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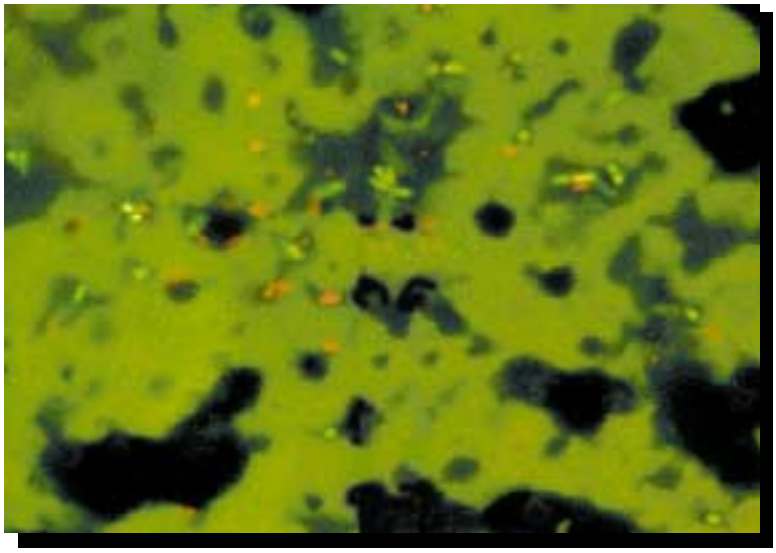
Application of Probiotic Bacteria to Functional Foods

C. Stanton, R.P. Ross, G. Fitzgerald and K. Collins

Probiotic strains and their key beneficial traits can withstand the technological stresses involved in the manufacture of Cheddar cheese and spray-dried powders.

Spray drying is a cost effective method for producing large quantities of some probiotic cultures in a form suitable for functional food applications.



This report is based on two complementary projects:

** Probiotic Bacteria: Analysis of Probiotic traits and development of functional foods (ARMIS No. 4527), and*

** Demonstration of Health Promoting Effects of Human Probiotic Bacteria Delivered in a Functional Food (ARMIS No. 4840).*

(Application of Probiotic Bacteria to Functional Foods)

Armis Nos. 4527 and 4840

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This project forms part of an ongoing collaborative research programme into Probiotic micro-organisms and their application in Functional Foods between Teagasc, Dairy Products Research Centre, Moorepark and University College, Cork.

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Summary and Conclusions

Probiotic cultures are described as live microbial feed supplements that improve intestinal microbial balance and are intended for maintenance of health or prevention, rather than the curing of disease. The demand for probiotic foods is increasing in Europe, Japan and the U.S. reflecting the heightened awareness among the public of the relationship between diet and health.

Traditionally, the most popular food delivery systems for these cultures have been freshly fermented dairy foods, such as yogurts and fermented milks, as well as unfermented milks with cultures added. However, in the development of functional foods, the technological suitability of probiotic strains poses a serious challenge since their survival and viability may be adversely affected by processing conditions as well as by the product environment and storage conditions. This is a particular concern, given that high levels (at least 10^7 per gram or ml) of live micro-organisms are recommended for probiotic products.

In previous studies (see DPRC No. 29) the successful manufacture of probiotic Cheddar cheese harbouring high levels (>10^8 cfu/g) of the probiotic Lactobacillus paracasei NFBC 338 *strain was reported.*

Hence, the overall objective of these studies was to continue the development and evaluation of Functional Foods containing high levels of viable probiotic bacteria, with particular emphasis on overcoming the technological barriers and the identification of strains suited to particular applications, such as incorporation into Cheddar cheese and spray-dried powders.

The Main Conclusions were as follows:

These studies demonstrate that probiotic strains and their key beneficial traits can withstand the technological stresses involved in the manufacture of Cheddar cheese and spray-dried powders.

Probiotic Cheddar:

* *A probiotic Cheddar cheese containing high numbers of probiotic* Lb. paracasei NFBC 338 *was manufactured at pilot scale before being successfully scaled up to commercial cheese plant production level.*

* *Sensory evaluation of the cheese by experienced commercial cheese graders indicated that the cheese was not adversely affected by the presence of the probiotic strain.*

* *The probiotic counts remained high (> 10^8 cfu/g) throughout the six-month maturation period and the cheese proved a good carrier of the probiotic strain to the intestine in model trials using pigs.*

* *A probiotic Cheddar cheese, harbouring large numbers of bifidobacteria was also manufactured successfully at pilot-scale, exhibiting improved Cheddar flavour during early ripening.*

Probiotic Spray-Dried Powders:

* *Spray drying of skim milk was evaluated as a means of preservation of* Lb. paracasei NFBC 338 *and* Lb. salivarius UCC 118. *It was demonstrated that* Lb. paracasei NFBC 338 *is more heat resistant than* Lb. salivarius UCC 118 *and exhibits superior survival during spray drying. Both spray-dried cultures appeared stressed following spray drying.*

* *The heat tolerance and survival of probiotic lactobacilli during spray drying was significantly improved by tempering of the cultures with mild heat or salt.*

* *The survival of both* Lactobacillus *strains during powder storage was inversely related to storage temperature. Maximum survival was achieved at 4°C.*

* *Probiotic spray-dried powder containing* Lb. paracasei NFBC 338 *was an effective inoculum, as an adjunct for probiotic Cheddar cheese, without adversely affecting product quality.*

Technique Development:

** A technique was developed using LIVE/DEAD® BacLight™ Viability Kit in conjunction with Confocal Scanning Laser Microscopy (CSLM) to enumerate viable bacteria in situ in probiotic dairy products.*

** RAPD PCR was found to be a useful method for discriminating between different bifidobacteria species and for confirmation of the identity of particular bifidobacteria strains in dairy products.*

Research and Results

Probiotic Strain Selection and Detection

To incorporate bifidobacteria into Cheddar cheese, a high inoculum (10^9 cells/ml inoculum approx.) is necessary. Therefore, initially, effort was devoted to the generation of concentrated cultures of selected human isolates.

Growth curves of bifidobacteria in various media including:

- *TPY*
- *10% reconstituted skim milk (RSM) supplemented with 0.5% yeast extract, and*
- *MRS supplemented with 0.05% cysteine hydrochloride were performed.*

Consequently, MRS supplemented with 0.05% cysteine hydrochloride was chosen as the most effective medium for yielding high cell numbers (10^9 cells/ml) and growth of bifidobacteria strain UCC 35612 in a 5 L laboratory scale fermentor.

It was demonstrated that RAPD PCR may be a useful method for discriminating between different bifidobacteria species and for confirmation of the identity of particular bifidobacteria strains in dairy products. A selective medium was developed for bifidobacteria which allows for

discrimination of bifidobacteria from other microflora in food products, including lactobacilli, streptococci and *E.coli*.

Using this medium, retail 'probiotic' yogurts were found to contain from 10^5 to 10^7 viable bifidobacteria/ml of product, indicating that a number of so-called 'probiotic' products do not meet the minimum recommended level of 10^7 probiotic cells/ml.

Probiotic dairy products were examined microscopically, using Confocal Scanning Laser Microscopy (CSLM), with the LIVE/DEAD® BacLight™ Viability Kit *(Fig. 1)*.

The LIVE/DEAD® BacLight™ Viability Kit (Molecular Probes Inc., Eugene, OR, USA) was developed to differentiate live and dead bacteria based on plasma membrane permeability and has been used to monitor growth of bacterial populations. The Viability Kit comprises two fluorescent nucleic acid stains: SYTO 9 and propidium iodide (PI). SYTO 9 (excitation/emission maxima 480/500 nm) penetrates both viable and non-viable bacteria while PI (excitation/emission maxima 490/635 nm) penetrates bacteria with damaged plasma membranes, thereby quenching the green SYTO 9 fluorescence. Bacterial cells with compromised membranes thus fluoresce red and those with intact membranes fluoresce green.

All microscopy work was performed using a Zeiss LSM310 confocal scanning laser microscope (Carl Zeiss Ltd, Welwyn Garden City, Herts., UK) using the method involving the LIVE/DEAD® BacLight™ viability staining kit. The sensitivity of the *in situ* viability staining technique was established by direct comparison of the bacterial numbers obtained in an actively growing broth culture obtained by plate counting, and CSLM. *In situ* LIVE/DEAD® BacLight™ bacterial viability staining in conjunction with CSLM was then compared with Standard Plate Counts for enumeration and viability assessment of probiotic bacteria in a range of dairy products including Cheddar cheese and spray-dried powders.

Probiotic Cheese Using Commercial Bifidobacteria Strains

Commercial strains of bifidobacteria (*Bifidobacterium lactis Bb-12* and *Bifidobacterium longum Bb 536*) were used in the manufacture of probiotic cheeses. Two vats of Cheddar cheese were manufactured at pilot-scale, each with a different adjunct of the commercial probiotic bifidobacterial strains and their viability throughout ripening investigated in addition to Cheddar composition. The bifidobacteria were added during Cheddar cheese manufacture at levels of 10^8 cfu/ml of cheesemilk.

*One (*Bifidobacterium lactis Bb-12*) survived ripening to high numbers (≥ 10^8 cfu/g), while viability of the other strain was reduced to 10^5 cfu/g cheese after one month of ripening.*

The Cheddar cheese, in which the probiotic bifidobacterial strain survived to high numbers was examined microscopically, using CSLM and the probiotic bifidobacteria were identified in the cheese using the

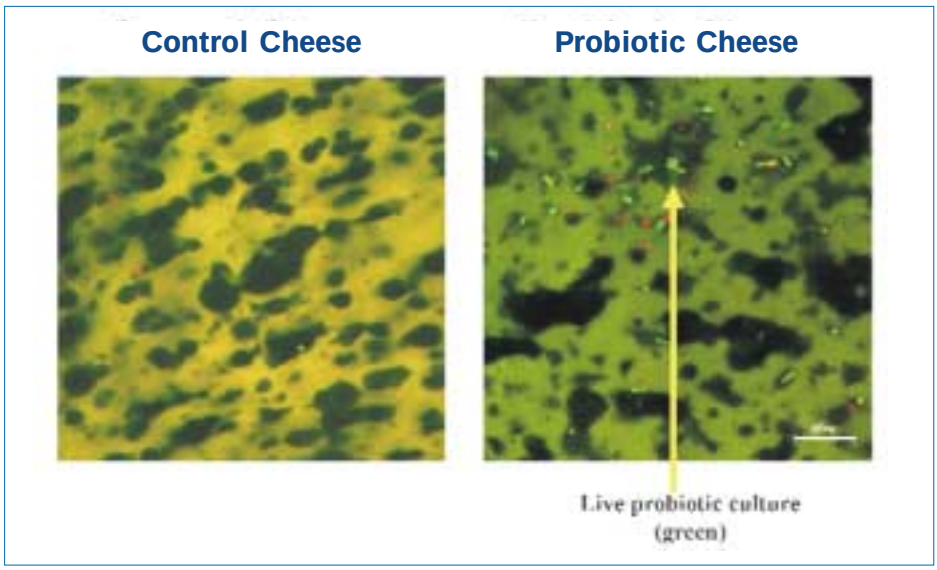

Fig. 1: LIVE/DEAD® BacLight™ viability staining and CSLM.

LIVE/DEAD® BacLight™ Viability Kit *(Fig. 1)*. The cheeses were analysed for composition and were generally found to be within normal limits.

The probiotic cheese containing 10^8 cfu/g cheese was found to have more extensive proteolysis and improved flavour in the early stages of ripening than the control cheese containing no added probiotic strain. Gas Chromatography Mass Spectroscopy of extracts of volatile compounds showed differences between the cheese containing the surviving probiotic strain compared with the control cheese.

Cheese-making trials with Lb. paracasei NFBC 338

The level of probiotic ingestion believed to be required by human subjects during feeding trials is in the order of 10^{10} cfu/day of the probiotic strain, a level equivalent to ingestion of 20 - 40 g of cheese containing 2.5 - 5 x 10^8 cfu/g per day. Although no guidelines exist in Europe at this time, current Japanese guidelines recommend that a probiotic food should contain at least 10^7 cells per g or ml of product. Hence the objective was to develop and manufacture sufficient quantities of probiotic Cheddar cheese harbouring high numbers of *Lb. paracasei NFBC 338* suitable for human clinical evaluation.

Mini cheese trials were performed to assess whether starter combinations used during Cheddar cheese manufacture had an effect on probiotic numbers in the cheese curd. The following starter combinations were used in conjunction with probiotic *Lb. paracasei NFBC 338* as adjunct, to mimic probiotic Cheddar cheese manufacture, under a variety of possible commercial applications: i.e. *Lactococcus lactis* subsp. *cremoris 303* and *227*, *Lactococcus lactis* subsp. *cremoris 223* and *227*, and *Lactococcus lactis* subsp. *cremoris R604*.

No effects on probiotic Lb. paracasei NFBC 338 *levels in the curd were obtained, as a result of employing the various combinations of starter cultures during manufacture. The cheesemilk was inoculated with probiotic adjunct at 3.9 x 10^6 cfu/ml, resulting in levels in the curd of 5.0 - 7.5 x 10^7 cfu/g, irrespective of starter combination.*

Subsequently, pilot-scale trials were performed which were aimed at identifying the minimum inoculum of the probiotic that would result in Cheddar cheese harbouring ≥10^8 cfu/g. As a result, it was confirmed that inocula of 1% (w/v) or higher were sufficient to yield probiotic counts in the cheese of > 10^8 cfu/g. Lb. paracasei NFBC 338 *numbers in one-day-old cheeses as a result of using 1 and 2% inocula of the adjunct probiotic culture during manufacture were 0.9 x 10^8 and 1.2 x 10^8 cfu/g, respectively which increased to 3.5 x 10^8 cfu/g after one month of ripening at 8°C.*

Having identified maximum numbers to which the probiotic strain grew (3.5 x 10^8 cfu/g), probiotic Cheddar cheese (180 kg) was manufactured at pilot-scale for clinical evaluation. The adjunct (2% inoculum of *Lb. paracasei NFBC 338*) was incorporated with the cheese starters *Lactococcus lactis* subsp. *cremoris 227* and *303* at 1.4%, while control cheese was manufactured using the same standardised milk as used for the probiotic cheese but without the addition of the starter adjunct. Probiotic counts in cheese reached 3.0 x 10^8 cfu/g after one month of ripening at 8°C.

Compositions of the control and probiotic cheeses were in the range typical for Cheddar, and the cheeses were classified as commercial grade with respect to sensory criteria for both flavour and texture, with the probiotic cheese scoring slightly higher. These results confirm that Cheddar cheese is capable of harbouring high numbers of Lb. paracasei NFBC 338 *and is also a suitable matrix for growth of the probiotic strain without adversely altering Cheddar cheese characteristics.*

Finally, Cheddar cheese manufacturing trials were undertaken at a scale similar to that employed in industry to demonstrate the viability of *Lb. paracasei NFBC 338* at industrial scale. In the first such trial, the probiotic adjunct culture was introduced into the cheese milk in 10% (w/v) RSM containing 0.5% (w/v) yeast extract. The yeast extract was prepared in an industrial pilot scale fermentor at 37°C for 24 hours, with the pH maintained constant at 6.5 throughout the fermentation by the addition of a concentrated solution of NaOH, using a pH stat. Cheddar cheese was manufactured in a 3000 L OST 4 vat using a 1% inoculum of the overnight culture of *Lb. paracasei NFBC 338*, which contained 1.1 x 10^9 cfu/ml.

Subsequently, the probiotic strain was present at a level of 2.0 x 10^8 in one-day-old cheese, which increased to 3.5 x 10^8 cfu/g after two week of ripening at 8ºC, and were at 6.0 x 10^8 after three months of ripening.

Commercial cheese manufacturing trials were undertaken in 22,000 litre vats in a commercial cheese manufacturing plant. On the day before probiotic cheese manufacture, the use of lactobacilli as adjuncts to the starters was avoided in the plant. This was done so that enumeration of the probiotic *Lactobacillus* strain in the cheese could be easily performed (without interference from other lactobacilli), using only selective media for enumeration of the probiotic strain. The inoculum for this commercial trial was initially grown in 100 litres MRS inoculated (at 10% v/v) into a bulk starter tank (1000 litres) containing pasteurised RSM (12.5% w/v) with 0.5% (w/v) yeast extract and incubated at 37ºC for ~20 hours. Probiotic counts after 20 hours of growth in RSM were 6.1 x 10^8 cfu/ml. This was used to innoculate the 22,000 litre vats at a rate of 0.1%. In all, the probiotic *Lb. paracasei NFBC 338* was added to 15 vats as an adjunct to the starter for probiotic cheese manufacture with one vat used as the control (to which no probiotic *Lactobacillus* adjunct was added). Probiotic counts in the cheese milk were 3.1 x 10^6 cfu/ml. Following one week of ripening at 8ºC, *Lactobacillus* counts in the probiotic cheese were 5.0 x 10^8 cfu/g, and remained high following one month of ripening (4.3 x 10^8 cfu/g). RAPD PCR was used to confirm the identity of the probiotic strain in the cheese, and counts remained stable throughout ripening for 6 months.

Sensory grading of the probiotic cheese was performed by commercial graders. The flavour of the probiotic cheese was compared with the control, throughout ripening, and was described as mature, with a sharp flavour at 6 months. A randomised, double blind, placebo-controlled clinical feeding study using healthy human subjects has been undertaken and the effects of ingestion of the probiotic cheese on various health parameters have been established.

In a separate study, the probiotic *Lactobacillus* culture was introduced into the cheese milk in the form of a spray-dried probiotic powder. *Lb. paracasei NFBC 338* (RifR®) was spray dried in a tall-form, Niro spray drier and contained 1 x 10^9 cfu/g powder. This was incorporated during Cheddar

cheese manufacture in the cheesemilk at 0.1% (w/v) inoculum. The probiotic strain grew in the cheese during ripening at 8ºC to reach levels of 1.3 x 10^8 cfu/g after one month. However, the probiotic had decreased to 7.7 x 10^7 cfu/g after 3 months of ripening.

This experiment demonstrated that adding a relatively low inoculum of the probiotic culture in spray-dried powder form (0.1% w/v) to the cheese vat at manufacture yielded Cheddar cheese which falls within the guidelines proposed for probiotic foods, i.e. containing at least 10^7 viable probiotic cells per gram or ml. Both the composition and sensory properties of this probiotic cheese were unaffected by the culture introduced in this way.

Cheddar cheese incorporating *Lactobacillus salivarius UCC 118* (RifR®) was also manufactured at pilot scale (450 L). This probiotic strain was incorporated at an inoculum of 1.6 x 10^6 cfu/ml and was present in one-day-old cheese at a level of 1.2 x 10^7 cfu/g. Following one month of ripening, the probiotic count has decreased to 7.1 x 10^6 cfu/g cheese. Therefore, this cheese cannot be described as 'probiotic', using current guidelines.

Pig Feeding Trials

It was confirmed that the process of cheese-making did not affect the ability of the probiotic Lb. paracasei NFBC 338 *culture to survive in the intestine and colonise intestinal epithelium through a feeding trial (lasting 3 weeks) involving the porcine model fed the strain in Cheddar cheese, containing high levels (~10^8 cfu/g) of the rifampicin resistant variant of* Lb. paracasei NFBC 338.

The probiotic strain was detected in faecal samples of probiotic-fed pigs during the feeding trial at levels of ~10^6 cfu/g, and the probiotic strain was not present in faecal samples from control pigs. This result demonstrated that the strain in Cheddar cheese survived passage through the mammalian GIT. Furthermore, the probiotic strain was detected in the small intestinal contents of the probiotic-fed animals (at levels of 10^4 - 10^5 cfu/ml) and not in intestinal contents of pigs fed the control cheese, at the end of the 3-week feeding period.

Spray-Dried Milk Powders containing Live Probiotic Bacteria

Spray drying of skim milk was evaluated as a means of preservation of *Lb. paracasei NFBC 338* and *Lb. salivarius UCC 118*. It was demonstrated that *Lb. paracasei NFBC 338* is more heat resistant than *Lb. salivarius UCC 118* and exhibits superior survival during spray drying. An air outlet temperature of 80 - 85ºC was optimal for spray drying, yielding powders with a moisture content of 4.1 - 4.2% and viable counts of 3.2 x 10^9 cfu/g *Lb. paracasei NFBC 338* and 5.2 x 10^7 cfu/g *Lb. salivarius UCC 118*. Thus, *Lb. paracasei NFBC 338* exhibited superior survival during spray drying compared with *Lb. salivarius UCC 118*. Both spray-dried cultures appeared stressed following spray drying, as evidenced by increased sensitivity of these cultures to NaCl.

Nonetheless, cellular activities such as bacteriocin production were unaffected by the spray drying process. Maximum survival of probiotic lactobacilli in the spray-dried powders was achieved by storage at 4ºC, and survival of both Lactobacillus *strains were inversely related to powder storage temperature. Survival of* Lb. paracasei NFBC 338 *remained constant at levels of ~1 x 10^9 cfu/g during 2 months of powder storage at 4ºC while a decline of approximately one log (from 7.2 x 10^7 to 9.5 x 10^6 cfu/g) was observed for* Lb. salivarius UCC 118 *stored under the same conditions.*

A number of approaches have been examined with a view to improving culture viability during spray drying, a process which costs up to six times less per kg of water removed than freeze drying. One such approach involves the exploitation of microbial stress responses. We examined the increased degree of thermotolerance and suitability for spray drying conferred on probiotic *Lb. paracasei NFBC 338* by adaptation to different stresses, such as heat, hydrogen peroxide and salt. Heat-adapted cells induced homologous tolerance and cross protection against NaCl, bile-salt toxicity and cold storage. Although not as efficient as the homologous stress, the levels of cross protection were in the order heat $\simeq$ salt > hydrogen peroxide > bile. Viability of the heat-adapted *Lb. paracasei NFBC 338* in RSM was enhanced 18-fold during spray drying at outlet temperatures of 95 to 105ºC, while salt-adapted cultures exhibited 16-fold greater viability than controls *(Fig. 2)*.

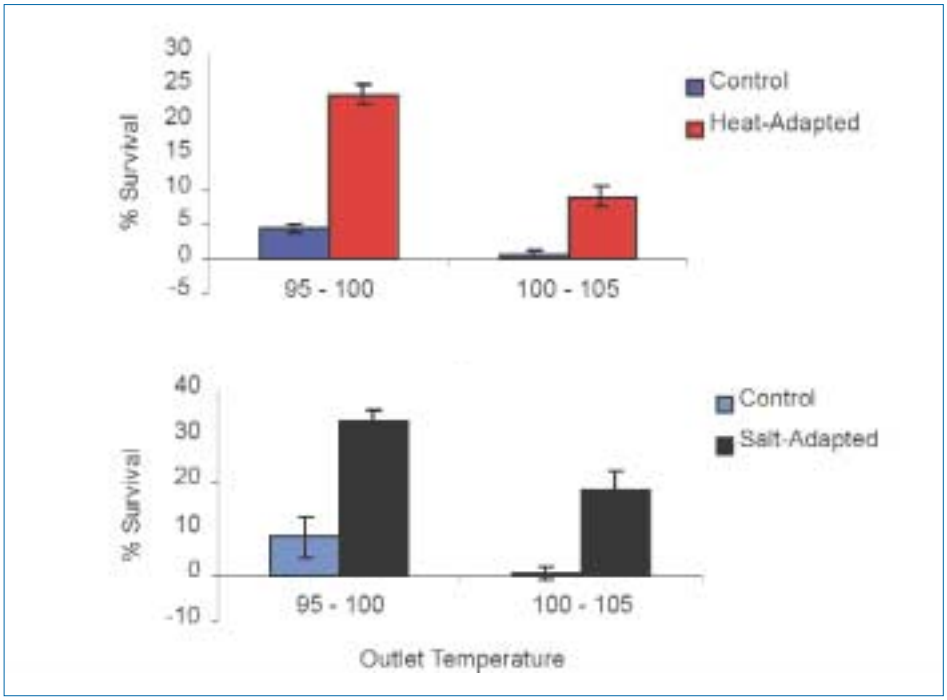

Fig. 2: Pre-adaption improves survival of Lb. paracasei NFBC 338 *during spray drying.*

These data highlight the effectiveness of pre-conditioning treatments of probiotic strains on the optimisation of viability during the harsh conditions encountered during the manufacture of spray-dried powders.

This approach offers potential for maximizing survival of probiotic bacteria during development of probiotic functional foods. The cross-protection afforded by salt against thermal stress may indicate that certain common protective proteins are induced in the cell by both heat and salt stress (Fig. 3).

Influence of Spray Drying on Probiotic Properties

The effect of spray drying on the capability of the probiotic bacterial strain *Lb. paracasei NFBC 338* to adhere to gut epithelial tissue was assessed. Adherence of probiotics to epithelial cells in culture is used as an indicator

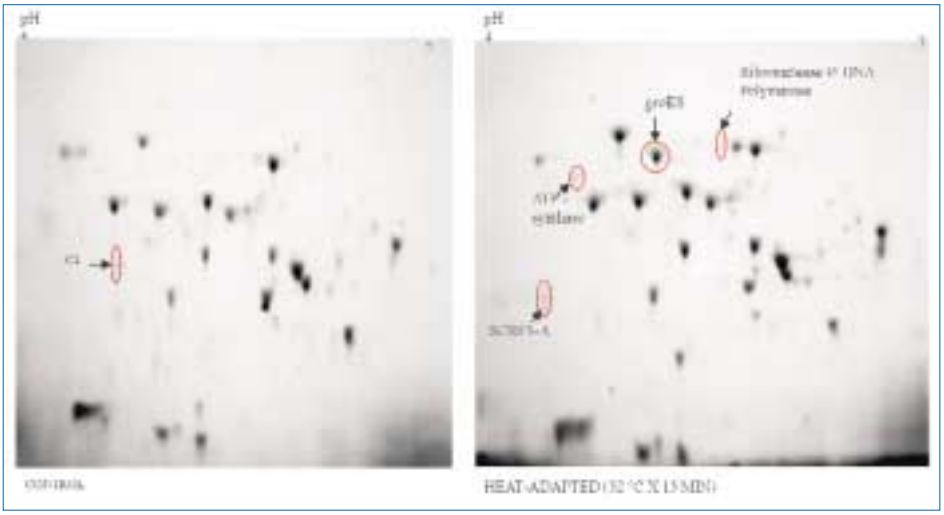

Fig. 3: Proteomic approach to identify stress proteins in Lb. paracasei NFBC 338.

of their ability to colonise the gastrointestinal tract. HT-29 cells are human intestinal cell-lines expressing morphological and physiological characteristics of normal human enterocytes. This cell-line has been previously exploited to elucidate the mechanisms mediating enteropathogen adhesion and have been employed in order to select for, and subsequently assess lactic acid bacteria on the basis of their adherence properties. In this study, the ability of *Lb. paracasei NFBC 338* to adhere to HT-29 monolayers was assessed following spray drying of the bacterial culture. Re-suspended spray-dried powder (containing 8.2 x 10^8 cfu/g of *Lb. paracasei NFBC 338*) was added to the eucaryotic cells, and following incubation at 37ºC, approx. 8.0 x 10^4 adherent lactobacilli were recovered using conventional microbiology techniques.

This result indicates that the ability of the probiotic bacterial strain to adhere to intestinal epithelial cells was not impaired by the process of spray drying.

For further information, please contact
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Main Publications

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