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The Production of Yoghurt with Probiotic Bacteria Isolated from Infants in Jordan

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Abstract: Cultures of presumptive lactobacilli and bifidobacteria were isolated from eight infants living in Amman, Jordan. After screening for the classic properties of probiotic organisms, three promising isolates were identified as *Lactobacillus casei*, *Lactobacillus gasseri* and *Bifidobacterium infantis*. These strains were employed to make yoghurt and, in order to achieve a short production time, a two-stage fermentation procedure was used with *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* providing the rapid acidification. Yoghurts containing counts of $> 1.0 \times 10^8$ cfu ml⁻¹ of the individual probiotics and high counts of the traditional species from yoghurt were produced, and storage trials at 4°C showed that the viability of the probiotic cultures was retained over 15 days.

Key words: Probiotic strains, *Lactobacillus*, *Bifidobacterium*, viability in yoghurt

Introduction

In general, commercial cultures of probiotic bacteria are species of *Lactobacillus* and *Bifidobacterium* that inhabit the human intestine and impart, through their presence, unique and beneficial effects on the health of the individual (Holzapfel *et al.*, 1998). Amongst the alleged benefits are modulation of intestinal health and the immune system, as well as anti-carcinogenic, anti-diarrhoeal and hypocholesterolaemic effects (Sanders, 1999; Steer *et al.*, 2000). However, the impact of a given species on the health of an individual within a specific probiotic species appear to be strain/host-dependent (Sellars, 1991). For example, a strain of *Lactobacillus acidophilus* isolated in North America may well be genetically different from a species recovered in Europe or the Middle East, and this difference could well be reflected in the reaction of a host ethnic population remains a controversial topic, for the health-promoting properties of. Thus, Hotta *et al.* (1989) showed that, while a culture of bifidobacteria administered to a patient with severe diarrhoea could colonize the colon and restore normal function, the foreign strain was replaced over time by strains already present in the patient's intestine.

Obviously the patient described by Hotta *et al.* (1989) did benefit from the administration of the culture, but a more compatible strain might have remained active for a much longer period of time. Thus, at present, it is usually assumed that, for strains of bacteria to be effective as probiotics, they must simply be of human origin and meet a number of *in vitro* physiological and biochemical criteria. For example, the strains should be resistant to gastric acidity and bile salts, adhere to gut epithelial tissue, produce antimicrobial substances and have the ability to influence human metabolic activities, e.g. reduce cholesterol levels in the blood (Dunne *et al.*, 2001).

However, this crude characterization of a probiotic

culture would appear to be in urgent need of modernization, and the potential importance of obtaining precise genetic fingerprints for probiotic strains employed in commercial bio-yoghurts or health supplements has long been a matter for speculation (Robinson and Samona, 1992). Certainly the instrumentation necessary for the DNA and 16S RNA analysis of bacterial strains is available, and there is a strong argument for suggesting that DNA fingerprinting should be introduced as one of the selection criteria for commercial cultures. Thus, if all the evidence implying that humans can derive a health benefit from the regular ingestion of authentic probiotic cultures is correct, consumers have the right to expect that the bacteria in a 'bio-yoghurt' have the potential to influence beneficially their health. Yet a purchaser may well take home a "yogurt" fermented with a "bio-culture" and have no idea at all which bacteria are present and whether the implied 'health-promoting' properties will be manifest.

Obviously reputable culture suppliers are seeking to operate in the interests of the consumer but, as a more specific 'step in the right direction', a long-term programme has been established in Jordan to evaluate the responses of selected hospital patients to the regular ingestion of strains of probiotic bacteria isolated in Jordan in comparison with imported commercial strains. Natural yoghurt was selected as the vehicle for the probiotic bacteria, and hence the aims of this stage of the project were:

1. to isolate some new strains of potentially probiotic bacteria from infants born to Jordanian nationals;
2. screen the same strains for the basic probiotic characteristics mentioned above; and
3. assess whether any strains that met the initial selection criteria would survive in a fermented milk.

Materials and Methods

Isolation of Probiotic Species: Eight faecal samples

from breast-fed Jordanian infants (aged three to six months) were collected by swabbing, and the individual swabs were kept in an ice bag during transport to the laboratory for processing (Hartemink and Rombouts, 1999). Pre-reduced sterile peptone water (1.0 g l^{-1} , Code No. CM9, Unipath Ltd., Basingstoke, Hampshire, UK) containing L(+) cysteine-HCl (5.0 g l^{-1} , Sigma-Aldrich, Poole, Dorset, UK) was used to release the bacteria from the swabs, and serial dilutions (10^{-1} - 10^{-7}) were made in the same medium (9 ml amounts in universal bottles). Duplicate aliquots (0.1 ml) of these serial dilutions were spread onto pre-poured plates of de Man, Rogosa and Sharpe (MRS) Agar (Code No. CM361) supplemented with cysteine-HCl (5.0 g l^{-1}) for isolation of lactobacilli, and onto Beerens Agar for the recovery of bifidobacteria (Beerens, 1990 and 1991). The plates were then incubated at 37°C under anaerobic conditions for two days (MRS Agar) and four days for the Beerens Agar (Kimura *et al.*, 1997).

For each of the original eight samples, ten colonies showing different morphologies and/or colours on each of the two media were sub-cultured onto plates of MRS Agar (Kimura *et al.*, 1997). After further purification on the same media, seventy-nine isolates were found to be Gram-positive, catalase-negative, non-motile bacilli or coccibacilli and were kept for further study (Charteris *et al.*, 1997). The isolates were maintained by sub-culturing monthly into culture bottles of MRS Broth (Code No. CM359, 50 ml), followed by 18-20 hours incubation at 37°C in an anaerobic jar (Unipath Ltd., Basingstoke, Hampshire, UK). The cultures were stored at 2°C after incubation, and each culture was sub-cultured prior to every test.

Assessment of probiotic potential: The protocol employed to check that any given isolate had the potential to be classed as 'probiotic' was based upon the procedure reported by Haddadin *et al.*, 1997, and the five characteristics checked were: tolerance of acidic growing conditions, tolerance of bile salts, antagonism to selected pathogens, cholesterol assimilation and adhesion to intestinal cells. A 'selection by rejection' technique was used to select likely cultures, i.e. only isolates that met the criteria of one test, e.g. resistance to acid, were selected for the next test.

Identification of Selected Isolates: The best three isolates in terms of potential probiotic characteristics were identified by certain morphological, physiological and biochemical characteristics relevant to the lactobacilli and bifidobacteria, respectively (Kandler and Weiss, 1986; Scardovi, 1986; Hammes *et al.*, 1992; Biaviati *et al.*, 1992).

Viability of the probiotic isolates in yoghurt: The yoghurt was prepared according to the method of Shah (2000) in

order to avoid the poor texture and acid development sometimes associated with the use of probiotic cultures. Full-cream bovine milk, fortified with skim-milk powder to give a level of 14% solids-non-fat, was pasteurized at 85°C for 15 minutes, cooled to $40\text{-}42^\circ\text{C}$ and inoculated at a rate of 20 ml l^{-1} with a probiotic isolate previously sub-cultured in skim-milk. The milk was then incubated at 37°C for 2 hours and, at this point, a traditional yoghurt starter culture consisting of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* was added (20 ml l^{-1}). The product was then incubated at 42°C until the desired pH of 4.4-4.5 had developed (Dave and Shah, 1997).

Samples of yoghurt containing one of the selected isolates and *Str. thermophilus* and *Lb. delbrueckii* subsp. *bulgaricus* were then stored at 4°C to monitor the effect of storage on the viability of the probiotic species. The counts of the probiotic starter cultures were determined after 0, 1, 2, 3, 4, 5, 10 and 15 days of storage; 15 days is maximum shelf-life for yoghurt in Jordan. The entire procedure was performed twice.

Results and Discussion

Eighty isolates each from MRS Agar and Beerens Agar were recovered from the eight faecal samples and, as mentioned earlier, a total of seventy-nine of these isolates were found to be Gram-positive, catalase-negative, non-motile, bacilli and coccibacilli. Forty-two were recovered from MRS Agar (presumptive lactobacilli) and thirty-seven from Beerens Agar (presumptive bifidobacteria), and all seventy-nine isolates were retained for further assessment.

Identification of potential probiotic isolates: Out of the original isolates, only nine passed all five tests in the probiotic protocol and, even then, only two isolates of presumptive lactobacilli and one presumptive *Bifidobacterium* were regarded as promising candidates; i.e. showing high levels of tolerance to gastric acidity and bile salts, and the ability to assimilate cholesterol. The results of the standard physiological and biochemical tests identified the isolates as *Lactobacillus casei*, *Lactobacillus gasseri* and *Bifidobacterium infantis*, respectively. The isolation of these species, along with many other species of *Lactobacillus* and *Bifidobacterium* has been reported by many researchers (Tannock, 1997; Holzapfel *et al.*, 1998) and *Lb. gasseri* is one of the most common species of the *Lactobacillus acidophilus* group in the human intestine (Klein *et al.*, 1998). Similarly, *Bif. infantis* often dominates the anaerobic microflora in infants (Hartemink and Rombouts, 1999).

If DNA finger-printing confirms these identifications, then their use in probiotic foods for distribution in Jordan would merit serious consideration. A fermented milk like yoghurt would be the obvious vehicle, and hence it was

Table 1: Viability of *Bif. infantis*, *Lb. gasseri* and *Lb. casei* in the presence of a traditional yoghurt starter culture during the storage of a fermented milk at 4°C; all counts as colony-forming units ml⁻¹ and the means of four determinations

Day	<i>Bif. infantis</i>	<i>Lb. gasseri</i>	<i>Lb. casei</i>
0	2.6 x 10 ⁸	3.1 x 10 ⁸	2.5 x 10 ⁸
1	4.5 x 10 ⁸	8.5 x 10 ⁸	5.2 x 10 ⁸
2	7.4 x 10 ⁸	7.4 x 10 ⁸	1.4 x 10 ⁹
3	1.5 x 10 ⁹	7.5 x 10 ⁸	4.2 x 10 ⁸
4	2.0 x 10 ⁹	4.9 x 10 ⁸	4.2 x 10 ⁸
5	1.2 x 10 ⁹	4.5 x 10 ⁸	5.0 x 10 ⁸
10	4.0 x 10 ⁹	4.8 x 10 ⁸	5.0 x 10 ⁸
15	2.3 x 10 ⁹	2.9 x 10 ⁸	1.9 x 10 ⁸

important to determine whether or not the three species could be employed in the production of yoghurt, and survive during refrigerated storage.

Effect of storage on culture viability in yoghurt: The results of the current trial are shown in Table 1, and it is clear that the initial counts of all three species were well above the count of 1.0 x 10⁶ cfu ml⁻¹ which is often quoted as the 'therapeutic minimum' (Robinson, 1989; Gardiner *et al.*, 2002). During storage, the counts of *Lb. casei* and *Lb. gasseri* remained broadly stable over the 15 days but, with *Bif. infantis*, the counts increased to reach 4.0 x 10⁹ cfu ml⁻¹ by the tenth day; the highest count achieved by any of the probiotic isolates throughout the whole period of storage. Even at 15 days, the counts for *Bif. infantis* were significantly better ($p < 0.05$) than the initial count, but the reason for this unexpectedly high level of growth and survival needs to be established.

Conclusion: Although nine isolates passed all the tests for probiotic species, it is equally relevant that a further seventy presumptive lactobacilli and bifidobacteria were discarded on the basis of quite arbitrary criteria. Indeed, it could be that employment of the usual selection procedure needs to be revised, for while passage through the stomach is the first 'hurdle' that potentially probiotic bacteria face on their way to the intestines (Steer *et al.*, 2000) and acid tolerance is regarded as a very important prerequisite in the selection of commercial cultures, it was notable that, in this study, only 30% of the isolates were shown to be capable of growth at low pH. Yet all the isolates must have been viable at the time of recovery, and it might be assumed that isolates designated as 'acid intolerant' had survived passage through the stomach of the infant prior to survival and, perhaps, growth in the intestine.

In part, this survival of the acid-intolerant isolates may be because the stomach contents of an infant is around pH 4.5 - 5.0 but, even so, it may be that the requirement that a probiotic culture should be resistant to low pH is a

rather artificial one. In other words, it is assumed that a commercial culture must be tolerant of the pH (pH ~ 3.0) of the stomach contents of an adult but, in practice, the short residence time of food in the stomach may allow many acid-sensitive lactobacilli and bifidobacteria to pass into the intestines trapped in particles of food.

In addition, the isolate of *Lb. casei* could have been 'eliminated' on the grounds of low antibacterial activity, and yet it was significantly better than the other isolates at assimilating cholesterol. This pattern suggests that it may be desirable to consider selecting probiotic cultures for specific purposes, so that a patient with a high cholesterol problem could be fed *Lb. casei*, while a symptomless carrier of a *Salmonella* sp. might be treated with a species with marked antimicrobial activity. It could be of interest also to determine whether the strains of *Lb. casei*, *Lb. gasseri* and *Bif. infantis* isolated during this work are, in any way, physiologically different from strains that could be purchased from a culture supplier in Europe. If they are specific to mothers and infants of Jordanian origin, then their use as probiotics in Jordanian hospitals merits serious consideration.

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