

Short Communication

Salmonella detection in probiotic products

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Abstract

The presence of large amounts of probiotic bacteria in a sample may interfere with the detection of undesirable microorganisms. To illustrate this, infant formula with various strains of probiotic bacteria and the corresponding probiotic culture powders and premixes were artificially contaminated with low levels of *Salmonella*. Recovery of *Salmonella* was generally very poor when the conventional pre-enrichment procedure using buffered peptone water (BPW) was applied. However, this problem was overcome by adding antimicrobial compounds to selectively suppress the growth and/or metabolic activity of the probiotic bacteria and increasing the buffering capacity of the pre-enrichment broth. It is recommended that these analytical constraints are already addressed during the development phase of new probiotic products.

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1. Introduction

Probiotic foods have steadily gained popularity over the past decades and a wide variety of foods are nowadays used as carriers for probiotic cultures, including fermented milk products (Coeuret et al., 2004), cheese (Stanton et al., 1998), ice cream (Christiansen et al., 1996), fermented sausages (Andersen, 1998), fruit juices (Post, 2002), cereal bars (Ouwehand et al., 2004) and infant formulas (Todd, 2003). Strains are selected based on their specific health promoting effects, but it is obvious that the safety aspects are also carefully considered to ensure that they do not pose any health risk for the consumer. The safety evaluation usually includes screening for (transferrable) antibiotic resistance genes, undesirable metabolic activities and collecting of evidence to demonstrate a “history of safe use”, etc. (Salminen et al., 1998).

Once a strain has been approved, it must still be determined whether the analytical methods that are normally used to verify the microbiological safety of the ingredients and the finished products can also be used for the new product. For microbiological methods, this is not very obvious: finished products typically contain ca. 10^7 cfu probiotics/g and ingredients may contain more than 5×10^{11} cfu/g and it is not unthinkable that the presence of undesirable contaminants is masked by the abundance of

beneficial microorganisms. Theoretically, this problem is most likely to occur with non-selective cultural methods. However, even with selective methods this possibility should be considered, in particular because many of these methods include a non-selective pre-enrichment step. This is for instance the case for various internationally recognized protocols for the isolation and detection of *Salmonella* (Anon, 2002a,b, Andrews et al., 1995). Surprisingly, though, it appears that this putative analytical problem has not been recognized yet.

Since the reliability of the *Salmonella* detection method is paramount to verify the safety of infant formula, we have addressed this question by analysing artificially contaminated infant formula containing various types of probiotic bacteria. We also included probiotic culture powders and premixes that are used as ingredients in the production of infant formula.

Here we report that the presence of probiotic bacteria can indeed seriously impair the recovery of *Salmonella* and also provide solutions to resolve this problem.

2. Materials and methods

2.1. Bacterial strains

Capsules with *S. panama* (ca. 5 cfu/capsule) were supplied by the RIVM, Bilthoven, the Netherlands. The lenticules with *S. goldcoast* (ca. 8 cfu/lenticule) were purchased from the HPA,

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Newcastle Laboratory, United Kingdom, and bioballs with *S. typhimurium* ATCC14028 and *S. abaeetuba* ATCC 35640 (with resp. 29 and 31 cfu/bioball) were supplied by BTF Pty Ltd., North Ryde BC, Australia.

Fifty additional strains of *Salmonella*, each representing a different serotype commonly associated with food, came from the Nestlé culture collection.

Probiotic bacteria (*Bifidobacterium longum* NCC 3001, *Bifidobacterium lactis* NCC 2818, *Streptococcus thermophilus* NCC 2496, *Lactobacillus paracasei* NCC 2461, *Lactobacillus rhamnosus* NCC 4007 and *Lactobacillus johnsonii* NCC 533) were supplied as concentrated powders containing 1×10^{10} to 7×10^{11} cfu/g by the Nestlé Product Technology Centre in Konolfingen, Switzerland.

2.2. Maltodextrin premixes

Maltodextrin premixes were prepared by dry-mixing maltodextrin with one or two different culture powders to obtain the desired concentration of probiotic bacteria (ranging from 1 to 7×10^{10} cfu/g).

2.3. Infant formula

Milk-based infant formula without probiotic bacteria were purchased from a local supplier. Infant formula with probiotics was prepared in house by dry mixing the infant formula with the maltodextrin premixes described above.

2.4. Pre-enrichment and spiking

The pre-enrichment was carried out by adding samples of 25 g to 225 ml buffered peptone water (BPW, Oxoid, Basingstoke, UK), BPW supplemented with vancomycin (10 mg/l), or double strength BPW supplemented with vancomycin (10 mg/l), malachite green (100 mg/l) and milk (10 g/l) together with the *Salmonella* capsules, lenticules or bioballs and probiotic bacteria, followed by incubation for 18 ± 2 h at 37 °C.

2.5. Selective enrichment, isolation and confirmation

From the pre-enrichment culture, 0.1 ml was inoculated in 10 ml Rappaport Vassiliadis broth with Soya (RVS broth, Oxoid, Basingstoke, UK) and incubated for 24 ± 1 h at 42 °C, after which the RVS culture was streaked onto Brilliant Green agar (BGA, Oxoid) and Mannitol Lysine Crystal Violet Brilliant Green agar (MLCB, Oxoid). Suspect colonies were confirmed by biochemical and serological testing as outlined in the standard method ISO 6579:2002 (Anon, 2002a,b).

2.6. Determination of vancomycin resistance

Freshly grown BHI cultures of *Salmonella* were diluted with 1% saline and added at a level of ca. 100 cfu/ml in BPW, containing milk (10% v/v) and filter sterilized vancomycin at concentrations of 0, 5, 10 and 15 µg/ml. The suspensions were incubated at 37 °C and growth of *Salmonella* was monitored by

impedance measurements using the Bactometer model 128, Vitek Systems, Biomerieux (Marcy l'Etoile, France).

3. Results and discussion

Thirty-four samples of infant formula, containing various types of probiotic microorganisms, were artificially contaminated with three *Salmonella* serotypes to evaluate the reliability of the protocol for detection of *Salmonella* in this type of matrix. The results (Table 1) showed that *Salmonella* was only recovered from 6 samples. The high incidence of false-negative results can probably be explained by the metabolic activities properties of the probiotic bacteria, in particular the production of organic acids, whose accumulation could render the pre-enrichment broth into a hostile environment for *Salmonella*. An alternative explanation would be that the false-negative results were (also) due to the production of other antimicrobial substances or to exhaustion of nutrients, but no attempt was made to investigate this further.

Nevertheless, these results clearly show that the detection method applied was not suitable for infant formula containing probiotic bacteria. According to the procedure described in ISO standard 6579:2002 (Anon, 2002a,b), double-strength buffered peptone water should be used for acidifying matrices. This solution did indeed improve the recovery of *Salmonella*, but we still observed ca. 25% false-negative results (data not shown), and therefore decided to explore the addition of antimicrobial substances to specifically inhibit the gram-positive microorganisms in the pre-enrichment broth. However, the choice of a suitable antimicrobial substance for this purpose is not very obvious, since *Salmonella* can be present in a sublethally

Table 1
Detection of *Salmonella* in deliberately contaminated infant formula with probiotic bacteria using BPW as pre-enrichment medium

| Probiotic Strain(s) | Level (cfu/g) | Spiked with | Recovery | pH of pre-enrichment culture after incubation |
|---|--------------------------------|-----------------------|----------|---|
| – | – | <i>S. typhimurium</i> | 3/3 | 5.2 |
| | | <i>S. goldcoast</i> | 3/3 | |
| | | <i>S. panama</i> | 10/10 | |
| <i>S. thermophilus</i> NCC 2496 | 10^7 | <i>S. goldcoast</i> | 2/3 | 4.8 |
| <i>S. thermophilus</i> NCC 2496 | 10^7 | <i>S. typhimurium</i> | 2/3 | |
| <i>B. lactis</i> NCC 2818 | 10^7 | <i>S. goldcoast</i> | 0/3 | 4.3 |
| <i>B. lactis</i> NCC 2818 | 10^7 | <i>S. typhimurium</i> | 0/3 | |
| <i>B. longum</i> NCC 3001 | 10^7 | <i>S. goldcoast</i> | 0/3 | 4.4 |
| <i>B. longum</i> NCC 3001 | 10^7 | <i>S. typhimurium</i> | 0/3 | |
| <i>L. johnsonii</i> NCC 533 | 10^7 | <i>S. goldcoast</i> | 1/3 | 4.5 |
| <i>L. johnsonii</i> NCC 533 | 10^7 | <i>S. typhimurium</i> | 1/3 | |
| <i>B. lactis</i> NCC 2818 and <i>S. thermophilus</i> NCC 2496 | 10^7 and 10^7 respectively | <i>S. panama</i> | 0/10 | 4.3 |

injured state, thereby rendering it susceptible to agents, which normally do not act against gram-negative bacteria. We therefore decided to select vancomycin, an antimicrobial compound that has already been used successfully as an additive to BPW for detection of stressed *Escherichia coli* O157 (Ogden et al., 2001).

Before incorporating this antibiotic in the pre-enrichment protocol, it was first verified that *Salmonella* is indeed resistant to vancomycin. This was investigated by determining the growth behaviour of 50 different serotypes of *Salmonella* with impedance measurements in BPW containing 0, 5, 10 and 15 µg vancomycin/ml. With all of the tested strains, it was found that the shape of the growth curves was not affected by the concentration of vancomycin (data not shown), from which it was concluded that *Salmonella* is indeed resistant at this concentration range.

Subsequent trials with artificially contaminated infant formula showed that this modification eliminated the problem of false-negative results (Table 2). However, the same table shows that this enrichment protocol failed to recover *Salmonella* from most of the maltodextrin premixes, probably because these contain up to 10,000-fold higher levels of probiotic bacteria than infant formula. Attempts were made to improve the recovery by adding other antibiotic substances that are specifically active against gram-positive microorganisms, including erythromycin (2 mg/l), mupirocin (100 mg/l), novobiocin (22 mg/l) and malachite green (100 mg/l), either alone or in combination. The cocktail of vancomycin and malachite green

proved to be the most effective, in particular when it was used in conjunction with double strength buffered peptone water containing non-fat dry milk powder (10 g/l). The latter was added to reduce the toxicity of malachite green for *Salmonella*, as recommended by Van Schothorst and Renaud (1985). With this combination, it was indeed possible to recover *Salmonella* from all the deliberately contaminated premixes and also from most culture powders (Table 3). The only false-negative results were obtained with culture powders of *B. lactis* NCC 2818, which was probably due to the extremely high cell count of this ingredient (7×10^{11} cfu/g). No further attempts were made to resolve this by modifying the composition of the pre-enrichment broth. Instead, it was investigated at which dilution false-negative results were no longer obtained. It was shown that this was already the case when this culture powder was diluted at a proportion of 1:20 in the pre-enrichment broth.

These results clearly demonstrate that the presence of large numbers of probiotic bacteria in a sample may interfere with the detection of *Salmonella*. It is conceivable that the detection of other microorganisms will pose similar problems, in particular when methods are used that are based on a non-selective pre-enrichment. The examples presented in this paper show that this problem can be overcome by modification of the composition of the pre-enrichment broth, i.e. by adding antimicrobial compounds to selectively suppress the growth and/or metabolic activity of the probiotic bacteria. It is obvious that such modified pre-enrichment broths can not be referred to as being non-selective, but apparently they still allow the detection of sublethally stressed *Salmonella*. It

Table 2
Detection of *Salmonella* in deliberately contaminated infant formula and premixes containing probiotic bacteria using BPW+vancomycin (10 µg/ml) as pre-enrichment medium¹

| Matrix | Probiotics | | Spiked with | Recovery |
|--|--|--|-----------------------|----------|
| | Strain(s) | Level (cfu/g) | | |
| Infant formula | – | – | <i>S. typhimurium</i> | 3/3 |
| | | | <i>S. goldcoast</i> | 3/3 |
| | | | <i>S. panama</i> | 10/10 |
| | <i>S. thermophilus</i> NCC 2496 | 10 ⁷ | <i>S. goldcoast</i> | 3/3 |
| | <i>S. thermophilus</i> NCC 2496 | 10 ⁷ | <i>S. typhimurium</i> | 3/3 |
| | <i>B. lactis</i> NCC 2818 | 10 ⁷ | <i>S. goldcoast</i> | 3/3 |
| | <i>B. lactis</i> NCC 2818 | 10 ⁷ | <i>S. typhimurium</i> | 3/3 |
| | <i>B. longum</i> NCC 3001 | 10 ⁷ | <i>S. goldcoast</i> | 3/3 |
| | <i>B. longum</i> NCC 3001 | 10 ⁷ | <i>S. typhimurium</i> | 3/3 |
| | <i>L. johnsonii</i> NCC 533 | 10 ⁷ | <i>S. goldcoast</i> | 3/3 |
| | <i>L. johnsonii</i> NCC 533 | 10 ⁷ | <i>S. typhimurium</i> | 3/3 |
| Maltodextrin premix | <i>B. lactis</i> NCC 2818 and <i>S. thermophilus</i> NCC 2496 | 10 ⁷ and 10 ⁷ , respectively | <i>S. panama</i> | 10/10 |
| | – | – | <i>S. panama</i> | 10/10 |
| | | | <i>S. goldcoast</i> | 6/6 |
| | | | <i>S. typhimurium</i> | 6/6 |
| | <i>B. lactis</i> NCC 2818 | 7 × 10 ¹⁰ | <i>S. goldcoast</i> | 0/3 |
| | | | <i>S. typhimurium</i> | 0/3 |
| | <i>B. longum</i> NCC 3001 | 1.2 × 10 ¹⁰ | <i>S. goldcoast</i> | 1/3 |
| | | | <i>S. typhimurium</i> | 2/3 |
| | <i>B. longum</i> NCC 3001 and <i>S. thermophilus</i> NCC 2496 | 1.2 × 10 ¹⁰ and 3.6 × 10 ¹⁰ , respectively | <i>S. goldcoast</i> | 0/3 |
| | | | <i>S. typhimurium</i> | 0/3 |
| | <i>B. longum</i> NCC 3001 and <i>L. paracasei</i> NCC 2461 | 1.2 × 10 ¹⁰ and 2.2 × 10 ¹⁰ , respectively | <i>S. goldcoast</i> | 2/3 |
| | | <i>S. typhimurium</i> | 2/3 | |
| <i>B. longum</i> NCC 3001 and <i>L. rhamnosus</i> NCC 4007 | 1.2 × 10 ¹⁰ and 2 × 10 ¹⁰ , respectively | <i>S. panama</i> | 1/10 | |
| | | <i>S. goldcoast</i> | 1/3 | |
| | | <i>S. typhimurium</i> | 1/3 | |

Table 3

Detection of *Salmonella* in deliberately contaminated premixes and culture powders of probiotic bacteria using double strength BPW+vancomycin (10 µg/ml), malachite green (100 µg/ml) and non-fat dry milk powder (10 g/l) as pre-enrichment medium

| Matrix | Probiotics | | Dilution | Spiked with | Recovery | |
|------------------------------|--|--|-----------------------|-----------------------|-----------------------|-----|
| | Strain(s) | Level (cfu/g) ⁴ | | | | |
| Maltodextrin | – | – | 1:10 | <i>S. typhimurium</i> | 3/3 | |
| | | | | <i>S. goldcoast</i> | 6/6 | |
| | <i>B. lactis</i> NCC 2818 | 7×10^{10} | 1:10 | <i>S. goldcoast</i> | 3/3 | |
| | <i>B. longum</i> NCC 3001 | 1.2×10^{10} | 1:10 | <i>S. goldcoast</i> | 3/3 | |
| | <i>B. lactis</i> NCC 2818 and <i>S. thermophilus</i> NCC 2496 | 7×10^{10} and 3.6×10^{10} , respectively | 1:10 | <i>S. goldcoast</i> | 3/3 | |
| | <i>B. longum</i> NCC 3001 and <i>S. thermophilus</i> NCC 2496 | 1.2×10^{10} and 3.6×10^{10} , respectively | 1:10 | <i>S. typhimurium</i> | 3/3 | |
| | | | | <i>S. goldcoast</i> | 3/3 | |
| | <i>B. longum</i> NCC 3001 and <i>L. paracasei</i> NCC 2461 | 1.2×10^{10} and 2.2×10^{10} , respectively | 1:10 | <i>S. typhimurium</i> | 3/3 | |
| | | | | <i>S. goldcoast</i> | 3/3 | |
| | <i>S. thermophilus</i> NCC 2496 and <i>L. paracasei</i> NCC 2461 | 3.6×10^{10} and 2.2×10^{10} , respectively | 1:10 | <i>S. typhimurium</i> | 3/3 | |
| | | | | <i>S. goldcoast</i> | 3/3 | |
| <i>L. johnsonii</i> NCC 533 | 1×10^{10} | 1:10 | <i>S. typhimurium</i> | 3/3 | | |
| | | | <i>S. goldcoast</i> | 3/3 | | |
| Culture powder | – | – | 1:10 | <i>S. typhimurium</i> | 4/4 | |
| | | | | <i>S. abaetetuba</i> | 4/4 | |
| | | | | <i>S. goldcoast</i> | 6/6 | |
| | <i>B. lactis</i> NCC 2818 | 7×10^{11} | 1:10 | <i>S. typhimurium</i> | 0/2 | |
| | | | | <i>S. abaetetuba</i> | 0/2 | |
| | | | | <i>S. goldcoast</i> | 0/3 | |
| | | | | 1:20 | <i>S. typhimurium</i> | 2/2 |
| | | | | | <i>S. abaetetuba</i> | 2/2 |
| | | | | | <i>S. goldcoast</i> | 2/2 |
| | | | | 1:40 | <i>S. typhimurium</i> | 2/2 |
| | | <i>S. abaetetuba</i> | 2/2 | | | |
| | | <i>S. goldcoast</i> | 2/2 | | | |
| | <i>B. longum</i> NCC 3001 | 1.2×10^{11} | 1:10 | <i>S. typhimurium</i> | 2/2 | |
| | | | | <i>S. abaetetuba</i> | 2/2 | |
| | | | | <i>S. goldcoast</i> | 3/3 | |
| | <i>S. thermophilus</i> NCC 2496 | 3.6×10^{11} | 1:10 | <i>S. typhimurium</i> | 2/2 | |
| | | | | <i>S. abaetetuba</i> | 2/2 | |
| <i>S. goldcoast</i> | | | | 3/3 | | |
| <i>S. typhimurium</i> | | | | 2/2 | | |
| <i>L. rhamnosus</i> NCC 4007 | 3.5×10^{11} | 1:10 | <i>S. goldcoast</i> | 3/3 | | |
| | | | <i>S. typhimurium</i> | 3/3 | | |
| <i>L. paracasei</i> NCC 2461 | 2.2×10^{11} | 1:10 | <i>S. goldcoast</i> | 3/3 | | |

should also be emphasized that the modifications that are described here do not necessarily provide a solution for strains that were not covered by this study. It is therefore recommended that these analytical constraints are already addressed during the development phase of new probiotic products.

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