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Survival and metabolic activity of the GanedenBC^{30} strain of *Bacillus coagulans* in a dynamic *in vitro* model of the stomach and small intestine

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Abstract

We have investigated the survival and activity of GanedenBC^{30} during passage through the upper gastro-intestinal tract. GanedenBC^{30} was tested in a dynamic, validated, *in vitro* model of the stomach and small intestine (TIM-1) on survival and its potential to aid in digestion of milk protein, lactose and fructose. The survival of GanedenBC^{30} was high (70%), although germination of the spores was minimal (<10%) under the conditions tested. Survival of the strain in the presence of lactose and fructose was markedly lower (56-59%) than in the absence of the sugars. The amount of digested milk protein available for absorption was somewhat higher (+0.2 g) when GanedenBC^{30} was added to the milk. When GanedenBC^{30} was tested with lactose or fructose added to the meal, the cumulative amount of lactate produced was slightly higher (+0.12-0.18 mmol) compared to the GanedenBC^{30} alone. In conclusion, although the differences in survival of GanedenBC^{30} are small, these results show the potential of GanedenBC^{30} to aid in protein digestion and in the digestion of lactose and fructose. If a larger fraction of the *Bacillus coagulans* cells had germinated, the influence on protein and carbohydrate digestion would probably have been much greater. Importance of the findings: the potential of GanedenBC^{30} to aid in the digestion of lactose and fructose could be used to prevent occurrence of intestinal symptoms in individuals sensitive to these carbohydrates.

Keywords: probiotic, *Bacillus coagulans*, GanedenBC^{30}, survival, metabolic activity, fructose intolerance, lactose intolerance

1. Introduction

Probiotics are live bacteria that show a documented beneficial effect to the host (Sanders, 2008). Probiotics exhibit a variety of effects: they may be used to prevent and treat antibiotic-associated diarrhoea and acute infectious diarrhoea; they also may be effective in relieving symptoms of irritable bowel syndrome, inflammatory bowel disease, and in treating atopic dermatitis in children. Other documented effects include such overlapping mechanisms as the regulation of intestinal microbial homeostasis, the stimulation of local and systemic immune responses, the prevention of pathogens infecting the mucosa, the stabilisation or maintenance of the gastrointestinal barrier function, the inhibition of procarcinogenic enzymatic activity, and the competition for limited nutrients (Boirivant and Strober, 2007; Rolfe 2000). However, probiotic effects appear to be strain specific (De Vecchi and Drago, 2006). Regardless of the strain and its potential effect, probiotics intended for the gastro-intestinal tract must survive gastric and bile acids (De Vecchi and Drago, 2006) in order to reach that intestinal tract, colonise the host epithelium, and exhibit a beneficial effect (Hyronimus *et al*., 2000). *Bacillus coagulans* is a Gram-positive spore-forming rod, which is aerobic to microaerophilic. Due to the formation of spores, these bacilli can withstand the acidic environment of the stomach to reach the intestine where they germinate and proliferate, producing the favoured L (+) optical isomer of lactic acid (Breed *et al*., 1957). *B. coagulans* GBI-30, PTA 6086 (GanedenBC^{30}) is a strain of lactic-acid producing bacteria, with self-affirmed GRAS (generally recognised

as safe) status, that can sustain the low pH of stomach acid (De Vecchi and Drago, 2006; Hyronimus *et al*., 2000).

Once active in the small intestine after germination, *B. coagulans* may aid in digestion of proteins and sugars from the diet. This may be beneficial to the host, especially in the case of lactose and fructose intolerance: once the sugars are digested in the upper gastro-intestinal (GI) tract, they will no longer cause the symptoms associated with the intolerance in the lower part of the gut, such as bloating, diarrhoea, etc.

As mentioned above, an important criterion for the selection of probiotic bacteria to be used in (functional and medical) food products or formulations in relation to health promotion is the survival and activity of these micro-organisms in the gastro-intestinal tract of the consumer. In order to investigate the survival of probiotic bacteria in the stomach and small intestine in humans, intubation methods are available. However, these *in vivo* methods are laborious, expensive and meet ethical constraints. Instead, validated *in vitro* methods should be performed, at least for the first selection of strains. The TNO *in vitro* gastro-Intestinal Models (nick-named TIM) are computer controlled, dynamic models that simulate to a large degree the successive dynamic processes in the stomach, the small intestine (TIM-1; Minekus *et al*., 1995; Havenaar and Minekus, 1996) and in the large intestine (TIM-2; Minekus *et al*., 1999; Venema *et al*., 2000). During the experiments samples from different sites of the GI tract can be taken in time. These models are unique tools and give good insight into the stability and activity of functional ingredients, such as probiotics. The results obtained in these models were validated and showed a very good resemblance to the results obtained in studies with humans and animals with respect to the digestibility and the availability for absorption of a broad scope of nutrients (e.g. proteins/amino acids, carbohydrates, minerals, vitamins, nutrients; Minekus, 1998; Larsson *et al*., 1997; Smeets-Peeters, 2000), the release, absorption and function of bioactive food compounds (e.g. functional proteins, anti-mutagenic compounds; Krul *et al*., 2002) and the survival of lactic acid producing bacteria (Marteau *et al*., 1997).

The aim of the study was to determine the survival and activity of a particular strain of *B. coagulans* known as GanedenBC^{30}, a commercial product, in the upper GI tract. This was tested in TIM-1, the model for the stomach and small intestine. Survival was evaluated on the basis of production of L-lactic acid, a metabolite produced by *B. coagulans* (Breed *et al*., 1957). In addition, the effect of the addition of GanedenBC^{30} to milk was studied, to evaluate whether the probiotic would aid in digestion of lactose and milk protein. Similarly, the probiotic was added to a fructose solution (mimicking apple-juice) to evaluate whether the strain would aid in the digestion of fructose.

2. Materials and methods

Test products

GanedenBC^{30} was provided by Ganeden Biotech (Mayfield Heights, OH, USA). This *B. coagulans* containing product contained 2.15×10^{10} colony forming units per gram powder (cfu/g). Lactose and fructose were obtained from Sigma (Zwijndrecht, the Netherlands) and low-fat, pasteurised milk was bought fresh in the local supermarket the day before the experiment was carried out.

Test system

The study was performed in the TNO dynamic, multi-compartmental system of the stomach and small intestine (TIM-1) as described in detail by Minekus *et al*. (1999) and schematically presented in Figure 1. Briefly, the model comprises four connected compartments, representing the stomach, duodenum, jejunum and ileum. Each compartment is made of a glass outer wall with a flexible silicone inner wall. The space between the inner and outer walls is filled with water at body temperature (37 °C). By periodically pumping the water, the flexible inner walls are squeezed, which simulates peristaltic movements of the GI tract. In the four compartments the pH is measured continuously and regulated by 'secretion' of hydrochloric acid in the gastric compartment and sodium bicarbonate in the intestinal compartments. Hollow fibre membrane systems continuously dialyse the digested and dissolved low-molecular weight compounds from the jejunum and ileum compartments, which simulates absorption of nutrients in the body. The set-points of pH, gastric emptying and intestinal transit time were controlled by a computer and are displayed in Figure 2. As a result, over the course of 3 hours, approximately 95% of the gastric contents were gradually delivered into the small intestine through the 'pyloric sphincter' (at B in Figure 1). Over the course of 6 hours, approximately 90% of the small-intestinal contents were gradually delivered into the 'large intestine' (sampling bottle) through the 'ileo-caecal sphincter' (at H in Figure 1). The gastric pH decreased from 6.5 to 2.0 in 120 minutes. In the small-intestinal compartments the pH was set at 6.5 in the duodenum compartment, 6.8 in the jejunum compartment and 7.2 in the ileum compartment (Minekus *et al*., 1999).

Study design

Throughout the study, the experiments in TIM-1 were performed under the average physiological conditions of healthy human adults (Figure 2). Duplicate experiments were performed to determine the survival of GanedenBC^{30}. For this, 2×10^{9} cfu of the *B. coagulans* spores were introduced into the gastric compartment in a mixture of

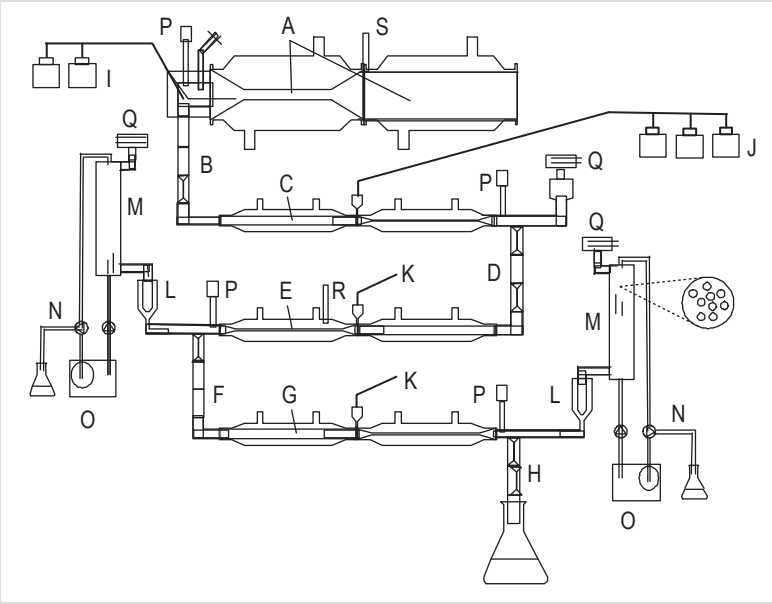

Figure 1. Schematic diagram of the dynamic, multi-compartmental model of the stomach and small intestine (TIM-1): A. stomach compartment; B. pyloric sphincter; C. duodenum compartment; D. peristaltic valve; E. jejunum compartment; F. peristaltic valve; G. ileum compartment; H. ileo-caecal sphincter; I. stomach secretion; J. duodenum secretion; K. jejunum/ileum secretion; L. pre-filter; M. semi-permeable membrane; N. water absorption; O. collected dialysate; P. pH electrodes; Q. level sensors; R. temperature sensor; S. pressure sensor.

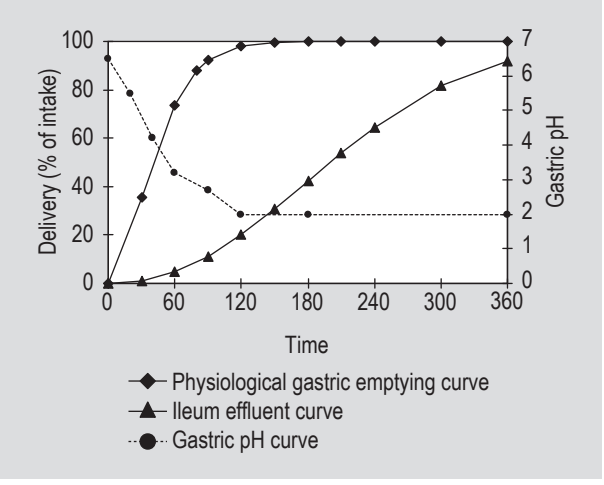

Figure 2. Physiological gastric emptying curve, ileum effluent curve and gastric pH curve for human adults ingesting milk.

200 ml of sterile demi-water and 100 ml artificial saliva (Minekus *et al*., 1999). In addition, duplicate experiments were performed by adding the same amount of spores to 200 ml of a lactose solution (5% w/v in water), a fructose solution (5% w/v in water), or milk. The outcome of these latter experiments revealed information on the effect of GanedenBC^{30} on the digestion of lactose, fructose and milk protein, respectively. It should be noted that in these experiments, there was no full complement of substrates that bacteria need for their growth, such as carbon and nitrogen sources, due to reasons of interference with the analysis on survival and more importantly lactose, fructose and milk protein utilisation. For each experiment the secretion products (i.e. saliva and gastric juice with enzymes and electrolytes, dialysis liquids, bile, pancreatin) were freshly prepared, the pH electrodes calibrated and the dialysis membranes (hollow fibre units) replaced (Minekus *et al*., 1999).

Sampling

The 'ileo-caecal sphincter' delivered the intestinal contents according to a computer-controlled ileal effluent curve (Figure 2). Over the course of 6 hours the ileal effluent was collected on ice in three 2 hourly aliquots by changing the sampling bottle (at H in Figure 1) at 2, 4 and 6 hours, except for evaluation of survival of the strain, where hourly fractions were taken. For each sample the volume was measured and samples were stored at -18 °C prior to analysis. At the same time intervals the dialysed and absorbed liquids of the jejunum and ileum compartments (at O in Figure 1) were collected. The total volumes were measured and individual samples were taken in time and stored as above.

Analyses

Total Kjeldahl nitrogen (Isaac and Johnson, 1976) was determined in the samples collected in the milk experiments. Lactate was measured as a parameter for fermentation of lactose and fructose. Lactate was measured using a Cobas Mira plus autoanalyser (Roche, Almere, the Netherlands) as previously described (Venema *et al*., 2005).

Samples collected at the ileal-caecal sphincter (at H in Figure 1) were evaluated for survival. *B. coagulans* in the samples was cultured on Glucose Yeast Extract Agar Medium (Tritium Microbiology, Veldhoven, the Netherlands) at 37 °C for 48 hours, under aerobic conditions. For this, samples were serially diluted 10-fold in sterile physiological saline solution (9 g/l; pH 7.0). The dilutions were plated immediately for viable *B. coagulans* (germinated cells and spores) and after heating (spores only). During the heating step, the dilutions were heated for 30 minutes in a 70 °C water bath, and then cooled immediately to approximately 45 °C before pipetting. Survival was determined in duplicate experiments under three conditions: GanedenBC^{30} only; GanedenBC^{30} + lactose; GanedenBC^{30} + fructose. Survival was expressed as a percentage of intake.

3. Results and discussion

Survival

GanedenBC^{30} spores are protected by a hardened coating primarily consisting of proteins, called the integument, that can withstand gastric acid and bile salts for delivery to the small and large intestines (Ara *et al*., 2002). Figure 3 shows the cumulative survival of GanedenBC^{30} after passage through TIM-1 for the spores. After 6 hours, the mean cumulative survival of the GanedenBC^{30} (spores only) as a percentage of the intake without a sugar added was 70.4%±9.4%. With lactose added to the meal it was 56.0%±2.5% and with fructose added it was 59.7±12.05%. The theoretical survival (assuming no changes occurred during passage, i.e. no death and no growth) after 6 hours was 87.1% (Figure 3). This indicated that a large number of spores were still present at the terminal ileum (approximately 81% of the theoretical amount). Germinated cells (data not shown) could not be counted in the 4-6 hour sample due to overgrowth of the endogenous microbiota in the system. However, after 4 hours 13.9% more viable cells were present in the samples that were not heated (65.6% versus 51.7% respectively). Assuming that after this time-point no more viable germinated cells reached the colon (sampling bottle), a minimum of approximately 85% (70.4% in terms of spores + 13.9% as germinated cells) of the cells introduced in the gastric compartment survived passage through the stomach and small intestine.

Survival of the strain in the presence of lactose and fructose was markedly lower than in the absence of the sugars. This may mean that more spores germinated in the presence of these sugars, and since germinated cells are more sensitive to the GI conditions, survival was lower.

Kjeldahl nitrogen

The amount of milk protein available for absorption during the experiment with milk only was 3.5 g. The addition of GanedenBC^{30} to the milk gave a somewhat higher result of 3.7 g (Figure 4). Although the difference is small, this result shows the potential of GanedenBC^{30} to aid in protein digestion. This is even more likely given the low germination of the spores (as discussed above). If more spores had germinated, the influence on protein digestion would probably have been greater.

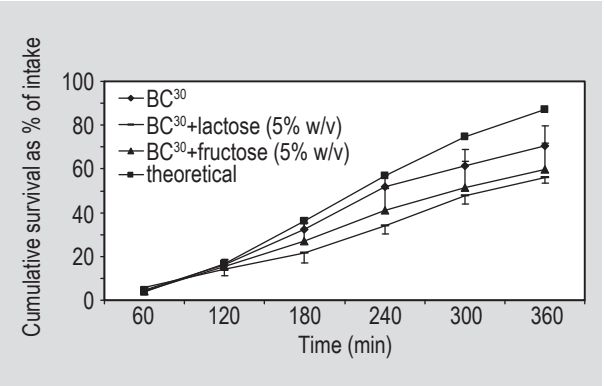

Figure 3. Cumulative survival of *Bacillus coagulans* spores (BC^{30}) under the three different conditions, as a percentage of the intake, after passage through TIM-1. The theoretical survival is the survival with the assumption that there would not be any growth or death of bacteria during passage through TIM-1.

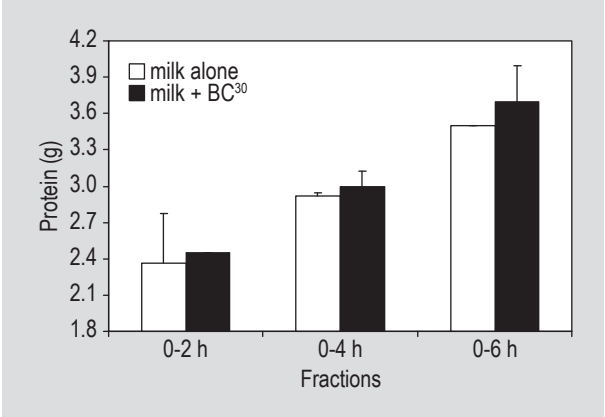

Figure 4. Cumulative amount of the bioaccessible fraction of protein in TIM-1 with and without the addition of GanedenBC^{30}.

Lactose and fructose digestion

Lactose and fructose digestion were measured by analysing the production of lactate, produced after fermentation of the sugars by *B. coagulans*. Lactate is only produced by vegetative cells, and thus is a measure of germination. In addition, the amount of lactate produced in comparison to an experiment without the addition of sugars was an indication of the digestion of the sugars, and therefore indicated that GanedenBC^{30} aided in digestion of the sugars. Figure 5 shows the cumulative amount of lactate produced under the various conditions. Lactate was measured in the dialysis liquid collected from the jejunum and ileum compartments (at O in Figure 1). Normally there is no lactate present in the dialysates of TIM-1. In the TIM-1 trial with GanedenBC^{30} alone there is already lactate present. This indicated that the germinated *B. coagulans* cells already produced lactic acid from endogenous materials such as bile and/or pancreatic juice or from the maltodextrin that was used as an excipient in the powder. In the TIM-1 trials with lactose or fructose added to the meal, the cumulative amount of lactate produced was slightly higher compared to the GanedenBC^{30} alone (0.12 and 0.18 mmol more for lactose and fructose, respectively). Although only minimally higher, the slightly higher lactate production in TIM-1 with lactose and fructose added implied that GanedenBC^{30} has the potential to aid in the digestion of these sugars and to prevent the occurrence of symptoms in individuals sensitive to these carbohydrates. As discussed above, most of the cells were recovered in the form of spores. If a larger fraction of the *B. coagulans* cells had germinated, the effect on lactose and fructose digestion would probably have been much greater. It should be realised that *in vivo* the interaction between *B. coagulans* and the intestinal microbiota may be much more complex than in the *in vitro* model, including various competitions, mutual inhibition, synergies *et cetera*. The model has a certain level of approximation to the *in vivo* conditions. However, the biggest advantage of TIM-1 is that individual parameters can be studied separately to determine their influence on the survival and/or activity of *B. coagulans*. For instance, in its real use, GanedenBC^{30} would be consumed as part of a standard diet. Due to reasons of interference with the analysis on survival and more importantly lactose, fructose and milk protein utilisation, no meal was added to TIM-1 besides the test product. This meant that the substrates available in the model (endogenous secretion fluids) were not sufficient for optimal bacterial growth. If the bacteria were added in the matrix of a complete meal, greater vegetative growth and metabolism of the strain would be expected. In that case, the assumed increase in germination would give an increased utilisation of lactose, fructose and protein. Furthermore, germination triggers such as L-alanine or inosine could be added to increase germination of the cells in GanedenBC^{30} (Hornstra *et al*., 2005), which will be investigated in future experiments.

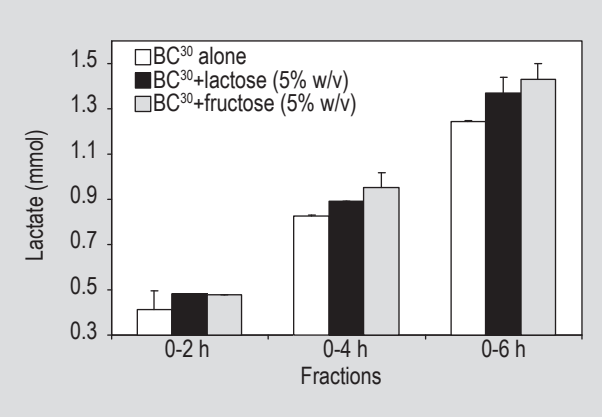

Figure 5. Cumulative amount of lactate produced in TIM-1 under the three different conditions.

The TIM-1 system simulates to a large degree the successive dynamic physiological conditions in the stomach and the small intestine. The model offers the possibility to simulate very closely the pH curves and the concentrations of enzymes in the stomach and small intestine, the concentrations of bile salts in the different parts of the gut, and the kinetics of passage of food through the stomach and intestine. In this model survival studies were performed with *Bifidobacterium* spp., *Lactobacillus* spp., *Streptococcus thermophilus*, *Lactococcus* strains and several pathogenic and food-borne bacteria under different gastro-intestinal conditions. The *in vitro* results showed good resemblance with the results obtained in studies with human volunteers (Marteau *et al*., 1997). This means that the results can be used to predict the survival of probiotic bacteria in the human intestinal tract. Therefore, the results obtained in the current study point into the direction of a good survival of GanedenBC^{30} in the GI tract of the human adult, as well as the potential to aid in digestion of protein carbohydrates. Specifically for lactose and fructose, this would be beneficial for those individuals that experience symptoms when ingesting these sugars due to a genetic or acquired intolerance. The benefit of ingestion of GanedenBC^{30} would be significant. Especially if the cells could be triggered into germination at the beginning of the small intestine, e.g. by ingesting them together with a diet containing L-alanine and/or inosine, or by including these in the powder formulation. In addition, in patients with pancreatic exocrine deficiency disorders such as chronic pancreatitis and cystic fibrosis, in which only small amounts of the normal amount of pancreatic enzymes are excreted (Ferrone *et al*., 2007), GanedenBC^{30} could contribute to the digestion of the protein and carbohydrates in their meal.

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