ARTICLE

Enhanced microbiological safety of acidified infant formulas tested *in vitro*

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Objectives. To determine growth behaviour of pathogens and spoilage micro-organisms in infant formulas after reconstitution, and comparison of the bacteriostatic effect of acidified formulas obtained through fermentation or by direct addition of lactic acid.

Design. Four commercially available infant formulas were deliberately contaminated with eight different pathogens and stored at 4, 25 or 37°C. Growth of the micro-organisms was followed by enumeration after 0, 3 and 6 hours. In a second challenge test the fate of pathogens added to a fermented infant formula was compared with that of pathogens in a non-fermented acidified formula.

Results. After a lag phase of a few hours, most of the examined micro-organisms grew well in the pH-neutral products at 37°C. At 25°C growth was clearly retarded and at 4°C no significant growth was detected within 6 hours. Fermented formula exerted an inhibitory effect on all micro-organisms. The same effect was observed with a non-fermented acidified formula.

Conclusions. Because pH-neutral reconstituted infant formulas may support rapid growth of many undesirable micro-organisms, including pathogens, utmost care should be taken to prevent contamination, and storage under conditions favouring growth of pathogens should be avoided. Alternatively, prevention of rapid microbial proliferation in infant formulas may be achieved by acidification, either through fermentation or by direct addition of lactic acid, provided that the pH is lower than 5.0.

Diarrhoea remains one of the leading causes of mortality among infants in developing countries. Although the estimated incidence has decreased over recent decades, it still accounts for about 1.5 million deaths per year among children under 5 years of age.[1] Very often it is caused by poor hygienic conditions and contamination of infant foods with bacterial pathogens such as enteropathogenic and enterotoxigenic strains of *Escherichia coli, Salmonella, Shigella*, etc. However, viruses (rotaviruses, caliciviruses) and parasites (in particular *Cryptospridium*) are also frequently associated with infant diarrhoea.[2,3] Breast-feeding reduces the exposures to these pathogens and also supplies the infant with protective antibodies against food-borne pathogens, resulting in a lower incidence of diarrhoea among breast-fed children, which is one of the main reasons why it is preferred to infant formula feeding.[4] If, however, infant formula has to be used, several measures can be taken to prevent contamination with and growth of pathogens. Examples of such measures include boiling the water used for reconstitution of the product, disinfecting the feeding bottles before filling, and avoiding prolonged storage at ambient temperature so that pathogens do not have time to proliferate.

Unfortunately these precautions are not always taken and the presence of additional interventions to prevent microbial contamination would increase the safety of infant formulas. Studies of traditional African weaning foods have shown that natural fermentation can serve as such an additional hurdle by impeding the growth of pathogens or even providing a bactericidal effect.[5] It has also been demonstrated that feeding biological acidified (fermented) milk-based infant formula reduced the incidence and severity of diarrhoea among children in Chile compared with a control group that received non-acidified formula.[6] However, few data are available on the growth characteristics of the major pathogens in fermented milk- or whey-based infant formula, nor is it known whether fermentation has any advantage in this respect compared with acidification through direct addition of lactic acid.

Here we describe the results of a challenge test performed on three commercially available infant formulas with a neutral pH and an acidified formula and

show that acidified products have a clear bacteriostatic effect on the major pathogens. We also show that acidification through direct addition of lactic acid provides the same degree of protection as acidification through fermentation with lactic acid bacteria.

Methods

Infant formulas

The infant formulas used for this study are listed in Table I. They were obtained from South Africa where they are commercially available, with the exception of the acidified non-fermented formulas which were produced for the purpose of this study by the Nestlé Product Technology Centre in Konolfingen, Switzerland.

Table I.	Infant formulas used in the challenge tests	
Product	**Main characteristics**	**pH***
A	Whey-adapted	6.8
B	Casein-predominant	6.8
C	Soy-based	6.9
D	Whey-adapted fermented	4.7
E	Whey-adapted Acidified, non-fermented	4.5
F	Whey-adapted Acidified, non-fermented	5.0
G	Whey-adapted Acidified, non-fermented	5.2

* The pH was measured after reconstitution of the product according to the instructions of the manufacturer using demineralised water.

Microbial cultures

Escherichia coli 0157:H7 FSM 140, 141 and 177, *Pseudomonas aeruginosa* FSM 1, 2 and 4, *Staphylococcus aureus* FSM 59, 61 and 62, *Bacillus cereus* FSM 148, C11 and C13, *Salmonella typhimurium* FSM 273, 274 and 447, *Shigella dysenteriae* FSM 1, 2 and 3, *Enterobacter sakazakii* FSM 155, 156 and 158, *Vibrio cholerae* O:139 FSM 63 and 64, *Candida albicans* FSM 172, 174 and 216 and rotavirus WA (human rotavirus serotype 1), Hochi (human rotavirus serotype 4) and SA11 (simian rotavirus corresponding to human rotavirus serotype 3) were all from the Nestlé Research Centre culture collection.

Challenge test

Bacterial strains were grown individually in brain heart infusion (BHI, Oxoid CM225) for 18 - 20 hours at 37°C. After dilution with tryptone salt (0.1% tryptone (Oxoid LR42) + 0.85% NaCl) to a concentration of approximately 10^5 CFU/ml, suspensions of strains belonging to the same species were pooled and aliquots of 1 ml were subsequently added to bottles containing 100 ml of the freshly prepared reconstituted infant formula, thus yielding an initial concentration of approximately 10^3 CFU/ml. The same procedure was followed for *Candida* except that it was grown in yeast and malt extract broth (YM, Difco 0711-17-1).

After inoculation the bottles were incubated in a water bath at 4, 25 or 37°C and growth or inactivation was monitored by enumeration after 0, 3 and 6 hours. For this purpose the following media were employed: violet red bile glucose agar (VRBG, Oxoid CM 485) for the Enterobacteriaceae, MYP (Merck 5267) for *B. cereus*, Baird-Parker agar (Oxoid CM 275) for *S. aureus*, cholera medium (TCBS, Oxoid CM 333) for *V. cholerae*, KF Streptococcus agar (Oxoid CM 701) for *E. faecalis*, DG18 (Oxoid CM729) for *C. albicans*, and *Pseudomonas* agar (Oxoid CM559) for *P. aeruginosa*. In order to determine the reproducibility of the results some of the challenge tests were performed in triplicate.

The antirotavirus activity was determined in an alpha-type neutralisation test against two rotavirus serotypes. In this test a high titred virus sample was mixed with the reconstituted infant formula sample, incubated (for 1 hour at 37°C) and then tested for residual viral infectivity on a susceptible cell line (MA104). For this test a dilution series is prepared for the formula-virus mixture, and the last dilution yielding infectious virus is determined by an immunohistological staining technique. This yields the residual viral titre. Antirotavirus activity would result in a reduction of the residual titre.

Results and discussion

Microbiological quality

Several microbiological tests were performed to verify that the starting material itself was not contaminated. The results (data not shown) demonstrate that all the infant formulas were free (< 1 CFU/ml of reconstituted product) of Enterobacteriacae, *P. aeruginosa, B. cereus, S. aureus*, enterococci, yeasts and moulds. Total plate counts were less than 50 CFU/ml.

Fate of microbial pathogens in reconstituted infant formula

In the first challenge test, four commercially available infant formulas were separately inoculated with eight different bacterial pathogens and one spoilage yeast (Fig. 1). As expected, the temperature was found to have a major effect on the behaviour of the various pathogens. At 4°C the tested pathogens apparently did not grow or die in any of the products during the 6 hours following reconstitution. At 25°C, most bacteria showed some (albeit slow) growth in the pH-neutral products, but in the fermented product 'D' no growth occurred. The yeast *C. albicans* did not grow or die off in any of the products.

At 37°C, all bacteria grew quite well in the pH-neutral products, but no or only very little growth occurred in the infant formula with a low pH. For *P. aeruginosa*, exposure to the fermented product at 37°C had a clear bactericidal effect. *C albicans* counts again showed little variation.

Exposure of rotavirus to reconstituted infant formula for 1 hour at 37°C did not lead to significant differences in the residual titre among the tested products (data not shown), suggesting that the acidified product does not have more (or less) antirotaviral activity than the pH-neutral products.
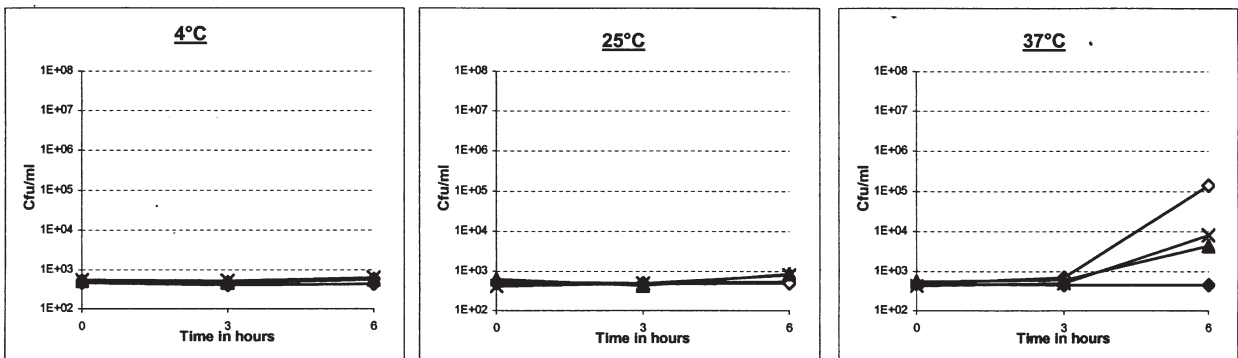
E. coli O157:H7

S. dysenteriae

S. typhimurium
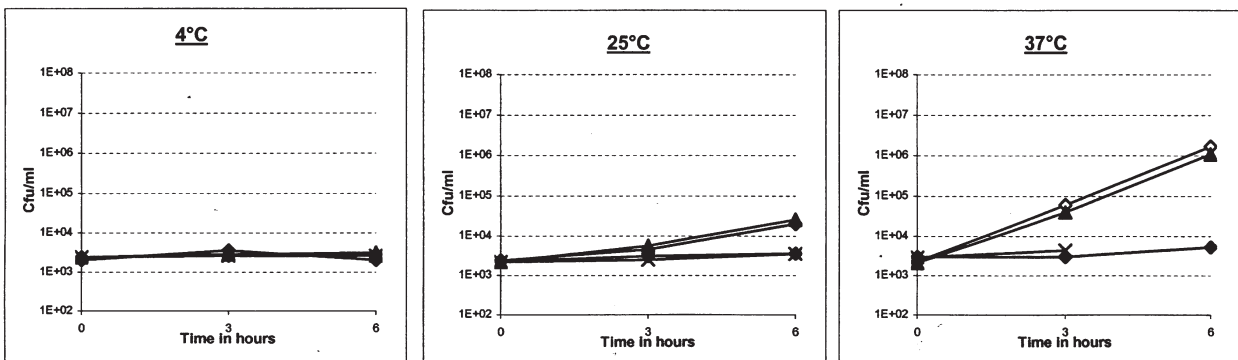

E. sakazakii
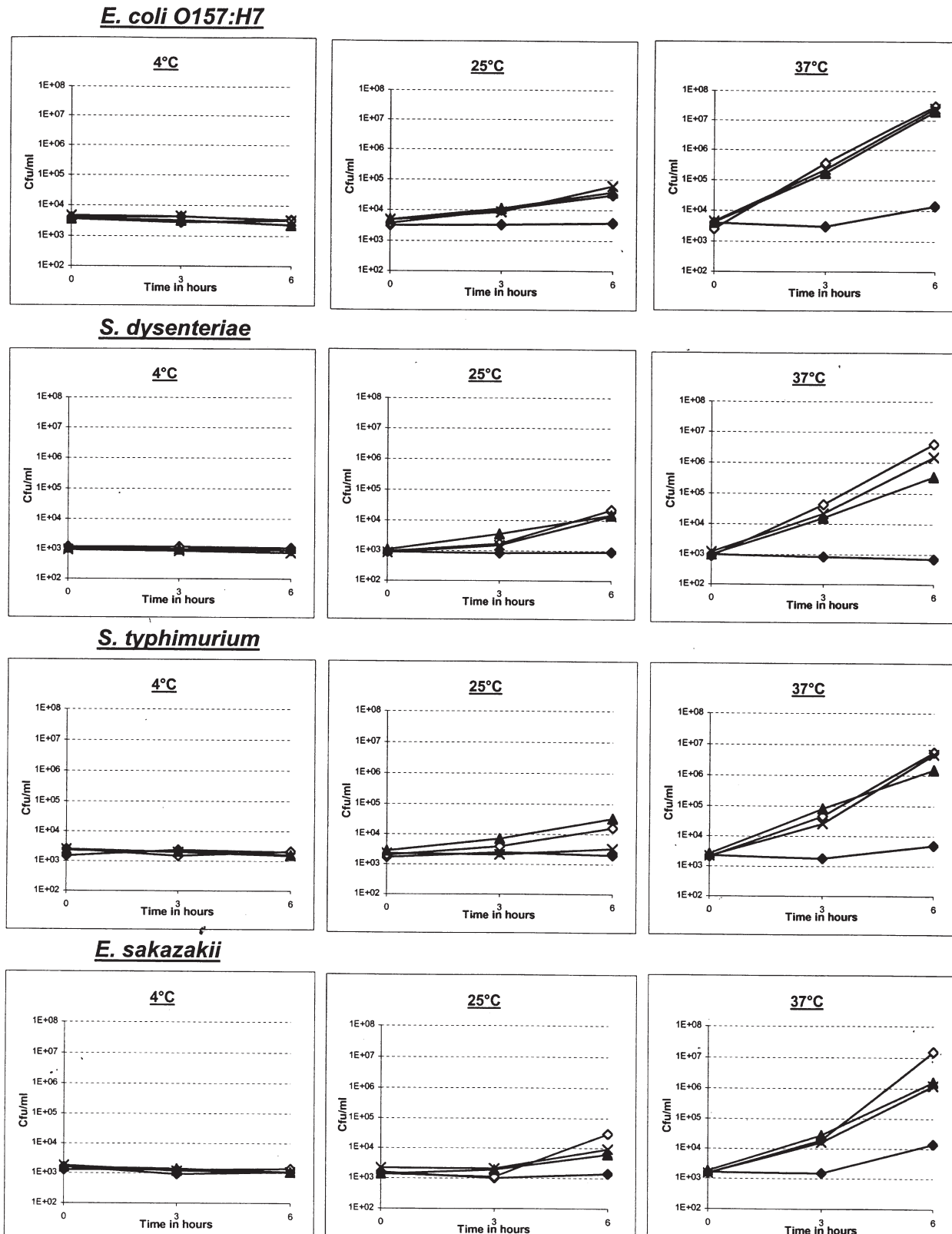
P. aeruginosa
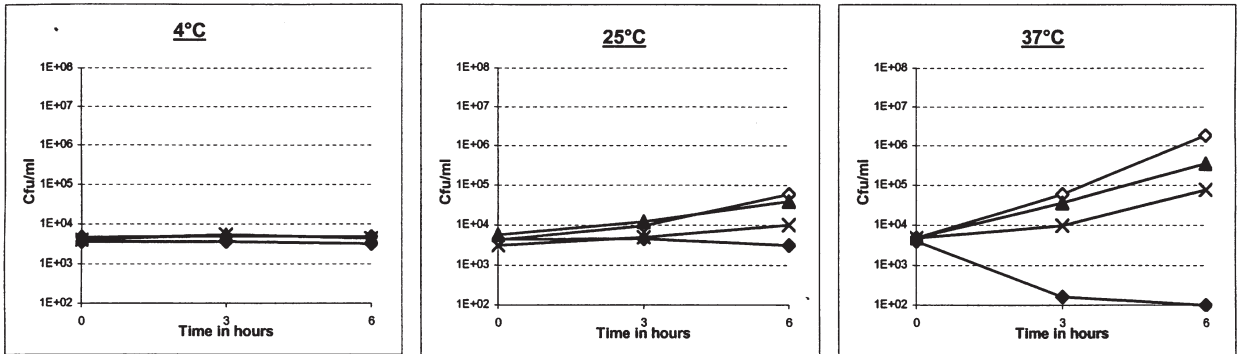
B. cereus
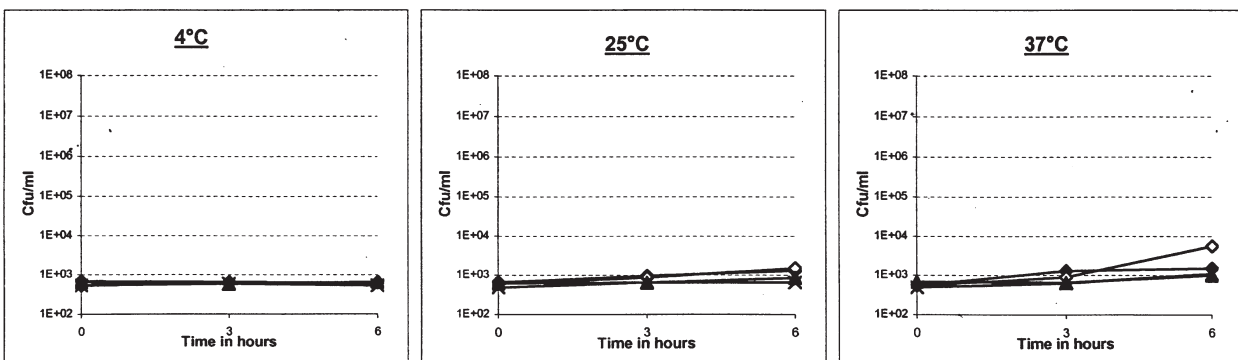

S. aureus

C. albicans

Fig. 1. Growth behaviour of pathogenic and spoilage micro-organisms in various commercially available infant formulas. Dehydrated infant formulas were reconstituted with demineralised water and deliberately contaminated with micro-organisms at a level of approximately 1 000 CFU/ml. After 3 and 6 hours' incubation at 4, 25 or 37°C the micro-organisms were enumerated. Products: ◇ 'A' (whey-adapted, pH 6.8), ▲ 'B' (casein-predominant, pH 6.8), ✕ 'C' (soy-based, pH 6.9), ♦ 'D' (whey-adapted, fermented, pH 4.7).
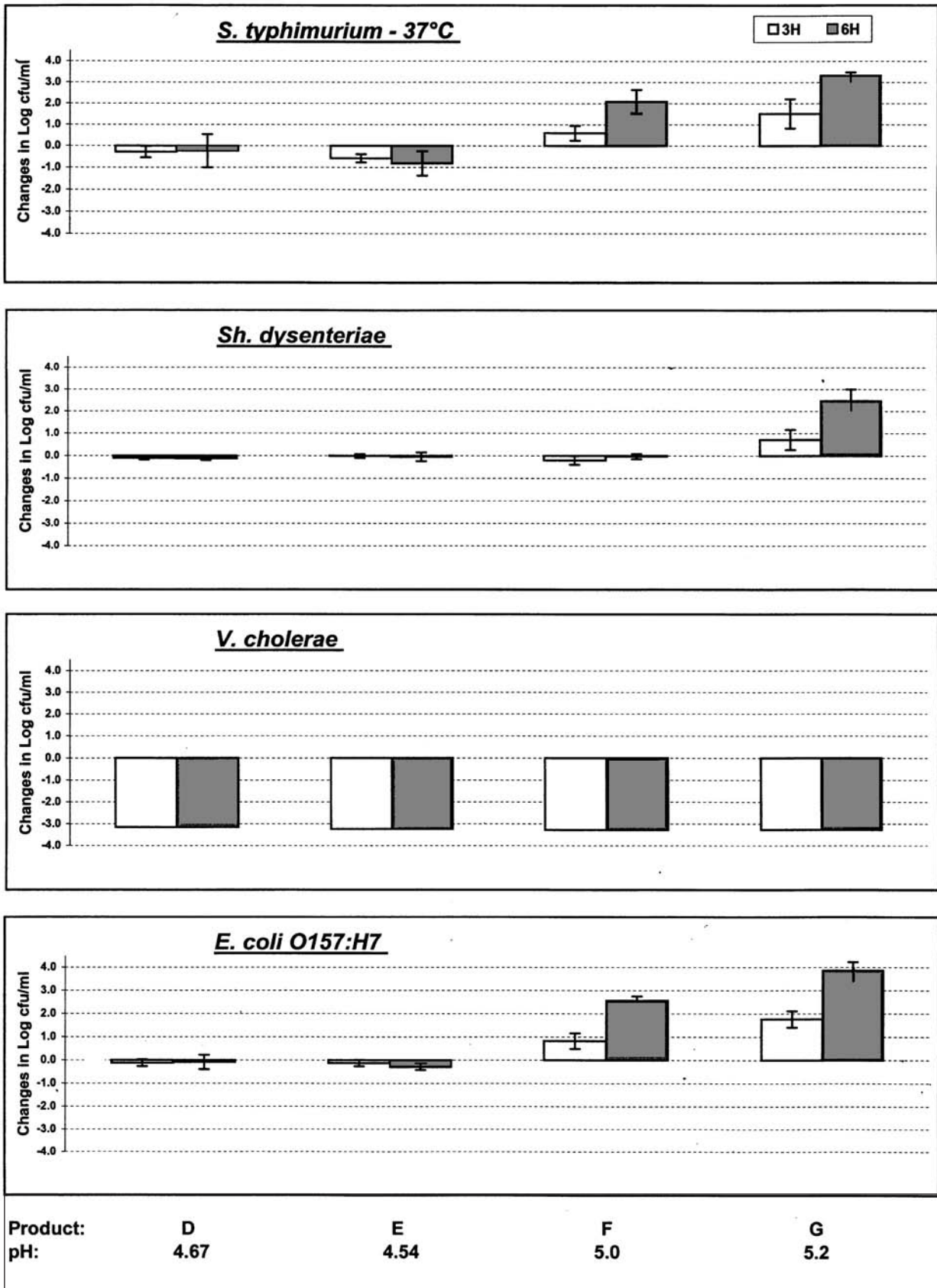
Fig. 2. Growth behaviour of pathogenic micro-organisms in acidified infant formulas obtained through fermentation with lactic acid bacteria ('D') or through direct addition of lactic acid ('E', 'F' and 'G') (log CFU/ml, mean ± standard deviation). Dehydrated infant formulas were reconstituted with demineralised water and deliberately contaminated with pathogenic bacteria at a level of approximately 1 000 CFU/ml. After 3 and 6 hours' incubation at 37°C the bacteria were enumerated. With the exception of the challenge test with Vibrio, the experiments were carried out in triplicate.

As these results showed that the fermented product had a clear bacteriostatic effect on pathogenic bacteria the question was raised whether this was solely due to the presence of lactic acid and the reduced pH or whether other inhibitory factors were involved. To address this a second challenge test was performed in which the antimicrobial properties of the fermented product (D) were compared with those of acidified non-fermented products (E, F and G).

It was found that exposure to the non-fermented acidified products or to the fermented product did not make any difference to the fate of the pathogens (Fig. 2). The Enterobacteriaceae (*S. typhimurium, Sh. dysenteriae* and *E. coli* O157:H7) resisted fairly well under the conditions tested (6 hours at 25 and 37°C), but for *V. cholerae* the exposure to this environment quickly became lethal at both temperatures. Within 3 hours it was already no longer detectable, which is in agreement with the reported acid sensitivity of this species.[7]

In the same experiment the influence of the pH was further investigated; it was found that already at pH 5.0 (product F) *Salmonella* and *E. coli* O157 were no longer inhibited, whereas *Shigella* started to grow at pH 5.2 (product G). For *Vibrio*, on the other hand, this pH was still bactericidal.

Conclusions

This study shows that pH-neutral formulas may support rapid growth of enteric pathogens when stored at 25 or 37°C after reconstitution. To prevent the risk of such products becoming hazardous, they should only be stored for a short time or under refrigeration. For acidified formulas, the most relevant pathogens cannot grow at a pH of 5.0 or lower. Products that have been acidified through fermentation with lactic acid bacteria have similar bacteriostatic properties to formulas that have been acidified through direct addition of lactic acid and both may provide a safe alternative for the feeding of infants in situations where breast-feeding may not be possible. However, further work is necessary to confirm the clinical relevance of these findings.

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