance of Enterogermina isolates for conjugated salts was comparable with that of *B. clausii* type strain (DSM 8716^T), and resulted higher than that observed for *B. subtilis* (ATCC 6051^T). All the considered *B. clausii* strains demonstrated microaerophilic growth, but only some grew anaerobically in a nitrate medium.
Conclusions: The ability of *B. clausii* spores to germinate after an acid challenge and grow as vegetative cells both in the presence of bile and under limited oxygen availability is consistent with the beneficial health effects evidenced for spore-forming probiotics in recent clinical studies.
Significance and Impact of the Study: The experimental evidence from this study emphasizes some functional properties of *B. clausii* strains regarding their use as probiotics.

Introduction

Bacillus clausii is a gram-positive, aerobic, endospore-forming, facultative alkaliphilic rod bacterium. Its relevant characteristics are catalase and oxidase production, gelatine and starch hydrolysis, nitrate reduction, growth from 30 to 50 °C and in NaCl (up to 10%). The organisms of this species did not hydrolyse pullulan or Tween. The guanine plus cytosine content of their DNA is around 43 mol % (Nielsen *et al.* 1995). *Bacillus clausii* strains are frequently found as components of soil microflora and some properties can be utilized for important applications in industrial and biotechnological fields, namely alkaline protease and xylanase production (Nielsen *et al.* 1995; Denizci *et al.* 2004; Joo *et al.* 2003; Kumar *et al.* 2004). Furthermore, *B. clausii* is an important human probiotic, together with other spore-forming bacilli (*Bacillus subtilis, Bacillus pumilus, Bacillus coagulans, Bacillus cereus* var. *vietnami*) (Sanders *et al.* 2003). A recent taxonomic revision based on molecular analyses (Green *et al.* 1999; Hoa *et al.* 2000; Senesi *et al.* 2001) has included in the *B. clausii* species the four antibiotic-resistant strains, formerly classified as *B. subtilis*, which represent the active principles of the pharmaceutical product Enterogermina, used in oral bacterio-prophylaxis and gastrointestinal tract therapy. Enterogermina was employed as a spore suspension for about 40 years in Italy and at present is also sold in other countries.

For many years, it was thought that the spores of *Bacillus* species were unable to germinate in a low redox environment, such as the intestinal tract. However, the adaptation to oxygen limitation and the anaerobic growth of *B. subtilis* have recently been demonstrated (Nakano and Zuber 1988; Marino *et al.* 2000). The spore germination of bacilli in the small intestine was later observed in a murine model, using culture and molecular approaches (Hoa *et al.* 2001; Casula and Cutting 2002; Duc *et al.* 2003). In literature, the probiotic activity of Enterogermina deals with prevention and treatment of acute diarrhoea and intestinal infections (Mazza 1994), gastrointestinal side effects due to antibiotic therapy (Mazza *et al.* 1992; Nista *et al.* 2004), immunomodulatory activity by stimulating systemic immunoglobulin (IgA, IgG) regulation, and by affecting the cytokine pattern in humans (Muscettola *et al.* 1992; Duc *et al.* 2003; Ciprandi *et al.* 2004). Experimental studies show that the O/C strain, has antimicrobial activity against gram-positive species (e.g. *Staphylococcus aureus* and *Clostridium difficile*) and is able to induce both the proliferation of murine CD4+ T cells and the activation of murine spleen and peritoneal leukocyte populations (Urdaci *et al.* 2004). Moreover, the ability of *B. clausii* to inhibit the genotoxic effect of 4-nitroquinoline-1-oxide, a powerful carcinogen capable of causing DNA adducts, has been evidenced *in vitro* by short-term bacterial assay (Caldini *et al.* 2002).

This evidence, both from clinical and laboratory research, clearly demonstrated the probiotic role of *B. clausii*, but some mechanisms regarding the fate of these bacteria in the gastrointestinal tract need further investigations.

The survival ability of vegetative cells, originated by spore germination in the gut after gastric transit is an important field of study. Bile acids in the intestine exert a potent antimicrobial activity, so that enteric flora have developed mechanisms to resist the action of bile (Gunn 2000). The gram-positive bacteria, compared with gram-negative ones, are usually less tolerant to bile acids excreted into the duodenum, such as *N*-acyl compounds conjugated with glycine or taurine (Ahn *et al.* 2003). Many studies have pointed out the ability of lactobacilli and bifidobacteria to tolerate gastric acidity and bile salts present in the intestinal tract with variability in strain behaviour (Charteris *et al.* 1998; Prasad *et al.* 1998; Haller *et al.* 2001). The acid resistance of *Bacillus* spores is known, and the role of acidity in activating spore germination has been evidenced (Ciffo *et al.* 1987; Mazza 1994; Faille *et al.* 2002). Data on the bile resistance of spore-forming probiotics are also available (Hyronimus *et al.* 2000; Spinosa *et al.* 2000), and a total survival rate of Enterogermina spores was confirmed in simulated gastric fluid, whereas a lower rate (77–83%) was observed by exposing them to simulated intestinal fluid for 3 h (Duc *et al.* 2004). In spite of this, there is not sufficient evidence about the acid and bile tolerance of vegetative cells.

In this paper, we investigated acid and bile tolerance of four probiotic *B. clausii*, studying the effect of conjugated and free bile salts (CBS-FBS) and comparing their behaviour with that of collection strains of the same species and *B. subtilis* reference strains. The considered bacteria were also analysed to identify traits such as alkaliphilic growth.

Materials and methods

Bacterial strains

Four probiotic *B. clausii* strains (O/C, N/R, SIN and T), and the relative mixed form (MIX) characterizing the commercial product Enterogermina, were obtained from Sanofi-aventis OTC (Milan, Italy) as spore suspensions (preparation date: 21 January 2002; the MIX contained the same proportion of the four strains). Reference collection strains were supplied in lyophilized form by Deutsche Sammlung Mikroorganismen (DSM, Germany) and American Type Culture Collection (ATCC, USA). They were: *B. clausii* DSM 8716^T, DSM 2512, DSM 9783; *B. subtilis* ATCC 6051^T, ATCC 33677. Spores and lyophilized forms were revitalized in Luria-broth (10 g l^{-1} of tryptone, 5 g l^{-1} of yeast extract, 5 g l^{-1} of NaCl) and incubated at 37 °C for 24–48 h.

Culture media

Growth kinetics was examined in nutrient broth (NB) and vital counts on nutrient agar (NA). The media were supplied by Oxoid (England). Spores of collection strains were produced on soil extract agar (5 g of peptone, 3 g of beef extract, 15 g of bacto-agar, 750 ml of tap water, 250 ml of autoclavated soil extract) (Gordon *et al.* 1973).

Bile salts

Bile extract L55, Oxoid, containing conjugated glycholate and taurocholate sodium salts (CBS); bovine bile B-8381, Sigma-Aldrich (St Louis, MO, USA), containing CBS-FBS; bile salts B-8756, Sigma-Aldrich, containing free [1:1] cholate and deoxycholate sodium salts (FBS). Bile salts were dissolved in distilled water, filtered through a 0·45-μm membrane (Sartorius, Germany) and added to sterile medium.

Procedure

Spores and vegetative cells were examined for their tolerance to experimental challenges (follows next). In the

different challenges, bacteria were added to liquid medium or saline (depending on the challenge) and incubated for 24–48 h at 37 °C, together with controls. Growth kinetics was studied by a biophotometer (Bonet-Maury, Paris, France), with automatic reading of absorbance percentages, with respect to time zero absorbance, at 620 nm for 24 h. Initial and final cell viability were assayed by plating suitable dilutions on agarized medium and incubating at 37 °C and pH 8, if not otherwise specified.

Alkaline tolerance

The growth kinetics in NB at pH 8 and 10 were studied. The pH values were adjusted with 0·1 mol l^{-1} of sodium sesquicarbonate (Fritze *et al.* 1990).

Acid tolerance

The survival of spores and vegetative cells after 1–2-h exposure to acid stress in saline was evaluated. pH 2, 4 and 6 were adjusted with 0·2 mol l^{-1} of HCl, 0·2 mol l^{-1} of citrate buffer and 0·2 mol l^{-1} of phosphate buffer, respectively (Ciffo *et al.* 1987; Gotcheva *et al.* 2002). Viability was determined by plating on agarized medium.

Tolerance to bile

Bile tolerance was tested by incubating vegetative cells in NB containing CBS or CBS-FBS mixture at the final concentrations of 0·5 and 1% (w/v) and evaluating growth kinetics and viability over 24 h. Bile levels were higher than those usually chosen for probiotic tolerance (Charteris *et al.* 1998; Prasad *et al.* 1998; Hyronimus *et al.* 2000). The pH was adjusted to 8·0 according to experimental protocols for lactobacilli (Charteris *et al.* 1998; Haller *et al.* 2001) and propionibacteria (Huang and Adams 2004). In fact, the pH of bile and pancreatic juice secreted in the gut is about 8, even though it will be below 8 for most of the time during intestinal transit.

Minimum inhibitory concentrations (MIC) were determined in NB tubes containing CBS or CBS-FBS (0·3–0·5–1·0–1·5–2·0%, w/v) and FBS (0·1–0·2–0·3–0·4–0·5%, w/v). Readings were performed after 24 and 48 h of incubation at 37 °C.

Tolerance to consecutive exposure to acid and bile

A simulation of gastrointestinal transit was performed (Haller *et al.* 2001) at different times analysing the growth, in NB plus 1% bile (CBS), of spores preincubated for 1–2 h at pH 2 and 3 in saline. The spore concentration at time zero was *c.* 10^7 ml^{-1} (Ciffo *et al.* 1987).

Microaerophilic and anaerobic growth

Experiments were performed in a Gaspak jar using the commercial gas-generating kits CampyGen CN25 (microaerophilic growth) and AnaeroGen AN25 (anaerobic growth) obtained from Oxoid (England). Spores were seeded in NB or NA and incubation protracted for 24–48 h at 37 °C. Anaerobic growth was also tested using the same media added with 1 g l^{-1} of $NaNO_3$.

Results

pH effect on the growth kinetics

The *B. clausii* strains were examined as regards ability to grow in alkaline conditions, an important taxonomic characteristic of the species. The alkali tolerance of all examined bacteria was confirmed and slight differences among strains were evidenced. Table 1 indicates that both the lag-time and the growth rate in the exponential phase were influenced by pH. Unlike N/R and T, the strains O/C and SIN showed a short lag-time at pH 7, whereas all the strains showed a shortening of generation time in passing from neutral to alkaline conditions.

In alkaline medium (pH 8 and 10) growth yields of O/C, N/R, SIN and T were comparable, whereas at pH 7

Table 1 Influence of pH on germinability and growth parameters of probiotic *Bacillus clausii* spores*

Strain	pH	Lag-time†‡ (h)	μ_{max}†§ (h^{-1})	g¶ (min)
N/R	7	5·07 ± 0·58	1·06 ± 0·51	56·60
	8	4·15 ± 0·98	1·99 ± 0·36	30·15
	10	4·30 ± 0·25	1·99 ± 0·86	30·15
T	7	7·23 ± 0·29	0·80 ± 0·54	75.00
	8	5·15 ± 0·17	0·93 ± 0·04	64·52
	10	7·27 ± 2·14	1·46 ± 1·20	41·10
O/C	7	3·50 ± 0·71	1·66 ± 0·50	36·14
	8	6·33 ± 1·15	1·93 ± 0·99	31·09
	10	6·10 ± 1·01	1·99 ± 0·26	30·15
SIN	7	4·43 ± 0·51	1·06 ± 0·33	56·60
	8	7·17 ± 2·80	1·13 ± 0·30	53·10
	10	6·30 ± 0·24	1·33 ± 0·44	45·11
MIX	7	4·12 ± 0·48	1·15 ± 0·50	52·17
	8	5·03 ± 0·46	1·52 ± 0·42	39·47
	10	4·15 ± 0·71	2·39 ± 0·15	25·10

*, Cultures started with spores (*c.* 10^5 ml^{-1}), in nutrient broth, at 37 °C.

†, Mean values with SE from three–four biophotometric experiments.

‡, Time associated to the intercept of the inoculum level absorbance with the tangent drawn to the exponential phase of the growth curve.

§, Maximum specific growth rate calculated by the slope of the tangent drawn to the linear tract of growth curve.

¶, Generation time estimated from mean μ_{max}.

the O/C and SIN rates were higher (Fig. 1). After 24 h of incubation, cultures obtained with MIX inoculum presented comparable yields in alkaline and neutral media indicating that the growth of MIX at pH 7 seems to be chiefly supported by O/C and SIN. Cell yields of the reference strain DSM 8716^T were similar to those of probiotic strains and confirmed pH 8 as optimal for its growth.

Tolerance to bile

A different degree of tolerance was observed considering CBS-FBS. The MIC of conjugated salts and CBS-FBS mixture were greater than 2·0% for the four probiotic strains, whereas for *B. clausii* DSM 8716^T the MIC values were 2·0% and 1·5% for CBS and FBS, respectively. The inhibitory activity of free salts was tenfold higher than that of the CBS and CBS-FBS mixture. Free salts MIC resulted in 0·1% and 0·2% at 24 and 48 h, respectively. All the considered bacteria were able to grow in NB containing 0·5 and 1% CBS, even if the cell concentrations reached after 24 h of incubation were generally lower than those of control cultures without bile (Fig. 2).

Figure 3 indicates that in the presence of conjugated salts growth was not prevented, but only delayed, and not greatly diminished. The effect of bile concentration occurs both by lag-time increase and growth rate reduction in passing from 0% to 0·5% and 1% bile. The behaviours observed for probiotic strains are coherent with that observed for the reference DSM 8716^T. Similar growth profiles were obtained studying the effect of the CBS-FBS mixture (data not shown).

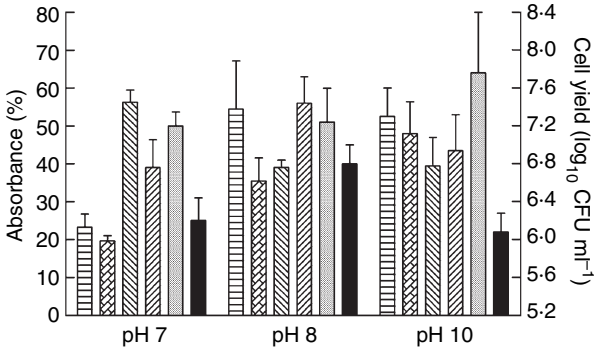

Figure 1 Cell yields of probiotic and collection *Bacillus clausii* strains grown at 37 °C for 24 h in nutrient broth at different pH (spore inoculum, Table 1). Data express mean absorbance percentages, compared with time zero absorbance, of biophotometric readings and the bars represent SD from three to four experiments. The right-hand scale shows cell concentration estimated by absorbance-plate count calibration curve. (▤), N/R; (▨), T; (▧), O/C; (▨), SIN; (■), MIX; (■), DSM 8716^T.

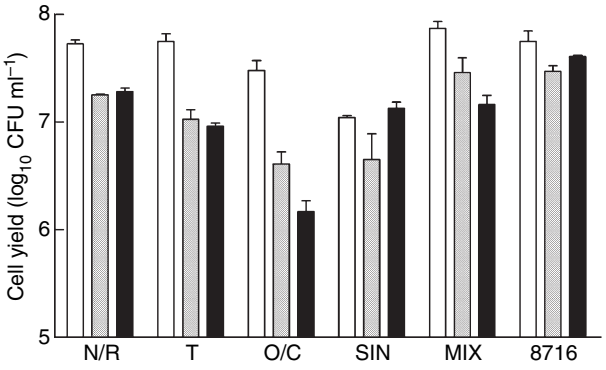

Figure 2 Cell yield of *Bacillus clausii* strains reached in nutrient broth medium (pH 8) containing 0·5% and 1% conjugated bile salts after 24 h of incubation at 37 °C. Cultures were started with vegetative cells (*c.* 10^5 cells ml^{-1}). Bars represent SD from three to four experiments. (□), no bile; (■), 0·5% bile; (■), 1% bile.

Comparing the growth percentages with respect to controls without bile (Fig. 4), we confirmed that they were higher with CBS than FBS. The bile tolerance of probiotic strains did not differ from that of *B. clausii* DSM 8716^T, and resulted more evident than that observed for *B. subtilis* ATCC 6051^T.

Effect of combined acid-bile challenges on spore germination

A preliminary screening confirmed 2 h of acid tolerance of probiotic spores up to pH 2, while vegetative cells tolerated pH 6 and were labile at pH ≤ 4 (data not shown). Spore-germinative ability was then evaluated miming gastrointestinal conditions. The response to acid-bile challenges was similar for all the examined strains. In particular, Fig. 5 shows that cell counts determined after spore preincubation at pH 2 for 2 h were comparable with untreated control suspensions. The subsequent exposure to a mixture of CBS-FBS produced a temporary stress on spore germination. In fact, cell counts diminished after a 1-h challenge. The stress was eliminated by extending incubation time to 24 h.

Growth in microaerophilic and anaerobic conditions

Table 2 shows that all the considered *B. clausii* and *B. subtilis* strains were able to grow in microaerophilic conditions both in liquid and agarized medium. In the same media, the growth was not evidenced under anaerobic incubation, but it can be seen that the four probiotic *B. clausii* and one *B. subtilis* (ATCC 33677) were different from the other reference strains, and were also able to grow in anaerobic conditions when the media were added with sodium nitrate.

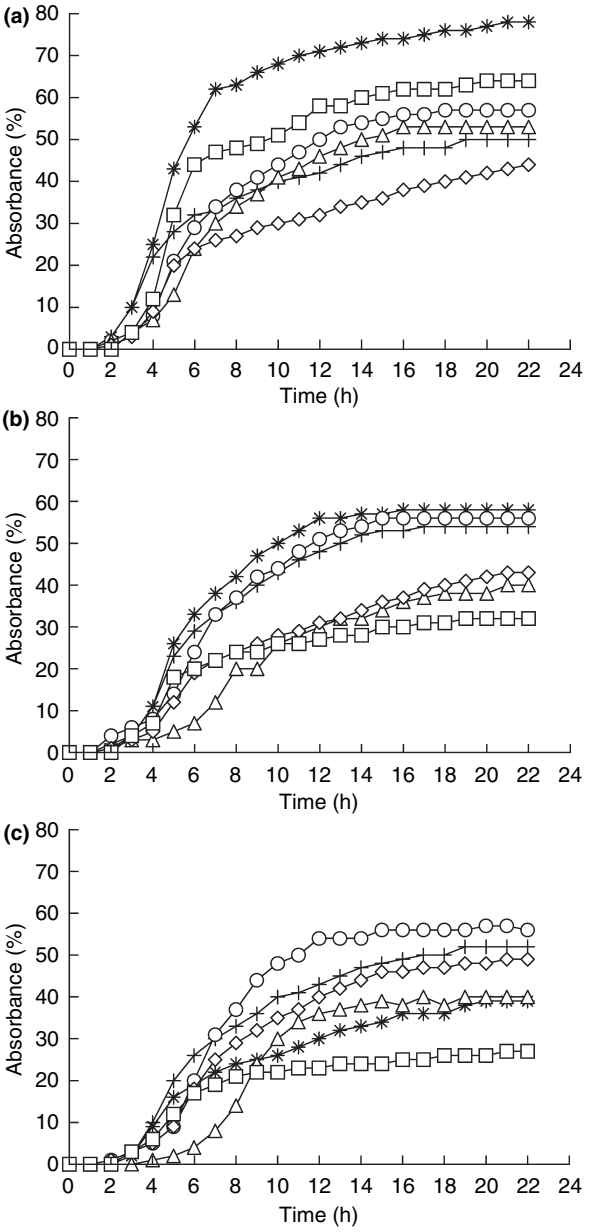

Figure 3 Representative growth kinetics of probiotic *Bacillus clausii* strains in nutrient broth medium (pH 8) containing 0% (a), 0·5% (b) and 1% (c) bile extract (L55, Oxoid). Cultures were started with vegetative cells (*c.* 10^5 cells ml^{-1}) and data express absorbance percentages, compared with time zero absorbance, of biophotometric readings transferred to Prism™ version 3·0 (GraphPad Software, Inc., USA). (○), N/R; (△), T; (◇), O/C; (□), SIN; (✳) MIX; (+) DSM 8716^T.

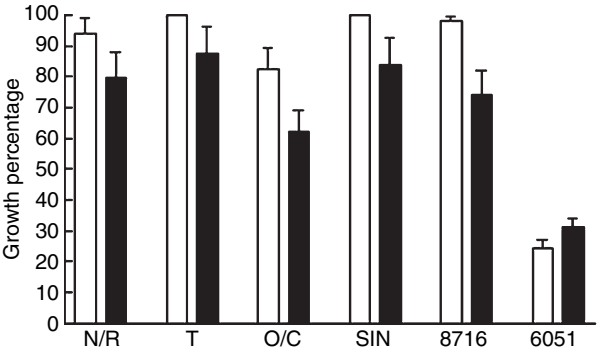

Figure 4 Comparative tolerance of the strains to 1% conjugated (CBS) and conjugated-free bile salts (CS-FBS)in nutrient broth medium (*Bacillus clausii*, pH 8; *Bacillus subtilis*, pH 7), after 24 h of incubation. Cultures were started with vegetative cells (*c.* 10^5 cells ml^{-1}). Data express percentage of counts calculated compared with that of control cultures (no bile). Bars represent standard deviation from three experiments. (□), CBS; (■), CBS-FBS.

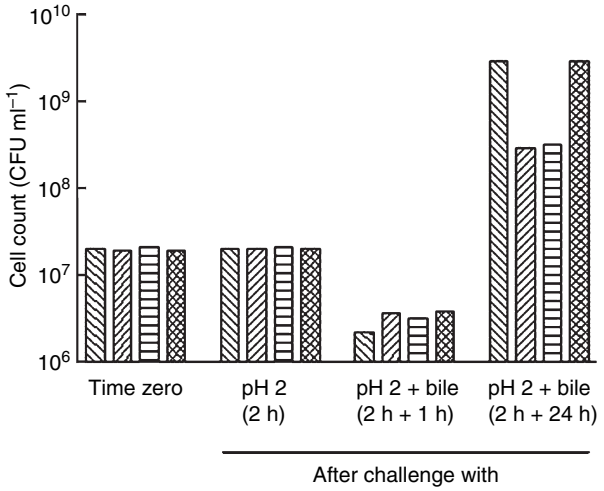

Figure 5 Development of tolerance to 1% conjugated bile salts of *Bacllus clausii* spores (*c.* 10^7 spores ml^{-1}) preincubated for 2 h at pH 2. Data express cell counts determined after incubation for 1 and 24 h in the presence of bile. Mean values from two experiments. (▨), O/C; (▨), SIN; (▤), N/R; (▨) T.

Discussion

As well as alkaliphilic growth of *B. clausii*, described as typical of the species (Fritze *et al.* 1990; Nielsen *et al.* 1995), our results confirmed spore germination as well as growth in a neutral environment. This behaviour was strain-dependent and growth yields obtained for O/C and SIN were higher than those for N/R, T and type strain (DSM 8716^T). Using MIX suspension containing the four strains, the growth was not influenced by a pH between 7 and 10, and so the yield at pH 7 seems to be chiefly supported by O/C and SIN. The adaptation of the multi-strain product to pH may be considered an important characteristic for probiotic function, as pH varies from

Table 2 Germinability of *Bacillus clausii* and *Bacillus subtilis* spores under limited or absence of oxygen availability*

		B. clausii								*B. subtilis*	
Medium	pH	N/R	T	O/C	SIN	MIX	8716^T	2512	9783	6051^T	33677
Microaerophilic growth											
NA†/NB‡	7	++/+	++/+	++/+	++/+	++/+	++/+	++/+	++/+	+/+	+/+
NA/NB	8	++/+	++/+	++/+	++/+	++/+	++/++	++/++	++/++	ND	ND
Anaerobic growth											
NA/NB	7	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–
NA/NB	8	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	ND	ND
NA/NB + nitrate	7	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	+/(+)
NA/NB + nitrate	8	±	±	±	±	±	–/–	–/–	–/–	ND	ND

*, Spores of reference strains (*B. clausii* DSM 8716^T, 2512, 9783 and *B. subtilis* ATCC 6051^T, 33677) were obtained in soil extract agar.
†, Nutrient agar.
‡, Nutrient broth.
++, high growth; +, growth; (+), poor growth; –, no growth; ND, not determined.

5·5 to 6 in the large intestine (Ahn *et al.* 2003) to around 8 in the small intestinal tract (Huang and Adams 2004).

According to well-known spore biology, *B. clausii* spores tolerate acidity, unlike vegetative cells, and their germination after a combined acid-bile challenge has been proved in this study. The ability to tolerate bile, usually considered a prerogative of enteric microflora, is important for survival of bacteria in the gut lumen. Therefore, bile tolerance of *B. clausii* must be considered of primary interest, in relation to the lack of univocal data for bacilli regarding their germination in the intestinal tract (Spinosa *et al.* 2000; Casula and Cutting 2002). All the strains showed considerable rates of growth when incubated with CBS and CBS-FBS mixture of bile salts. Note that the concentrations tested (up to 1%) were higher than 0·3% and 0·5%, which are considered critical levels for good and very good tolerance, respectively, for probiotics (Charteris *et al.* 1998; Hyronimus *et al.* 2000). Unlike what happens in lactobacilli and bifidobacteria, for which strain variation for bile tolerance were reported (Charteris *et al.* 2000), no relevant differences were found among probiotic and collection *B. clausii* strains. Moreover, bile tolerance observed in *B. clausii* was higher than that in *B. subtilis* and we consider this as an intrinsic characteristic of spore-forming bacilli, which varies among species. In fact, referring to literature data, the MIC of bile may be estimated as 0·1%, 0·7% and more than 1% for *Bacillus laevolacticus*, *Bacillus racemilacticus* and *Bacillus coagulans* strains, respectively (Hyronimus *et al.* 2000).

Regarding the high sensitivity of vegetative cells for FBS, we observed a MIC lower than 0·1% after 24 h and equal to 0·2% after 48 h, demonstrating that the initial stress had been overcome. However, it should be remembered that human bile contains chiefly CBS (De Boever and Verstraete 1999).

The microaerophilic growth and multiplication in anaerobiosis, when strains were cultured in medium containing nitrate, further underline the possible adaptation of *B. clausii* to the gut environment. This latter characteristic, which has also been evidenced for the 'strict aerobe' *B. subtilis* (Nakano and Zuber 1998; Cruz Ramos *et al.* 2000; Marino *et al.* 2000), may be interpreted on the basis of a high concentration of cytochromes in alkaliphilic bacilli, even if the exact functions of their respiratory chain is not yet completely clear (Yumoto *et al.* 1997).

In conclusion, the described physiological properties of the examined *B. clausii* strains validate both the ability of the spores to germinate during gastrointestinal transit and the possibility for vegetative cells to survive in the intestinal tracts, further demonstrating their probiotic function.

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