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Intrinsic tolerance of *Bifidobacterium* species to heat and oxygen and survival following spray drying and storage

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ABSTRACT

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Aims: This study examined the tolerance of various species of the genus *Bifidobacterium* to heat and oxygen and evaluated the survival of selected strains following spray drying and during storage.

Methods and Results: Nine *Bifidobacterium* species were considered to be relatively tolerant to both heat and oxygen and mostly segregated into two clusters within the 16S rDNA phylogenetic tree. Four species were tolerant to oxygen and 12 species were considered sensitive to oxygen and heat. Using a skimmed milk-based carrier good survival following spray drying and storage at 4°C correlated with tolerance to heat and oxygen. Viability was inversely related to storage temperature and at 15°C and 25°C, a significant decline was observed for all species. The inclusion of gum acacia had no significant affect on survival or viability. However, using a fluidized-bed spray dryer viability was greatly improved.

Conclusions: A group of closely related species tolerant to heat and oxygen had high survival following spray drying and maintained viability during prolonged storage at 4°C.

Significance and Impact of the Study: Spray drying is a suitable method for the production of skimmed milk powder enriched with high numbers of viable bifidobacteria.

Keywords: bifidobacteria, heat, oxygen, spray drying, survival, tolerance, viability.

INTRODUCTION

The manufacture of probiotic food containing bifidobacteria at the recommended level of 10^6–10^7 CFU per millilitre or gram of product represents a major technological challenge (see reviews by Ishibashi and Shimamura 1993; Tamime *et al.* 1995; Knorr 1998; Arunachalam 1999; Gomes and Malcata 1999; Saarela *et al.* 2000).

Spray drying technology has been used to manufacture powders containing high numbers of viable bacteria from a range of genera (Mary *et al.* 1993; To and Etzel 1997a,b; O'Riordan *et al.* 2001). It offers high production rates at relatively low operating costs and resulting powders are dry, stable and easily transported (Johnson and Etzel 1993). However, at the temperatures needed to produce powders with a moisture content of approx. 4%, required for powder stability and spoilage prevention (Písecký 1997; Masters 2002), low probiotic survival can often occur (Johnson and Etzel 1993; Teixeira *et al.* 1995a; To and Etzel 1997a,b; Gardiner *et al.* 2000). Loss of viability appears to be principally caused by cell membrane damage (Teixeira *et al.* 1995, 1995a; Gardiner *et al.* 2000) although the cell wall, ribosome and DNA are also affected at higher temperatures (Teixeira *et al.* 1997).

The intrinsic sensitivity of a given strain to heat appears to be an important factor in determining survival (Daemen and van der Stege 1982; Teixeira *et al.* 1995b; To and Etzel 1997a; Gardiner *et al.* 2000). Lian *et al.* (2002) found strains from the closely related species *Bifidobacterium longum* biotype *longum* and *B. longum* biotype *infantis* (Sakata *et al.* 2002) had distinctly different survival values irrespective of spray drying conditions and carrier medium, although these parameters did influence their overall survival. The authors

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suggested that the difference might reflect their intrinsic tolerance to heat. In two separate spray drying studies the survival of *Bifidobacterium animalis* ssp. *lactis* (Fávaro-Trindade and Grosso 2002) and *Bifidobacterium ruminantium* (O'Riordan *et al.* 2001) were markedly different from each other and to those for *B. longum* biotype *longum* and *B. longum* biotype *infantis*. Similarly, although oxygen tolerance is known to vary among *Bifidobacterium* species (Scardovi 1986; Shimamura *et al.* 1992; Talwalkar *et al.* 2001) it is unclear whether oxygen sensitivity plays a role in determining survival following spray drying.

Maintenance of probiotic viability during powder storage is an essential factor in determining the commercial success of a product (Espina and Packard 1979). For freeze-dried (Nagawa *et al.* 1988; Castro *et al.* 1995) and spray-dried powders (Mary *et al.* 1993; Teixeira *et al.* 1995; To and Etzel 1997b; Silva *et al.* 2002) the level of water activity appears to be important factor. Powders with a water activity value of approx. 0·2, equivalent to 4% moisture (Písecký 1997), appear to maintain the best viability. In addition, viability is generally inversely related to storage temperature (Abd-El-Gawad *et al.* 1989; Mary *et al.* 1993; Teixeira *et al.* 1995; Gardiner *et al.* 2000, 2002; Silva *et al.* 2002; Corcoran *et al.* 2004). For bifidobacteria little appears to be known about viability. Poor viability for *B. ruminatium* was reported following storage at ambient temperatures (O'Riordan *et al.* 2001).

In previous studies skimmed milk feed was found to give good protection to strains during spray drying compared with other carriers (Johnson and Etzel 1993; Lian *et al.* 2002; Corcoran *et al.* 2004) and stationary phase cultures were found to be more resistant than exponential phase cultures to heat (Teixeira *et al.* 1994) and spray drying (Teixeira *et al.* 1995a; Corcoran *et al.* 2004). In addition, the incorporation of gum acacia into skimmed milk was found to protect a probiotic *Lactobacillus paracasei* strain during spray drying and improve viability during storage (Desmond *et al.* 2002).

In the current study, stationary phase cultures of *Bifidobacterium* species were assessed for their tolerance to heat and oxygen. Selected strains were then spray dried in a skimmed milk carrier with and without gum acacia under conditions to attain powders with approx. 4% moisture at laboratory and large scale. Initial survival and viability after storage at specific temperatures were determined.

MATERIALS AND METHODS

Strains and growth conditions

The *Bifidobacterium* strains used in the study are listed in Table 1. The newly described and closely related genus and species *Aeriscardovia aeriphila* was also included. Strains were routinely cultured under anaerobic conditions (anaerobic jars with Anaerocult A gas packs; Merck, Darmstadt, Germany) at 37°C in modified MRS (mMRS), comprising Lactobacilli MRS medium (Difco, Detroit, MI, USA) supplemented with 0·05% (w/v) cysteine–HCl. Colony-forming units (CFU) were determined by serial dilutions of cultures and powders in maximum recovery diluent (MRD) (Oxoid) followed by pour plating with mMRS. Plates were incubated at 37°C for 3 days under anaerobic conditions.

Heat tolerance assay

The heat tolerance of each strain was determined from stationary phase cultures established from two successive 1% (v/v) inoculations in mMRS and incubation (as described above) for 20 h. Cultures were cooled on ice and 200 μl aliquots were heated for 5 min at 42°C, 52°C, 55°C, 57°C and 60°C in a gradient PCR thermal block. The samples were immediately cooled for 15 min on ice and CFU ml^{-1} counts were determined as above using a 100-μl sample in 9·9 ml of MRD (see above). The percentage survival was calculated as follows: % survivors $= N/N_o \times 100$, where N represents the CFU ml^{-1} in the mMRS at a selected temperature and N_o is the CFU ml^{-1} in mMRS at 42°C. All data are based on duplicate assays.

Oxygen tolerance assay

The ability of each strain to grow under aerobic conditions was determined as follows: cells cultured to stationary phase under anaerobic conditions as described above were inoculated at 1% (v/v) into mMRS, 10 ml (test-tube) and 100 ml (500 ml conical flask). The former was incubated under anaerobic conditions and the latter was continuously shaken at 150 rev min^{-1} on an orbital shaker. Both cultures were incubated at 37°C for 24 h. OD_{600} was used to measure growth. Tolerance to oxygen was calculated as follows: % OD $= N/N_o \times 100$, where N represents the OD under aerobic conditions and N_o is the OD under anaerobic conditions.

Spray drying

Laboratory-scale spray drying. From two successive overnight (o/n) mMRS cultures a 1% (v/v) inoculum was used to seed 100 ml of mMRS. Following incubation under anaerobic conditions for 20 h cells were harvested by centrifugation at 5000 g for 10 min and re-suspended in 200 ml of ice-cooled 20% (w/v) RSM. The RSM feed was mixed with compressed air in a two-fluid nozzle atomizer before drying at a constant air inlet temperature of 170°C and outlet temperature of 85–90°C in a mini spray dryer (model B-191, Büchi, Flawil, Switzerland). At least two

Table 1 *Bifidobacterium* species and their tolerance to heat and oxygen

Species	Mean % survival ± SD (CFU ml^{-1} at each selected temperature per CFU ml^{-1} at 42°C)				Heat tolerance rating	Mean % growth ± SD (OD aerobic/ OD anaerobic)	Oxygen tolerance rating
	52°C	55°C	57°C	60°C			
B. adolescentis NCMB 2204^T	ND	ND	0	0	Low/moderate	1·4 ± 0·35	Low
B. angulatum NCMB 2236^T	129 ± 9·2	0·35 ± 0·03	0	0	Low	3·4 ± 0·5	Low
B. animalis ssp. *animalis* DSMZ 20104^T	ND	ND	37·5 ± 17·1	1·9 ± 0·1	High	23·4 ± 1·3	Moderate
B. animalis ssp. *lactis* JCM 7117	ND	ND	77·8 ± 17·5	65·4 ± 6·9	High	25·8 ± 2·1	Moderate
B. animalis ssp. *lactis* DSMZ 20105	ND	ND	60·6 ± 3·4	1·3 ± 0·9	High	24·9 ± 5·3	Moderate
B. animalis ssp. *lactis* BB12	ND	84·4 ± 2·7	66·4 ± 3·4	1·2 ± 0·2	High	25·6 ± 0·85	Moderate
B. bifidum NCMB 795	3 ± 0·8	0	0	0	Low	0·85 ± 0·3	Low
B. breve NCMB 8807	26·9 ± 8·3	0·008 ± 0·005	0	0	Low	5·7 ± 0·5	Moderate
B. boum LMG 10736^T	ND	44·6 ± 6·9	0·08 ± 0·01	0	Moderate	58·2 ± 4·7	High
B. choerinum ATCC 27686	ND		78·5 ± 21·6	1·3 ± 0·9	High	29·2 ± 5·6	Moderate
B. cuniculi LMG 10738^T	14·1 ± 1·5	0·017 ± 0·004	0	0	Low	30·7 ± 1·8	Moderate
B. gallicum LMG 11596^T	51·1 ± 19·1	6·9 ± 0·9	0·004 ± 0·002	0	Low	1·65 ± 0·2	Low
B. gallinarum DSMZ 20670^T	ND	ND	0	0	Low	0	Low
B. longum biotype *infantis* NCMB 2205^T	ND	ND	0	0	Low/moderate	0·9 ± 0·3	Low
B. longum biotype *longum* NCIMB 8809	12·8 ± 1·3	0·002 ± 0·001	0	0	Low	0·85 ± 0·3	Low
B. magnum DSMZ 20222^T	ND	ND	46·7 ± 25·2	2·6 ± 0·3	High	7·1 ± 1·1	Moderate
B. merycicum LMG 11341^T	13·1 ± 1·1	5 ± 1·1	0	0	Low	0·5 ± 0·07	Low
B. minimum DSMZ 10105^T	92·3 ± 18	60·5 ± 6·4	0·08 ± 0·02	0	Moderate	55·6 ± 1·3	High
B. pseudolongum ssp. *globosum* JCM 5820^T	ND	97·4 ± 27·7	23 ± 12·2	0·16 ± 0·07	High	5·5 ± 0·7	Moderate
B. pseudolongum ssp. *pseudolongum* NCMB702244^T	ND	95·9 ± 21·4	14 ± 12·5	0·04 ± 0·001	High	25·7 ± 1·4	Moderate
B. pseudocatenulatum 8811	0·3 ± 0·15	0	0	0	Low	0	Low
B. pyschraerophilum	27·5 ± 8·9	0·009 ± 0·006	0	0	Low	80·9 ± 2·6	High
B. pullorum DSMZ 20433^T	9·6 ± 5·2	0	0	0	Low	0	Low
B. ruminantium DSMZ 6489^T	7·9 ± 7	ND	0	0	Low	0	Low
B. saeculare DSMZ 6531^T	79·9 ± 16·7	25 ± 6·2	0	0	Low	0	Low
B. longum biotype *suis* ATCC 27533^T	117·8 ± 53·1	3·6 ± 2·7	0	0	Low	6·7 ± 0·2	Moderate
B. thermacidophilum LMG 21395^T	70·7 ± 1·2	68 ± 2·2	69·7 ± 1·4	ND	High	20·9 ± 4	Moderate
B. thermophilum DSMZ 20209	79·7 ± 19·6	9·2 ± 0·18	0	0	Low	14·6 ± 1·3	Moderate
B. thermophilum NCIMB 702253^T	30·8 ± 3	3·2 ± 2·8	0	0	Low	7·9 ± 2·9	Moderate
B. thermophilum NCIMB 702554	40 ± 10·6	1 ± 0·5	0	0	Low	6·65 ± 0·26	Moderate
A. aeriphila LMG 21773^T	18 ± 7·6	0	0	0	Low	83·5 ± 8·4	High

Heat tolerance rating (compared with survival at 42°C); high >1% at 57°C, moderate >40% at 55°C, low <40% at 55°C. Oxygen tolerance rating (aerobic growth compared with anaerobic growth); high >30%, moderate >5%, low <5%. ND, not determined.

trials were performed per strain. Selected strains were also inoculated into a feed medium containing a mixture RSM (10%, w/v) and gum acacia (10%, w/v) and spray dried as described above.

Large-scale fluidized-bed spray drying. Cells were cultured as above except a 1% (v/v) inoculum was added to 32 l of mMRS. Anaerobic growth conditions were promoted by the use of fresh medium and screw-lid bottles. The cells were collected by centrifugation and added to 240 l of RSM (20%, w/v). The feed was pumped by a positive displacement pump through a scraped surface heater at 23°C and delivered to a high pressure pump at approx. 2·0 bar. Drying was performed in a tall form spray dryer (Niro A/S, Copenhagen, Denmark, model TFD20) with a nominal water vapour capacity of 70–100 kg h^{-1}, fitted with two external fluidized beds, set at air temperatures of 55°C and 25°C respectively. The feed was atomized using three nozzles and dried at an inlet and outlet temperature of 175°C and 68°C respectively. Two spray drying trials were performed at this scale.

Powder storage, initial survival and viability. Skimmed milk powders were stored in polythene bags and kept in aluminium-coated paper bags. Initially all powders were held at 4°C for 7 days before segregation and storage at 4°C, 15°C and 25°C. The percentage survival was calculated as follows: % survivors $= N/N_o \times 100$, where N_o represents

the number of bacteria in the RSM before drying (taken as an average of two samples taken at the start and end of the drying period) and N is the number in the spray-dried powder. Both N and N_o were expressed per gram of dry matter. Viability in powders was determined from 0·1 g samples taken 30 and 90 days after the day of powder manufacture. All results were compared by the Student's *t*-test (two-tail paired) with significance measured at a probability level of $P \leq 0{\cdot}10$ and $\leq 0{\cdot}05$ for survival following spray drying and storage respectively.

Moisture content in spray dried powders

The moisture content of spray-dried powders was determined by oven drying at 102°C. This involved determination of the difference in weight before and after drying, expressed as a percentage of the initial powder weight, according to the International Dairy Federation Bulletin (IDF 1993).

16S rDNA Phylogenetic tree construction

The 16S rDNA phylogenetic tree for the genus *Bifidobacterium* and several closely related genera was constructed as previously described (Simpson *et al.* 2003).

RESULTS

The inoculum and growth times used in the current study were considered to yield early to mid stationary phase cultures based on previous growth curves for selected strains (data not shown). Prolonged periods in stationary phase resulted in reduced cell counts. The culture pH for each strain was measured and compared with the minimum value attained after 48 h. Good growth was considered to occur when the pH value was within 0·5 units of the minimum.

Based on the relative survival at elevated temperatures compared with 42°C the following heat tolerance ratings were assigned: high, >1% survival at 57°C (none strains); moderate, >40% survival at 55°C (two strains); and low, <40% survival at 55°C (20 strains) (Table 1). The following oxygen tolerance ratings were assigned to the strains based on their relative growth under aerobic and anaerobic conditions: high, >30% growth (four strains); moderate, >5% growth (15 strains); and low, <5% growth (12 strains).

Eleven strains representing nine species and subspecies had either moderate or high tolerance to heat and oxygen. These included *B. animalis* ssp. *animalis*, *B. animalis* ssp. *lactis*, *Bifidobacterium pseudolongum* ssp. *pseudolongum*, *B. pseudolongum* ssp. *globosum*, *Bifidobacterium choerinum* and *Bifidobacterium magnum* that grouped within a single branch of the 16S rDNA phylogenetic tree for the genus and *Bifidobacterium thermacidophilum* and *Bifidobacterium boum* that also segregated together (Fig. 1). Eight strains had a low tolerance to heat but a moderate or high tolerance to oxygen and 10 strains had a low tolerance to heat and oxygen. Interestingly, no species had a high or moderate tolerance to heat but a low tolerance to oxygen.

Twelve species or subspecies with varying heat and oxygen tolerances were selected for spray drying. A total of 42 powder samples were produced and all had $>10^8$ CFU g^{-1} and powder moistures $\leq 4{\cdot}2\%$ (Table 2). The majority of species (5 from 6) with high heat tolerance and moderate oxygen tolerance had survival values ranging from 68% to 102% (Table 2). Only *B. thermacidophilum* from this tolerance group had a relatively poor survival value of 12%. The remaining five species with a low tolerance to heat and a variety of tolerances to oxygen had lower survival values ranging from 19·4% to 38% (Table 2). Only the latter group of species and *B. thermacidophilum* had survival values significantly lower than the highest observed for *B. choerinum*. Culturing *Bifidobacterium pyschraerophilum* under anaerobic and aerobic conditions prior to spray drying had no significant affect on percentage survival (Table 2).

The inclusion of gum acacia into the skimmed milk feed resulted in comparable initial survival levels and powder moisture contents for *B. animalis* ssp. *lactis*, *Bifidobacterium breve* and *B. pyschraerophilum* (Table 2). *Bifidobacterium thermophilum* NCIMB 702254 had comparable survival and powder moisture content following spray drying at either laboratory scale or large scale (Table 2).

All powder viability data is presented in Table 3. For the six heat- and oxygen-tolerant species no significant reduction in viability after 30 days at 4°C was observed except for *B. thermacidophilum*. However, after 90 days *B. choerinum* and *B. magnum* were significantly reduced. At 15°C *B. animalis* ssp. *animalis*, *B. animalis* ssp. *lactis* and *B. pseudolongum* ssp. *globosum* had no significant decline after 30 days but a significant decline was recorded by 90 days and at 25°C the affect was noted after 30 days. For the five low-heat-tolerant strains a significant fall in viability was seen at each temperature after 30 days storage. The only exceptions seen were *B. longum* and *A. aeriphila*. In both cases the discrepancies could be accounted for by the large difference in CFU counts within the skimmed milk feed used in the two spray-drying trials (Table 2). Although significant reductions occurred for all species at 25°C after only 30 days the highest viability correlated with heat and oxygen tolerance. For example, *B. animalis* ssp. *animalis*, *B. animalis* ssp. *lactis* and *B. pseudolongum* ssp. *pseudolongum* retained approx. 30%, 5% and 20% viability, respectively, compared with 0·01–0·2% viability for the other species.

Following the inclusion of gum acacia into RSM only *B. breve* appeared to show a consistent improvement in viability. However, the increases were insufficient to prevent significant reductions in viability at each storage temperature

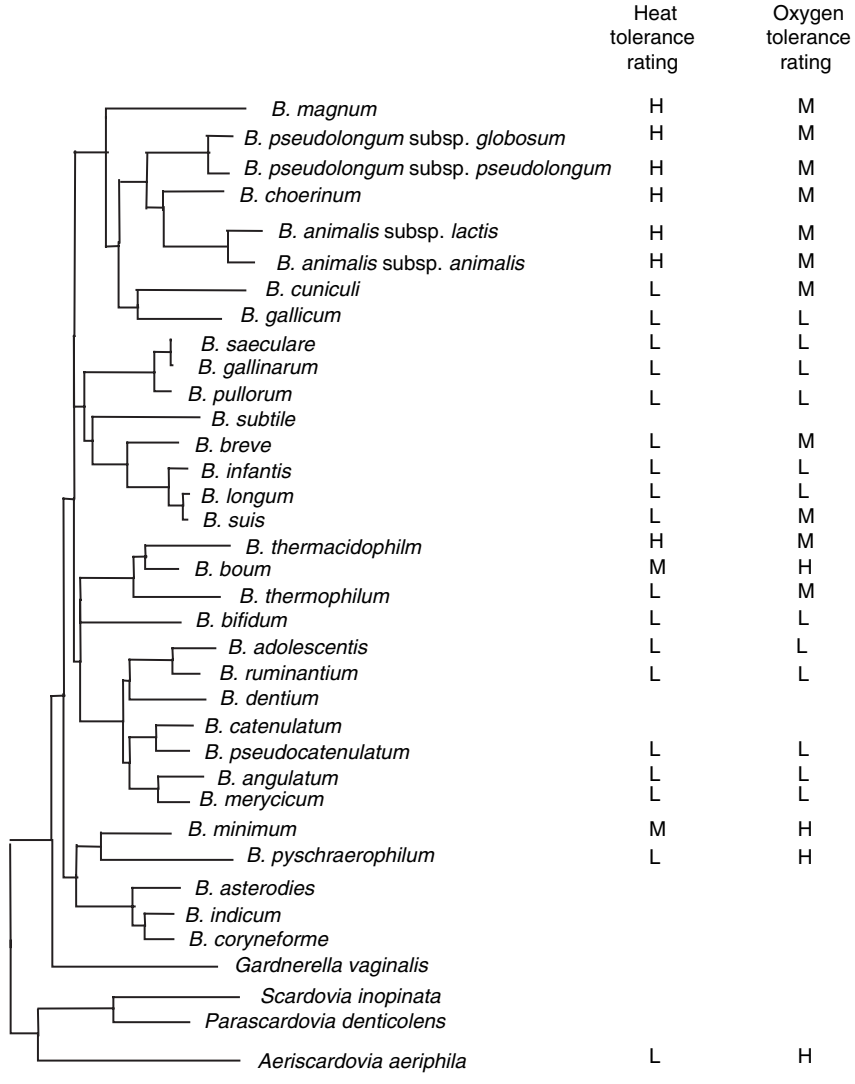

Fig. 1 16S rDNA phylogenetic tree of the genus *Bifidobacterium* and related genera showing the genetic relatedness of heat and oxygen tolerance species. H, high; M, moderate; L, low

after 30 days. The viability of *B. thermophilum* was markedly improved in powders produced at large scale compared with laboratory scale, with no significant decline after 90 days at 4°C or 30 days at 25°C (Table 3). Although significant declines were evident after 90 days at elevated temperatures clear improvements were evident. For example, at 25°C the approx. 10% viability seen with the large-scale powders represented a 160 000-fold increase over the laboratory-scale powders.

DISCUSSION

Considerable heterogeneity between *Bifidobacterium* species was observed and a number of distinct heat tolerance ratings were identified. Several species, characterized as having high or moderate tolerance, clustered into two distinct branches within the 16S rDNA phylogenetic tree. None of these species were previously considered to be heat tolerant although *B. thermacidophilum* can grow at 48·5°C (Dong *et al.* 2000). Interestingly, the closely related species *B. thermophilum* can reportedly survive a 30-min exposure to 60°C (Scardovi 1986). However, in the current study the species was considered to have a low heat tolerance rating.

Several *Bifidobacterium* species were considered to have some level of oxygen tolerance and most our findings agreed with previous studies (Scardovi 1986; Meile *et al.* 1997; Dong *et al.* 2000; Talwalkar *et al.* 2001; Simpson *et al.* 2004). We also considered *B. animalis* ssp. *animalis*, *Bifidobacterium cuniculi*, *B. magnum*, *B. pseudolongum* ssp. *pseudolongum* and *Bifidobacterium suis* to be oxygen tolerant. However, Talwalkar *et al.* (2001) found *B. animalis* and *B. pseudolongum* grew poorly and *Bifidobacterium infantis* grew well under aerobic conditions respectively. It is unclear whether these discrepancies reflect strain variation within the species.

Table 2 Laboratory-scale spray drying of bifidobacteria, feed and powder CFU counts, percentage survival and powder moisture values

Species	No. of trials	Mean log CFU ml^{-1} of feed ± SD	Mean log CFU g^{-1} powder ± SD	Mean % survival ± SD	Mean % moisture ± SD
B. animalis ssp. *animalis* DSMZ 20104^T	3	8·66 ± 0·04	9·27 ± 0·18	87 ± 34**	2·47 ± 0·35
B. animalis ssp. *lactis* BB12	4	8·65 ± 0·22	9·24 ± 0·23	79 ± 25**	3·16 ± 0·32
B. animalis ssp. *lactis* BB12	2†	8·70 ± 0·08	9·24 ± 0·11	72 ± 29**	3·35 ± 0·08
B. bifidum NCMB 795	3	8·61 ± 0·45	8·54 ± 0·42	21 ± 10*	3·83 ± 0·60
B. breve NCMB 8807	3	8·77 ± 0·12	9·03 ± 0·17	38 ± 9*	3·23 ± 0·21
B. breve NCMB 8807	2†	8·47 ± 0·01	8·53 ± 0·11	23 ± 6*	3·05 ± 0·64
B. choerinum ATCC 27686	2	8·17 ± 0·40	8·9 ± 0·13	102 ± 45	2·55 ± 0·07
B. longum biotype *longum* NCIMB 8809	2	8·11 ± 1·06	8·12 ± 1·07	20 ± 1*	4·18 ± 0·46
B. magnum NCIMB 20222^T	2	7·95 ± 0·15	8·43 ± 0·13	68 ± 11**	3·65 ± 0·07
B. pseudolongum ssp. *globosum* JCM 5820^T	2	8·13 ± 0·07	8·80 ± 0·03	94 ± 9**	3·35 ± 0·07
B. pyschraerophilum LMG 21775^T	4	9·36 ± 0·28	9·34 ± 0·28	19 ± 5*	3·18 ± 0·83
B. pyschraerophilum LMG 21775^T	2†	9 ± 0·20	9·14 ± 0·06	30 ± 17*	3·34 ± 0·51
B. pyschraerophilum LMG 21775^T	2‡	9·5 ± 0·50	9·44 ± 0·23	16 ± 9*	4·05 ± 0·21
B. thermacidophilum LMG 21395^T	2	9·07 ± 0·15	8·77 ± 0·20	12 ± 9*	3·18 ± 0·45
B. thermophilum NCIMB 702554	3	9·3 ± 0·21	9·38 ± 0·36	26 ± 9*	3·78 ± 0·25
B. thermophilum NCIMB 702554	2§	8·51 ± 0·09	8·54 ± 0·01	22 ± 4*	3·38 ± 0·10
A. aeriphila LMG 21773^T	2	8·36 ± 0·83	8·02 ± 0·61	9·5 ± 5*	3·83 ± 0·74

Feed, RSM (20%, w/v), outlet and inlet temperature 170°C and 85–90°C respectively.
†Feed RSM (10%, w/v) and gum acacia (10%, w/v).
‡Cells cultured under aerobic conditions prior to spray drying.
§Fluidized-bed spray dryer, outlet and inlet temperature 175°C and 68°C respectively.
*Significant and **nonsignificant difference ($P \leq 0·10$) compared with the highest mean survival rate (*B. choerinum*) respectively.

Table 3 Viability of bifidobacteria in spray-dried powders stored at selected temperatures for 30 and 90 days

	Mean log CFU g^{-1} ± SD						
		30 days			90 days		
Species	0 days	4°C	15°C	25°C	4°C	15°C	25°C
B. animalis ssp. *animalis* DSMZ 20104^T	9·27 ± 0·18	9·29 ± 0·19**	9·18 ± 0·11**	8·78 ± 0·23*	9·11 ± 0·33**	7·31 ± 0·59*	5·54 ± 0·04*
B. animalis ssp. *lactis* BB12	9·24 ± 0·23	9·28 ± 0·28**	8·67 ± 0·52*	7·98 ± 0·43*	9·16 ± 0·26**	5·84 ± 1·35*	4·50 ± 1·14*
B. animalis ssp. *lactis* BB12	9·24 ± 0·11†	9·26 ± 0·11**	8·93 ± 0·68**	8·17 ± 0·21*	8·72 ± 0·29*	5·99 ± 0·71*	4·91 ± 0·59*
B. bifidum NCMB 795	8·54 ± 0·42	7·78 ± 0·45*	5·91 ± 1·04*	5·41 ± 1·29*	6·64 ± 1·00*	<2*	<2*
B. breve NCMB 8807	9·03 ± 0·17	8·00 ± 0·39*	6·51 ± 1·06*	5·93 ± 0·9*	6·72 ± 0·74*	4·49 ± 1·22*	4·10 ± 1·16*
B. breve NCMB 8807	8·53 ± 0·11†	8·10 ± 0·02*	7·2 ± 0·54*	6·53 ± 0·09*	7·12 ± 0·60*	5·52 ± 0·56*	4·08 ± 1·32*
B. choerinum ATCC 27686	8·90 ± 0·13	8·70 ± 0·06**	6·48 ± 0·78*	5·97 ± 0·86*	7·35 ± 0·86*	4·71 ± 0·17*	4·30 ± 0·27*
B. longum biotype *longum* NCIMB 8809	8·12 ± 1·07	6·63 ± 1·91**	5·13 ± 2·26**	4·26 ± 1·66**	6·20 ± 1·43**	<2*	<2*
B. magnum NCIMB 20222^T	8·43 ± 0·13	8·16 ± 0·13**	7·35 ± 0·11*	6·32 ± 0·16*	6·27 ± 0·18*	3·88 ± 0·17*	<2*
B. pseudolongum ssp. *globosum* JCM 5820^T	8·80 ± 0·03	9·15 ± 0·12**	9·00 ± 0·09**	8·11 ± 0·14*	8·43 ± 1·03**	7·18 ± 0·78*	<2*
B. pyschraerophilum LMG 21775^T	9·34 ± 0·28	8·79 ± 0·06*	7·73 ± 0·27*	6·49 ± 0·07*	8·09 ± 0·61*	5·42 ± 0·19*	4·91 ± 0·15*
B. pyschraerophilum LMG 21775^T	9·14 ± 0·06†	8·80 ± 0·07*	7·45 ± 0·67*	5·77 ± 0·35*	7·86 ± 0·33*	4·49 ± 0·15*	3·41 ± 0·89*
B. pyschraerophilum LMG 21775^T	9·44 ± 0·23‡	8·81 ± 0·26*	8·27 ± 0·23*	6·72 ± 0·25*	8·38 ± 0·10*	6·21 ± 0·57*	5·03 ± 0·40*
B. thermacidophilum LMG21395^T	8·77 ± 0·20	8·06 ± 0·07*	7·45 ± 0·01*	6·13 ± 0·05*	8·31 ± 0·19**	5·41 ± 0·24*	4·72 ± 0·13*
B. thermophilum NCIMB 702554	9·38 ± 0·36	8·31 ± 0·46*	6·78 ± 0·39*	6·17 ± 0·08*	7·25 ± 0·46*	2·68 ± 2·36*	2·96 ± 2·57*
B. thermophilum NCIMB 702554	8·54 ± 0·01§	8·36 ± 0·51**	8·22 ± 0·30**	8·00 ± 0·42**	8·42 ± 0·47**	7·54 ± 0·28*	7·34 ± 0·56*
A. aeriphila LMG 21773^T	8·02 ± 0·61	7·59 ± 0·68**	6·64 ± 0·52**	4·74 ± 0·62*	6·33 ± 0·36**	<2*	<2*

†Feed RSM (10%, w/v) and gum acacia (10%, w/v).
‡Cells cultured under aerobic conditions prior to spray drying.
§Fluidized-bed spray dryer, outlet and inlet temperature 175°C and 68°C respectively.
<2, no colonies observed at the lowest dilution of 10^2.
*Significant and **nonsignificant difference ($P \leq 0·05$) in CFU g^{-1} compared with the initial count respectively.

Interestingly, all heat-tolerant species were also moderately oxygen tolerant. Although differences in survival and growth levels were evident, given their genetic relatedness, it is possible that they share some common elements in their tolerance mechanisms. Interestingly, Matsumoto *et al.* (2004) recently reported a marked tolerance to acid and bile with *B. animalis* ssp. *animalis* and *B. animalis* ssp. *lactis*. It is tempting to speculate that a general adaptive response mechanism operates within the heat and oxygen-tolerant species. Indeed, Park *et al.* (2002) identified the *rpoD* gene in *B. animalis* ssp. *lactis*, the main sigma A factor in *Bacillus subtilis* responsible for transcription from a majority of the promoters including those related to heat shock proteases (Abee and Wouters 1999).

Reported survival values following spray drying for *B. longum* biotype *longum* (Lian *et al.* 2002) and *B. animalis* ssp. *lactis* (Fávaro-Trindade and Grosso 2002) concurred well with those presented in the current study. A correlation between good survival and intrinsic tolerance to heat and oxygen was observed with the exception of *B. thermacidophilum*. In addition, previous survival values for *B. longum* biotype *infantis* (Lian *et al.* 2002) and *B. ruminantium* (O'Riordan *et al.* 2001) also correlated with their low heat and oxygen tolerance rating. Oxygen tolerance alone did not correlate with good survival but as none of the heat-tolerant species had a low oxygen tolerance rating it was not possible to assign good survival to heat tolerance alone.

No correlation between heat tolerance and spray drying survival was seen for *B. thermacidophilum* and Corcoran *et al.* (2004) noted a similar finding for several *Lactobacillus* species. It was suggested that survival was limited by sensitivity to other nonthermal stresses. Alternatively, heat tolerance observed with liquid heating may not be induced at the stationary phase but only in response to heating (Prasad *et al.* 2003) and may have a limited effect when cells are rapidly dried during heat exposure.

Although a clear difference in spray drying survival was observed between species all produced powders above the recommended 10^6–10^7 CFU g^{-1} at the required moisture content of ≤4%. However, only some of the species with an intrinsic tolerance to heat and oxygen maintained viability after prolonged storage at 4°C and only these were considered to have commercial potential.

Declining cell viability during storage, especially at ambient temperatures (Abd-El-Gawad *et al.* 1989; Teixeira *et al.* 1995; Gardiner *et al.* 2000; Silva *et al.* 2002; Corcoran *et al.* 2004), is a major limitation in the marketing of spray-dried powders. Moisture and oxygen are known to influence viability and aluminium-based packaging material can act as a barrier to both (Nagawa *et al.* 1988; Ishibashi and Shimamura 1993) and may in part account for the high viability seen at low temperatures for some species in the current study.

In a previous study the viability of *L. paracasei* was improved when exponential phase cells were spray dried in RSM containing gum acacia although a significant decline was observed after storage for 8 weeks (Desmond *et al.* 2002). In the current study using stationary phase cultures an improvement was noted for *B. breve* but not for two other species. However, for *B. breve* a significant decline in viability still occurred in the presence of gum acacia after 1 month in cold storage.

Improved viability can be achieved by reducing the spray drying outlet temperature (Fu and Etzel 1995; To and Etzel 1997b) and recently Prasad *et al.* (2003) observed a marked improvement when *Lactobacillus rhamnosus* stationary phase cultures were heat shocked prior to fluid-bed drying. Gardiner *et al.* (2002) greatly improved the viability of *L. paracasei* by using a fluidized-bed spray dryer. It was suggested that the improvement reflected the lower outlet temperature needed to produce powders with an equivalent moisture content (Písecký 1997; Masters 2002). In the current study, using the same spray dryer the viability of *B. thermophilum* was also greatly improved following storage, especially at elevated temperatures. However, it appears that good viability need not correlate with initial survival. Gardiner *et al.* (2000) and Corcoran *et al.* (2004) both reported similar relative declines in the viability for *Lactobacillus* strains with widely different initial survival values. In addition, in this and a previous study, initial survival for a fluidized-bed spray dryer was similar to that of a conventional spray dryer (Gardiner *et al.* 2002). Viability may depend on the induction of sub-lethal damage that is converted to lethal forms during storage by processes influenced by the level of activated water, oxygen and temperature.

In summary, the study identified a group of closely related *Bifidobacterium* species with a distinctive tolerance to heat and oxygen that included the commercial probiotic strain *B. animalis* ssp. *lactis* BB-12. These species had high initial survival following spray drying and maintained viability during storage at refrigerated temperatures. In addition, viability at ambient temperatures was greatly improved when powders were produced using a fluidized-bed spray dryer.

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