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# *Bacillus coagulans* GBI-30, 6086 increases plant protein digestion in a dynamic, computer-controlled *in vitro* model of the small intestine (TIM-1)

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**RESEARCH ARTICLE**



## Abstract

The aim of this study was to assess the potential of the probiotic *Bacillus coagulans* GBI-30, 6086 [GanedenBC$^{30}$] (BC30) to aid in protein digestion of alimentary plant proteins. To test this, three plant proteins, from pea, soy and rice, were digested in a validated *in vitro* model of the stomach and small intestine (TIM-1) in the absence and in the presence of BC30. Samples were taken from the TIM-1 fractions that mimic uptake of amino acids by the host and analysed for α-amino nitrogen (AAN) and total nitrogen (TN). Both were increased by BC30 for all three plant proteins sources. The ratio of TN/AAN indicated that for pea protein digestion was increased by BC30, but the degree of polymerisation of the liberated small peptides and free amino acids was not changed. For soy and rice, however, BC30 showed a 2-fold reduction in the TN/AAN ratio, indicating that the liberated digestion products formed during digestion in the presence of BC30 were shorter peptides and more free amino acids, than those liberated in the absence of BC30. As BC30 increased protein digestion and uptake in the upper gastrointestinal (GI) tract, it consequently also reduced the amount of protein that would be delivered to the colon, which could there be fermented into toxic metabolites by the gut microbiota. Thus, the enhanced protein digestion by BC30 showed a dual benefit: enhanced amino acid bioavailability from plant proteins in the upper GI tract, and a healthier environment in the colon.

**Keywords:** protein digestion, probiotic, *Bacillus coagulans*, BC30, *in vitro* gastrointestinal model

## 1. Introduction

Ingestion of plant proteins is increasing enormously nowadays. Sustainable meat-production in an overcrowded world is not feasible, and hence research focuses on alternative protein sources. Moreover, vegan products have gained immensely in popularity. However, plant proteins have been known to have lower digestibility than animal-based protein-sources.

The healthy pancreas secretes more than 20 zymogens in the duodenum of the gastrointestinal (GI) tract, which are, after activation into active digestive enzymes, responsible for the digestion of luminal proteins, lipids and carbohydrates. Despite the fact that most of the time a surplus of enzymes is excreted compared to the amounts of substrate present in the GI tract, not all protein, carbohydrate and lipids are completely digested in the upper GI tract and some of these macromolecules make it to the colon, where they may be fermented by the gut microbiota. Especially for proteins, there is a broad range of reported digestibilities, with plant protein generally being digested to a lower degree. Clinical signs of nutrient malabsorption, however, only occur when 75% or more of the pancreatic function is lost (Anonymous, 2004; Lankisch *et al.*, 1986). To increase e.g. protein digestion, usually exogenous enzymes are used, which can be extracted from porcine sources or from plants (Minevich *et al.*, 2015). These are added to the diet of subjects to boost protein digestion (Anonymous, 2004). An alternative strategy would be the use of enzymes from bacterial and/or fungal origin instead of enzymes from animal sources (Andriamihaja *et al.*, 2013; Layer *et al.*,

2001; Sikkens *et al.*, 2010). In the current study, we focused on the effects of addition of a probiotic culture (with its endogenous enzyme content) for its capacity to degrade alimentary plant proteins in conjunction with the pancreatic enzymes. Because the time-course of protein hydrolysis is known to be partly related to the specificity of enzymes for target amino acids, three different plant proteins were investigated: from pea, soy, and rice.

Probiotics are live microorganisms, which, when administered in adequate amounts, confer a health benefit on the host (FAO/WHO, 2001; Hill *et al.*, 2014). *Bacillus coagulans* GBI-30, 6086 (BC30) 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. The strain has been shown to be a probiotic in subjects with irritable bowel syndrome (IBS) (Dolin, 2009; Hun, 2009; Kalman *et al.*, 2009) and by showing immunomodulation in a number of clinical trials (Baron, 2009; Kimmel *et al.*, 2010; Mandel *et al.*, 2010). Recently, it was shown to reduce exercise-induced muscle damage and to increase recovery in recreationally-trained males undergoing strenuous exercise when co-administered with dietary protein (Jager *et al.*, 2016). As efficacy of probiotics is strain specific, BC30 was chosen to study, as it is the only *B. coagulans* strain that has been shown to have an effect on protein utilisation (Jager *et al.*, 2016).

The use of added enzymes or probiotic cultures to increase nutrient absorption has primarily been restricted to animal farming. For instance, in a study in 1 day old broiler chickens inclusion of pea meal reduced weight gain, feed conversion and nutrient digestibility. Enzyme addition partially ameliorated the detrimental effects of pea meal inclusion, although in this case the enzymes were carbohydrases and not proteinases (Cowieson *et al.*, 2003). As far as the authors are aware there have been no studies that focused on (increased) protein bioavailability in humans.

Validated *in vitro* models, that closely mimic the dynamic changing conditions in the gastrointestinal (GI) tract, are excellent tools to study the digestive processes in the mostly inaccessible GI tract. The computer-controlled, dynamic *in vitro* gastrointestinal model of the stomach and small intestine (TIM-1) developed by TNO (the Dutch Institute for Applied Scientific Research) is such a validated model, which simulates to a high degree the successive dynamic processes in the upper GI tract (Minekus, 1995, 1998). During the experiments samples from different sites of the GI tract can be taken in time. This gives good insight on the (rate of) digestibility and kinetics of bioaccessibility of nutrients and/or the stability and activity of functional ingredients. The model is a unique tool to study the stability, release, dissolution, absorption and bioconversion of nutrients, chemicals, bioactive compounds and pharmaceuticals in the gastrointestinal tract, under reproducible conditions. Specific protocols have been developed and tested to simulate the GI conditions of babies (Havenaar *et al.*, 2013), adults and elderly (all with their own physiological parameters), as well as dogs (Smeets-Peeters *et al.*, 1999), pigs and calves (Minekus, 1998; Venema *et al.*, 2009). This model was used previously to study germination of spores of *Bacillus* in the upper GI tract (Hatanaka *et al.*, 2012; Maathuis *et al.*, 2010).

The aim of the current research was to study whether *B. coagulans* GBI-30, 6086 would aid in protein digestion of three alimentary plant proteins, pea, soy or rice, in a validated model mimicking the human upper GI tract.

## 2. Materials and methods

### Plant proteins

The three plant proteins used in the study were Soy Protein Isolate NoGel Lovi (Gushen Biological Technology Group, Co., Ltd, Shandong, China P.R.) with ~90% protein, 5% ash and 5% moisture; Organic VegOtein P80 (Axiom Foods, Los Angeles, CA, USA), a yellow pea protein concentrate with 82% protein, 5.2% ash and 6.8% moisture, the remainder being composed of fat and fibre; and Organic Oryzatein Ultra 80 (Axiom Foods), a rice protein concentrate with 82.3% protein, 4% ash, 1.2% fat, 8.8% fibre and 3.7% moisture. All proteins were free flowing powders soluble in water.

### *In vitro* model of the stomach and small intestine

The TNO *in vitro* model of the stomach and small intestine (Minekus *et al.*, 1995), TIM-1 (Supplementary Figure S1), was used to study digestion of pea, soy or rice protein in the absence and presence of the probiotic BC30. The model was set-up and run for protein digestion as described before (Minekus, 1998), according to the validated protocol for survival of lactic acid bacteria and probiotics (Marteau *et al.*, 1997). In brief, experiments were performed in duplicate under the average physiological conditions as found in the human gastrointestinal tract for adults. The gastric emptying, intestinal residence time and gastric and intestinal pH-curves mimicked the situation as found in humans for semi-solid foods (Minekus *et al.*, 1995). The concentrations of electrolytes, enzymes, bile, and pancreatic juice were adjusted to the average concentrations as described for healthy humans. Pancreatic output was simulated by secreting 10% pancreatin (Pancrex V, Paines and Birne, Greenford, UK) in small intestinal electrolyte solution (containing NaCl 5 g/l, KCl 0.6 g/l, $CaCl_2$ 0.22 g/l) at 0.25 ml/min. Biliary output was simulated by secreting a 4% bile (porcine bile extract, Sigma) solution at 0.5 ml/min. Prior to the experiment the compartments were filled with start residues as described before (Minekus *et al.*,

1995), except for the gastric residue, which was mixed with the 'meal'. Hollow fibre membrane systems continuously dialysed the digested and dissolved low-molecular weight compounds from the jejunum and ileum compartments (Supplementary Figure S1 part M), which simulates absorption of nutrients in the body.

The test meals were prepared by mixing 15 g of test-products with 45 ml of saliva/gastric electrolyte solution containing $CaCl_2$ 0.22 g/l, KCl 2.2 g/l, NaCl 5 g/l and $NaHCO_3$ 1.5 g/l and adjusted to 300 ml with water. The gastric residue was added and the mixture was adjusted to a pH of 5.2 with 1M HCl, the starting pH of the run. The 'meal' was quantitatively introduced in the gastric compartment just prior to the start of the run. For a period of 6 h, samples were collected from the jejunal and ileal dialysate (Supplementary Figure S1 part M, mimicking absorption of nutrients), and the ileal efflux (Supplementary Figure S1 part H, mimicking the undigested fraction). Samples were also collected from the secretion fluids (pancreatic juice and bile; Supplementary Figure S1 part J). In addition, after termination of the run, the residues in the model were collected to be able to create a complete mass-balance. The total volumes of the samples were measured and recorded, to allow the calculation of absolute amounts of absorbed digestion products.

### Addition of *Bacillus coagulans* BC30

In those experiments where the probiotic culture was added, vegetative cells of BC30 were introduced in the jejunum compartment (Supplementary Figure S1 part E). Assuming regular intake of BC30 by the host, the amount of cells in the small intestine of a human volunteer is expected to remain in steady state (some cells will leave the small intestine, but those will be renewed by subsequent ingestion). Therefore, the concentration of vegetative cells of BC30 added to the jejunum compartment was approximately $1\times10^9$ cfu each time. It was hypothesised that addition of BC30 will increase the α-amino nitrogen (AAN) values (see next paragraph) compared to the experiments without BC30, indicating increased digestion.

### Analyses for α-amino nitrogen and total nitrogen

AAN was used as a proxy for free amino acids and short peptides. In the assay, the terminal amino group is measured and this indicates the extent of digestion that has occurred (Figure 1). AAN was determined with 2,4,6-trinitrobenzenesulfonic acid (TNBS) at 40 °C. Briefly, 260 µl 0.1 M $Na_2HPO_4$ buffer (pH = 8.0)+0.6 g/l sodium dodecyl sulphate + 20 mg/l $Na_2SO_3$ and 10 µl sample were mixed. Subsequently 6 µl of 0.25 M TNBS (dissolved in 25% methanol) + 50 µl water was added. After 7.5 min the absorbance was measured at 405 nm. A calibration curve was prepared with glycine as a reference. This procedure

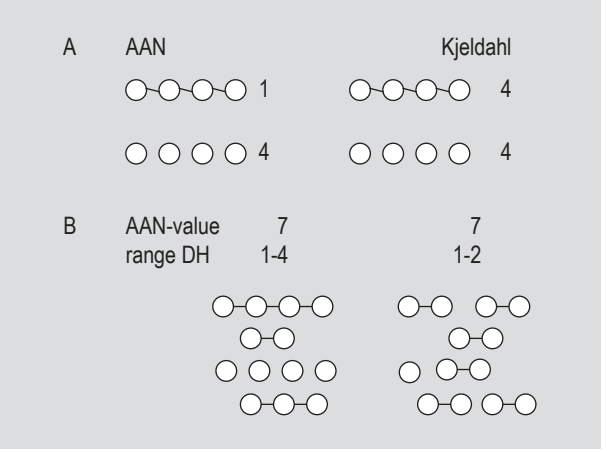


**Figure 1. α-amino nitrogen (AAN) as a proxy for the degree of digestion. (A) AAN for a polypeptide of 4 amino acids (a.a.) is 1. AAN for 4 free a.a. is 4. Kjehldahl (total nitrogen) is 4 for both. (B) AAN is not suitable for determining the distribution of oligopeptides: both mixture (left and right) have an AAN of 7, but a different distribution (DH) of free a.a. and oligo-peptides: left from 1 to 4 a.a.; right from 1 to 2 a.a.**

was fully automated using a Cobas Mira Plus autoanalyser (Roche Diagnostics, Almere, the Netherlands), and was performed by Bio-aNAlytiX (Zoetermeer, the Netherlands).

Moreover, total nitrogen (TN) was measured with a modified, miniaturised Biuret assay. Briefly, to 500 µl sample 100 µl 6 M NaOH was added. Samples were then boiled for 5 min at 100 °C. After cooling down to room temperature 450 µl of the boiled alkalinised sample was mixed with 150 µl 25 g/l $CuSO_4{\cdot}5H_2O$ and incubated for 5 min at room temperature. Samples were centrifuged at 12,000×*g* and the absorbance of the clear supernatant was measured at 500 nm. Bovine serum albumin (BSA) was used to prepare a standard calibration curve. This procedure was automated using a Cobas Mira Plus autoanalyser, and was performed by Bio-aNAlytiX. The ratio of TN/AAN reflects the degree of digestion of the proteins in the samples, where a lower ratio indicates a higher degree of digestion (due to a higher AAN). Data was expressed as percentage of protein intake where appropriate. Protein intake was the sum of protein in the 'meal' plus that in the digestive juices (pancreatic juice and bile). Data are corrected for total TN recovery, which ranged from 94 to 99.7% (not shown).

## 3. Results

### Digestion of total nitrogen in the absence and presence of BC30

Figure 2 shows the cumulative amount of total nitrogen (TN) for the three protein sources that was 'absorbed by the body' (in other words, present in the jejunum and ileum dialysate fractions), normalised (at 100%) to

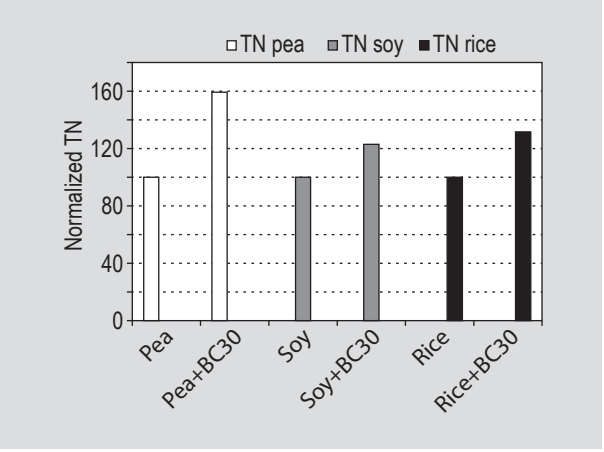


**Figure 2. Average normalised (%) total nitrogen (TN) in the jejunum + ileum dialysate fractions (normalised to 100% for the experiments without *Bacillus coagulans* (BC30)), representing the fraction that is taken up by the body.**

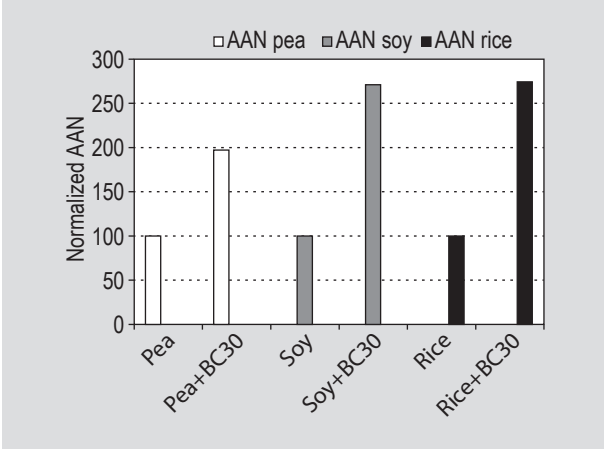


**Figure 3. Average normalised (%) α-amino nitrogen (AAN) in the jejunum + ileum dialysate fractions (normalised to 100% for the experiments without *Bacillus coagulans* (BC30)), representing the fraction that is taken up by the body.**

experiments without BC30. The absolute numbers are shown in Supplementary Table S1. The figure shows that in the presence of BC30 more total nitrogen is present in the dialysate fractions. This cannot be explained by the small addition of extra nitrogen due to the addition of the BC30 culture (which was on average only 0.025±0.005% of the protein intake), but is the beneficial effect of BC30 on protein digestion. The increase is biggest for pea protein (approx. +60%), then for rice (approx. +30%) and smallest for soy (but still approx. +20%).

These results indicate that BC30 increases the total amount of nitrogen that is absorbed by the host, indicating it aids in digestion of these proteins. If digestion in the absence of BC30 for these three proteins is evaluated, then digestion for the individual proteins is highest for soy (87.8% of the total nitrogen of the protein intake is present in the summed dialysate fractions), then pea (84.6%) and finally rice protein (79.4%). In the presence of BC30, digestion increase to 99.5, 98.5 and 92.6% for these three proteins, respectively.

### Digestion of α-amino nitrogen in the absence and presence of BC30

Similar to TN, BC30 also increased the cumulative amount of AAN for the three proteins (Figure 3, with absolute numbers in Supplementary Table S1). When expressed as normalised to the experiment without BC30, pea shows a 100% increase, whereas the other two proteins show a 170-175% increase.

### Degree of hydrolysis: ratio TN/AAN

As indicated in Figure 1, the ratio of TN and AAN is an indication of the 'degree of hydrolysis' of the peptides (and free amino acids) in the dialysate fractions. Figure 4 shows this ratio of TN/AAN. Although both TN and AAN increase in the presence of BC30 (see Figure 2 and 3, respectively), the increase of AAN is larger, which results in a lower TN/AAN ratio for all three protein sources, indicating smaller peptides (and/or more free amino acids). This reduction in the TN/AAN ratio is small for the pea protein, but approximately half for soy and rice protein (Figure 4). Despite that the increase in absorption of TN was largest for pea protein (Figure 2), a more or less similar TN/AAN ratio means that these peptides were not digested into smaller pieces to any great extent. However, this is the case for soy and rice protein. The increase in absolute TN (Figure 2) and the lower TN/AAN ratio (Figure 4) for these two proteins indicates that, not only is there more total nitrogen, on average these peptides are also smaller in the presence of BC30.

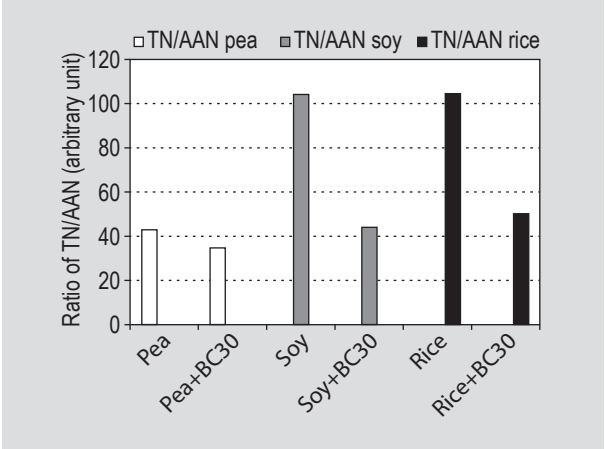


**Figure 4. Average ratio of total nitrogen (TN) over α-amino nitrogen (AAN) in the dialysate samples in the absence and presence of *Bacillus coagulans* (BC30).**

## 4. Discussion

As indicated before, the *in vitro* model used has been validated for a number of applications. These include amongst others carbohydrate digestion, lipid digestion, survival of probiotics, vitamin uptake. These validation experiments showed very high correlations between the *in vitro* and *in vivo* experiments. Also for protein digestion the model has been validated (Minekus, 1998). Correlation analysis showed the following correlation between *in vitro* (y) and *in vivo* (x) data: $y = 0.9882x$ ($R^2 = 0.9728$) for 8 proteins ranging in digestibility from 58.0 to 98.9% (unpublished data).

The genome of BC30 has been determined (Orru *et al.*, 2014). The BC30 genome contains a number of genes encoding peptidases, which could aid in digestion of the three plant proteins. For this, the peptidases, which are predicted to be intracellular (data not shown), need access to the plant proteins or partly digested proteins in the extracellular milieu. We believe this is possible because some of the cells will lyse in the lumen of the gut (model), due to the presence of e.g. bile, releasing the intracellular enzymes (unpublished data).

The increase in protein digestibility due to the addition of BC30 is clear for all three plant protein sources. However, this was measured with a crude analysis method, measuring α-amino nitrogen, which measures the terminal amino group of free amino acids and peptides. This value does not say anything about the quality of the digested protein. Certain essential amino acids cannot be produced by the host and should be obtained from the diet. Whether or not the increased protein liberated by BC30 also contains these essential amino acids is not known. Even when the plant proteins contain these amino acids, there is no guarantee for them being released by the natural digestion process and/or BC30. For this amino acid profiles need to be determined.

The amount of protein added to the upper GI tract model averaged between 8.4 and 14.9 g. The average daily requirement for protein is 56-91 g/day for the average male, and 46-75 g/day for the average female, depending on the lifestyle (sedentary vs athletic). Divided over 3 meals per day this amounts to approx. 20 grams, which is twice the amount tested in TIM-1. It should be noted that the volume in TIM-1 is scaled down slightly. Therefore, the amounts of protein tested are considered to be relatively physiological for a single meal. It should be noted that under the conditions tested, the three plant proteins were already fairly digestible in TIM-1, with amounts of total nitrogen 'taken up by the body' between 79 and 88%.

Excess protein that is not digested in the upper GI tract reaches the colon. Depending on the amounts of available fermentable carbohydrates present in the colon, proteins that reach the colon may be incorporated into microbial biomass of the gut microbiota, or be fermented by the gut microbiota (Venema, 2010). The latter leads to the production of all kinds of metabolites which are considered to be harmful for the host, such as ammonia, phenol, *p*-cresol, $H_2S$, etc. (Van Nuenen *et al.*, 2003). This mostly occurs in the absence of fermentable carbohydrate, when the microbiota switches to fermentation of proteins. The increased digestion of the plant proteins by BC30 therefore has the additional benefit of decreasing the amount of proteins that reach the colon and might be fermented by the gut microbiota into potentially toxic metabolites.

In summary, BC30 increased protein digestibility for all three plant proteins in a validated *in vitro* model of the upper GI tract. For pea protein, the increase was primarily more peptides, but not of decreased length (i.e. the degree of hydrolysis was more or less identical). For soy and rice protein, this was both an absolute increase in total nitrogen, as well as smaller peptides, given the drop in TN/AAN ratio.

## Supplementary material

Supplementary material can be found online at https://doi.org/10.3920/BM2016.0196.

**Figure S1.** Schematic diagram of the dynamic, multi-compartmental TNO *in vitro* model of the stomach and small intestine (TIM-1).

**Table S1.** Cumulative absolute total nitrogen and α-amino nitrogen in the jejunum+ileum dialysates.

## Conflict of interest

DK, HC and SF are employees of Ganeden Inc., KV has been consulting for Ganeden Inc. Authors confirm that all the research meets the ethical guidelines, including adherence to the legal requirements of the study country.

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