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Effect of accelerated natural lactic fermentation of infant food ingredients on some pathogenic microorganisms

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Accelerated natural lactic fermentation in mixtures of water and ground sorghum, millet and pigeon pea was obtained by gradual selection of lactic acid bacteria, through inoculum recycling. Weaning food prepared from ingredients fermented this way, contained approx. 0.7% lactic and 0.05% acetic acids and had a pH of about 3.8. In porridges, a pH of ≤ 4.0 was required to cause death of *Salmonella typhimurium* and *Staphylococcus aureus*. Several intestinal pathogenic bacteria were inoculated into sour porridge. The most resistant *Salmonella* sp. died at a rate of 1.2 log cycle/h; the most resistant *Shigella* sp. at 0.9 log cycle/h; and the most resistant *Escherichia coli* strain at 0.6 log cycle/h. A yeast, *Candida albicans*, could grow well in the sour product, whereas a bacteriophage (MS-2) was inactivated at a rate of 0.1 log cycle/h. In the acid-sensitive bacterial cultures, no gradual adaptation to acid environments could be observed. The survival studies were carried out at 30 °C.

Key words: Infant food; Weaning food; Lactic acid fermentation; *Salmonella*; *Shigella*; *Escherichia coli*; Pathogen; Diarrhea

Introduction

It has been estimated (Evans, 1986) that over 15 million infants and children below 5 years die annually in the tropical regions of the world. A major role in child mortality is played by combinations of diarrheal diseases, nutrient malabsorption and malnutrition. Based on 1980 data, it was estimated (Snyder and Merson, 1982) that acute diarrheal diseases accounted for approx. 10^9 episodes and 4.6 million deaths of children aged under 5 years in the developing world.

In addition to bacterial intestinal pathogens causing infectious diarrhea, infantile gastroenteritis is associated with viral (e.g. HumanRotaVirus) infections (Middleton et al., 1977). Also, parasitic infections (Itotia et al., 1978) may cause chronic or acute

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diarrhea (Oyerinde et al., 1977; Oyerinde et al., 1979) or may contribute to malnutrition (Stephenson et al., 1980). Although the genera *Salmonella, Campylobacter, Shigella, Vibrio, Yersinia* and *Escherichia* are mostly associated (Snyder and Merson, 1982; Itotia et al., 1978; Agbonlahor and Odugbemi, 1982) with bacterial diarrheal disorders, other enterotoxigenic genera including *Pseudomonas, Enterobacter, Klebsiella, Serratia, Proteus, Providencia, Aeromonas, Achromobacter* and *Flavobacterium* have also been implied (Jiwa et al., 1981).

That food and food handlers play an important role as vectors for infective diarrhea has been demonstrated (Itotia et al., 1978; Jiwa et al., 1981; Rowland et al., 1978).

Most weaning food is prepared by boiling the ingredients in water. Although the majority of microbial contaminants will be inactivated as a result, heat-resistant bacterial endospores may survive. Even more important, recontamination of the prepared weaning food is very likely to occur from utensils, handling, insects, faeces, etc. before it is consumed (Rowland et al., 1978). Whether this contamination pressure from the environment will lead to microbial growth and turn wholesome food into a health hazard, depends on: the nature of the contamination, the environment created by the food and the storage conditions.

Rowland et al. (1978) stressed that weaning foods for tropical use must be 'reasonably resistant to bacterial overgrowth for at least the 1–2 h when the infant is likely to be feeding'. The formulation of weaning foods according to the nutritional requirements of 9–12 months old children has been described elsewhere (Nout et al., 1989).

Recycling of inoculum achieves a natural selection of lactic acid bacteria (Nout et al., 1987b) in fermenting mixtures of cereals, tubers, legumes, and water (Nout et al., 1989). Acidification results in pH $\leq$ 4.0 within 8–10 h at 25–30 °C. If children's food (porridge) is prepared from such fermented material, a porridge pH of $\leq$ 4.0 can be realized with most weaning food ingredients. The microbiological stability of acidified porridge has been mentioned earlier (Nout et al., 1989), using strains of *Salmonella typhimurium* and *Staphylococcus aureus* as test organisms. Antimicrobial influences in lactic fermented foods can be due to the production of various compounds, including organic acids, H_2O_2 and antibiotic-like substances (Gibbs, 1987). The present paper deals with the mechanism of the microbiological stability of fermented cereal porridge, and the behaviour of a number of pathogens responsible for gastroenteritis and closely related diseases.

Materials and Methods

Ingredients

Cereals and legumes used included white sorghum (*Sorghum bicolor* ssp. *caffrorum*) and Japanese millet (*Echinodoa frumentacea*); and pigeon pea (*Cajanus cajan*), respectively. Malt was prepared from white sorghum as described below.

Microbial cultures

Candida albicans 240, *Citrobacter* 3465/69, *Enterobacter cloacae, Escherichia coli* strains 055, 0111, 0125, and 0126, *Salmonella abony* KO 10, *S. branderup* No. 49, *S. eastbourne, S. havana* No. 134, *S. senftenberg* KO 36 (group E), *S. typhi* KO 120, *S. typhimurium* II 505 (resistant to 200 ppm nalidixic acid), *S. zanzibar* No. 82, *Shigella boydii* 748, *Sh. dysenteriae* II ALM 59 (var. *Schmitzii*), *Sh. flexneri* II 850143, *Sh. flexneri* III 850161, *Sh. sonnei* 763, *Staphylococcus aureus* S6 (SEA, SEB), *Yersinia enterocolitica* P1272/11 6.30 + and phage MS-2 (F-specific RNA phage; host strain *Salmonella typhimurium* WG-49 phage-type 3 Nal^R F′lac : Tn5) were from the culture collection of the National Institute of Public Health and Environmental Protection, Bilthoven, The Netherlands. *Citrobacter, Enterobacter, Escherichia, Salmonella* except *S. typhimurium, Shigella, Staphylococcus*, and *Yersinia* were grown and maintained on slopes of Nutrient Agar (NA, Oxoid CM 3), and *Candida* on Malt Extract Agar (MEA, Oxoid CM59). *S. typhimurium* was grown and maintained in Brain Heart Infusion broth (BHI, Gibco, U.K., No. 152-0680) with 200 ppm nalidixic acid (Sterling-Winthrop, Guildford, U.K.). Phage MS-2 was maintained at −70 °C in Tryptone Yeast Extract Glucose broth (TYG, prepared with trypticase peptone (BBL, 11921) 8 g, Yeast Extract (Difco, 0127) 0.8 g, NaCl (Merck, 6404) 6.4 g in 800 ml distilled water, pH 7.2, sterilized 15 min at 121 °C, followed by addition of 8 ml of filter sterilized (0.2 μm) solution of $CaCl_2 \cdot 2H_2O$ (Merck, 2382) 3 g, and glucose (Difco, 0155) 10 g, in 100 ml distilled water) to which 20% glycerol (Merck, 4094) was added.

Preparation of ingredients

Cereals and pigeon pea were cleaned dry and ground to whole-grain meals using a laboratory impact mill (Pallmann type REF L18, Zweibrücken, F.R.G.). Malt was prepared from white sorghum by subsequently soaking 6 h at 20 °C, discarding the water, aerating 15 min, rinsing with fresh tapwater, soaking 18 h at 20 °C, discarding the water, rinsing the grain and spreading it 1 cm thick on a wet cloth, germinating 3 days at 20 °C until root length reached 1.5 cm, drying in a hot air oven at 50 °C for 2 h, removing rootlets by sieving and grinding the malted grain by impact milling.

Composition of formulated porridges:

We used two porridges, each designed to contain 4.20 kJ energy/ml and a minimum of 7.8% net protein energy in order to cover the nutritional demands of children of the second semester (6–12 months). Formula 1 contained 23% ground Japanese millet, 4.3% ground pigeon pea, 1% ground sorghum malt, and 71.7% water. Formula 2 contained 22.8% ground white sorghum, 4.6% ground pigeon pea, 1% ground sorghum malt, and 71.6% water (percentages on weight basis).

Accelerated natural lactic fermentation

The principle of gradual selection of lactic acid bacteria by recycling of inoculum was described earlier (Nout et al., 1987b). Mixtures of flour and tapwater (40 : 60, w/w) were incubated at 30 °C. Fermentation cycles were 24 h. At the start of each new cycle, material of the previously fermented batch was added as a starter at an inoculum rate of 10%. The fermented mixture is referred to as 'concentrate'.

Preparation of porridge

Using the above-mentioned formulas as a basis, an appropriate volume of tapwater was brought to a vigorous boil and a corresponding quantity of concentrate was added gradually while stirring to avoid lumps. After boiling 10 min at 100 °C, the viscous mass was allowed to cool to 45 °–55 °C; subsequently, the specified weight of malt was added and mixed by stirring. Liquefaction was allowed for 30 min and the product was boiled again 5 min to reduce the microbial load introduced by the malt, to inactivate the malt enzymes and to remove the gritty mouthfeel otherwise caused by the uncooked malt, and allowed to cool. The resulting final product is referred to as 'porridge'.

Determination of death kinetics

Batches of porridge were prepared as described above and distributed in 100 g portions in polyethylene bags which were heat-sealed and stored at −20 °C until use. After defrosting and warming up till 30 °C, porridges were inoculated with appropriate aliquots of BHI-cultures of the tested bacteria, Malt Extract broth (ME, Oxoid CM57) cultures of *Candida*, or phage MS-2 suspension in TYG broth. Subsequently, the inoculum was distributed through the porridge by mixing in a Colworth type 400 stomacher. Determination of viable cells or phages was carried out immediately after inoculation and after appropriate periods of incubation at 30 °C.

Analysis

Sampling: samples for chemical and microbiological analysis were 50 g and 10 g, respectively. *pH*: A pH meter (Electrofact, Sweden) with Schott N61 electrode was used. *Titratable acidity*: 10 g sample was mixed with 90 ml distilled water and 0.1000 M NaOH was added up to pH 8.50, while stirring continuously. After 10 min stirring, more NaOH was added to adjust the pH to 8.50, and this was repeated after another 10 min stirring. Acidity was reported as follows: ml NaOH $\times 10^{-3} \times 90 =$ % (w/w) lactic acid. *Organic acids*: L(+)-lactic acid and D(−)-lactic acid were determined by enzyme assay (Cat. No. 139084, Boehringer, Mannheim, F.R.G.). Acetic acid was also determined by enzyme assay (Cat. No. 148261, Boehringer, Mannheim, F.R.G.). In addition, lactic and acetic acids were determined by HPLC (SpectraPhysics SP8000) after clarification with Carrez A (0.1 M $K_4Fe(CN)_6 \cdot 3H_2O$)

and B (0.2 M $ZnSO_4 \cdot 7H_2O$) solutions. Eluent used was 0.01 N H_2SO_4 at 30 °C and 80 bar, precolumn AG50W/X4 (BioRad), column HPX-87-H (BioRad), and detector RI2 × shodex RI SE-61.

Microbiological analysis: Sample preparation and determinations of total aerobic count, yeasts and lactic acid bacteria were as described earlier (Nout et al., 1987a). In all enumerations of Enterobacteriaceae, resuscitation on solid medium was carried out by spreading 0.1 ml of appropriate sample dilution on thin Tryptone Soya Agar (TSA, Oxoid CM 131) plates and incubating 2 h at 37 °C. Subsequently, a thick overlay of Violet Red Bile Glucose (VRBG, Oxoid CM485) agar was applied followed by incubation for 24 h at 37 °C. Enumeration of the nalidixic acid resistant strain of *Salmonella typhimurium* was carried out in pour plates of Plate Count Agar (PCA, Oxoid CM 325) containing 200 ppm nalidixic acid at 37 °C for 24 h. If sublethal injury was expected, resuscitation was carried out by spreading 0.1 ml diluted sample on thin Tryptone Soya Agar plates (TSA, Oxoid CM 131) and incubating 2 h at 37 °C. Subsequently, a thick overlay of PCA + 200 ppm nalidixic acid and incubation at 37 °C for 24 h was applied. If numbers of *S. typhimurium* were below the detection level of the pour plates with PCA, a qualitative method was used to detect the presence or absence of viable cells. In that case, pre-enrichment from 25 g sample in 225 ml Buffered Peptone Water (BPW, Oxoid CM 509) for 20 h at 37 °C was followed by selective enrichment of 0.1 ml BPW culture in 100 ml Rappaport Vassiliadis medium (RV, Oxoid CM 669) with 150 ppm nalidixic acid for 48 h at 42.5 °C and surface streaking of plates of Brilliant Green Agar (BGA, Oxoid CM 329) after 24 h and 48 h. BGA was incubated for 24 h at 37 °C. Enumeration of *Staphylococcus aureus* using Baird-Parker medium was carried out as reported elsewhere (Nout et al., 1988). Plaque-forming units of phage MS-2 were determined after 18 h incubation at 37 °C of Tryptone Yeast Extract 2% Agar (TYG with 2% agar (Sobigel)) plates, with an overlay consisting of a mixture of 2.5 ml Tryptone Yeast Extract Glucose 1% Agar (TYG with 1% agar (Sobigel)), 1 ml host suspension containing $1-3 \cdot 10^8$/ml *Salmonella typhimurium* WG-49 in TYG broth, and 1 ml of sample dilution. Reporting of counts and detection limits were described earlier (Nout et al., 1987a).

Results and Discussion

The simple method of recycling inoculum has long been known and is still used at present in, for example, the maintenance of sourdough. The establishment of a stable microflora in sourdough through natural selection is well documented (Sugihara, 1985). Recently, we found (Nout et al., 1987b) that this recycling technique can be employed successfully to enhance the pre-fermentation of soyabeans for tempe manufacture, resulting in improved bacteriological safety and acceptability of tempe. Table I summarizes the changes in microbiological composition and acidity when the recycling inoculum method is applied to the fermentation of a mixture of sorghum and pigeon pea meals in water. Compared with a spontaneous fermentation occurring during overnight incubation (Table I, situation

TABLE I

Fermentation of sorghum/pigeon pea porridge base (33.3% sorghum, 6.7% pigeon pea, 60.0% water), by recycling of inoculum (incubation at 30 °C, cycles of 24 h, inoculum rate 10%)

Fermentation cycle	pH	Total aerobic bacteria	Lactic acid bacteria	Yeasts	Enterobacteriaceae
0 [a]	6.3	5.95 [c]	4.67	3.65	4.15
1 [b]	4.8	9.69	9.61	7.17	5.98
2	3.9	9.24	9.18	5.74	< 2.70
3	3.9	9.34	9.31	6.51	< 2.70
4	3.8	9.47	9.41	7.84	< 2.70
5	3.7	9.67	9.61	7.23	< 2.70
6	3.7	9.15	9.61	6.78	< 2.70

[a] Measured at the start of the first cycle.
[b] Measured at the end of cycle 1, 2, 3, etc.
[c] Counts expressed as ^{10}log N/g porridge base.

at the end of the first cycle), repeated recycling of inoculum results in an accelerated acidification of inoculated fresh batches after 2–4 recyclings. Due to the dominance of lactic acid bacteria and rapid acidification, Enterobacteriaceae are absent in the fermented material, even though they are introduced with each new batch of cereal and legume meal.

To determine the effect of the extent of fermentation on the microbiological stability of weaning food, porridge was prepared from different ratios of fermented and non-fermented ingredients. Table II shows that *Salmonella typhimurium* and

TABLE II

Influence of initial pH of millet/pigeon pea (formula 1) on microbiological stability in challenge tests with *Salmonella typhimurium* and *Staphylococcus aureus* at 30 °C

Ratio F/NF [a]:	0/100	40/60	60/40	80/20	100/0
Initial pH [b]:	6.00 [c]	5.08	4.16	3.96	3.82
S. typhimurium					
t_0 (initial)	4.44	4.92	4.44	4.74	4.44
t_{10h}	7.70	5.39	3.77	< 2.4 + [d]	< 2.4 +
t_{24h}	8.06	7.52	< 2.4 +	– [d]	–
t_{48h}	8.10	7.50	–	–	–
S. aureus					
t_0 (initial)	4.44	4.44	4.44	4.44	4.44
t_{10h}	7.22	6.04	3.59	2.44	< 2.4
t_{24h}	8.53	7.96	< 2.4	< 2.4	< 2.4

[a] F/NF = fermented/non-fermented millet/pigeon pea base.
[b] Obtained by cooking porridge from different mixing ratios of fermented (pH 3.82) and non-fermented (pH 6.00) concentrates.
[c] Counts expressed as ^{10}log N/g porridge.
[d] + presence still detectable by liquid selective enrichment, – absent by liquid selective enrichment.

TABLE III

Effect of non-fermentative and fermentative acidification and dehydration on antimicrobial properties of sorghum/pigeon pea (formula 2) at 30 °C

^{10}Log N/g porridge	Non-fermented with added acids (fresh)	Fermented	
		Fresh	Dehydrated reconstituted
Salmonella typhimurium II 505			
t_0	7.00	7.24	7.15
t_{3h}	3.59	3.04	2.80
t_{6h}	3.00	< 2.70	< 2.70
t_{24h}	< 2.70	< 2.70	< 2.70
Yersinia enterocolitica P 1272/11 6.30+			
t_0	7.32	7.19	7.26
t_{3h}	3.30	4.74	< 2.70
t_{6h}	< 2.70	< 2.70	< 2.70
Escherichia coli			
t_0	7.34	7.26	7.15
$t_{2.5h}$	6.86	7.04	7.09
t_{5h}	6.26	6.35	6.69
t_{7h}	4.59	4.57	4.34
t_{24h}	< 2.70	< 2.70	< 2.70
Citrobacter 3465/69			
t_0	7.03	7.15	6.18
t_{3h}	3.33	< 2.70	2.81
t_{6h}	< 2.70	< 2.70	< 2.70
Enterobacter cloacae			
t_0	7.18	7.10	7.18
t_{3h}	< 2.70	< 2.70	2.78

Staphylococcus aureus could grow very well in porridges of pH 6.00 made with 100% non-fermented ingredients, as could be expected. The higher the proportion of fermented ingredient base, the lower was the porridge pH after preparation. At pH $\leq$ 4.16, porridges caused a reduction of inoculated salmonellae and staphylococci. The highest anti-microbial activity was found in porridges of pH $\leq$ 3.96. These pH levels could only be obtained using the system of accelerated natural lactic fermentation (Table I).

We investigated whether anti-microbial effects other than acidity were present in the fermented ingredients by simulating the conditions of the fermented material in non-fermented ingredients by the addition of 0.7% (w/w) lactic and 0.05% (w/w) acetic acids and adjustment of the pH to 3.80 corresponding to the results obtained by analysis of the fermented ingredients. Table III shows the effect of acidity in sorghum/pigeon pea porridges on selected bacterial cultures. There appeared to be no significant difference between their behaviour in fermented porridge or acid-supplemented non-fermented porridge, which implies that the anti-microbial effect is

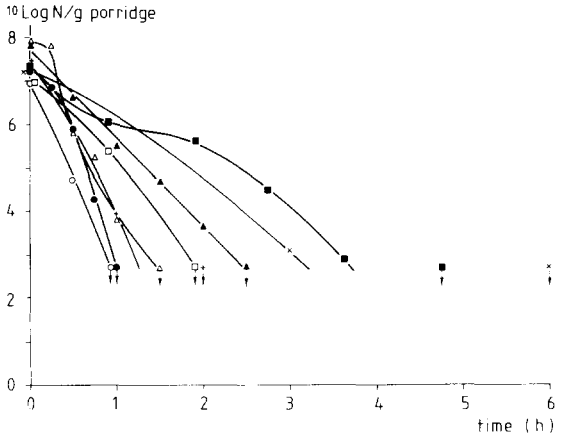

Fig. 1. Death kinetics of *Salmonella* strains inoculated into porridge (formula 2, pH 3.80) made from fermented sorghum/pigeon pea and incubated at 30 °C. *Salmonella abony* ●———●, *Salmonella branderup* ○———○, *Salmonella eastbourne* ▲———▲, *Salmonella havana* △———△, *Salmonella senftenberg* ■———■, *Salmonella typhi* □———□, *Salmonella typhimurium* ×———×, *Salmonella zanzibar* +———+.

due to the presence of lactic and acetic acids at reduced pH, and that other anti-microbial substances did not play a detectable role.

Cooked fermented porridge was dehydrated by drum-drying and reconstituted with tapwater at 30 °C to the original porridge dry matter content. No significant influence of this treatment on the death rate of the inoculated bacteria was observed, which indicates that losses of organic acids due to evaporation during drum-drying are negligible. Since some loss of volatile fatty acids, e.g. acetic acid had been expected, chemical analysis of organic acids in fresh and drum-dried porridge was carried out by HPLC. It was confirmed that there are no significant losses of organic (i.e. lactic and acetic) acids resulting from the drum-drying process (data not included).

Figs. 1–3 show the destruction curves in sorghum/pigeon pea porridge (pH 3.80) at 30 °C of a number of bacteria associated with acute diarrhea. In Fig. 1, *Salmonella* species are shown to vary with respect to death rate and shape of the death curve. The most resistant strain, *S. senftenberg*, died at a rate of approx. 1.2 log cycle/h. Fig. 2 illustrates the behaviour of some *Shigella* strains. Except for the sensitive *Sh. dysenteriae* strain, the other shigellas had a death-curve with a slow on-set followed by a higher death rate. The most resistant strains died at an average rate of 0.9 log cycle/h. In Fig. 3, strains of *Citrobacter, Escherichia coli* and *Yersinia enterocolitica* are shown. *E. coli* strains 055 and 0111 were most resistant (average rate of death of strain 0111 was 0.6 log cycle/h) and showed curves similar to the *Shigella* strains.

Fig. 4 shows that the tested yeast *Candida albicans* was perfectly able to grow at 30 °C in the sour porridge, as would be expected. The same figure also illustrates the fate of bacteriophage MS-2 in the fermented porridge, as well as in a neutral,

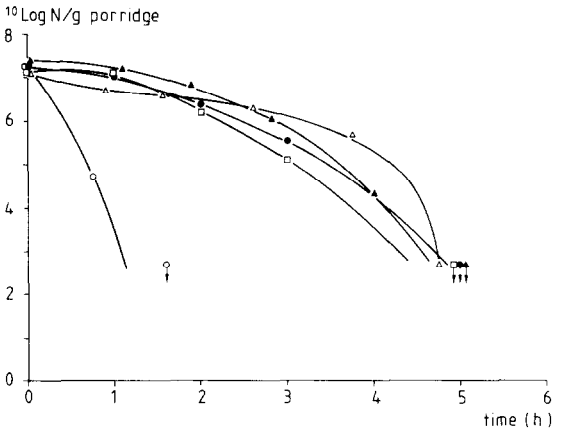

Fig. 2. Death kinetics of *Shigella* strains inoculated into porridge (formula 2, pH 3.80) made from fermented sorghum/pigeon pea and incubated at 30 °C. *Shigella boydii* ●———●, *Shigella dysenteriae* ○———○, *Shigella flexneri* II ▲———▲, *Shigella flexneri* III △———△, *Shigella sonnei* □———□.

non-fermented control porridge. The acid conditions resulted in a (slow) inactivation rate of approx. 0.1 log cycle/h. Although it is not known whether the behaviour of this phage is representative for other viruses, this result indicates a slight viricidal activity of fermented porridge. It should be realized that several viruses (e.g. the enteroviruses) are less sensitive to low pH.

All bacteria died rather quickly in the sour porridge. With *E. coli* 0125, *E. coli* 0126 and *Shigella flexneri* II we investigated whether a gradual adaptation to the

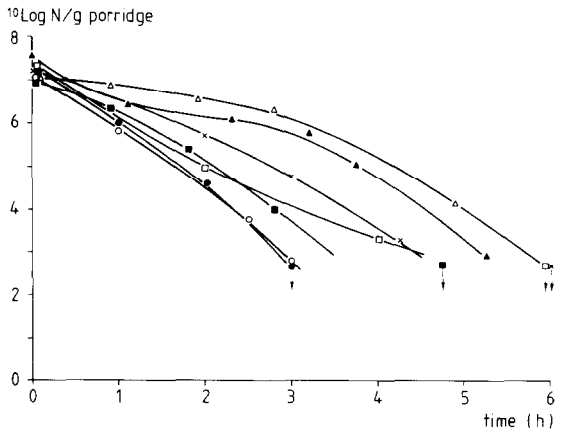

Fig. 3. Death kinetics of *Citrobacter, Enterobacter cloacae, Escherichia coli* and *Yersinia enterocolitica* inoculated into porridge (formula 2, pH 3.80) made from fermented sorghum/pigeon pea and incubated at 30 °C. *Citrobacter* ●———●, *Enterobacter cloacae* ○———○, *Escherichia coli* 055 ▲———▲, *Escherichia coli* 0111 △———△, *Escherichia coli* 0125 ■———■, *Escherichia coli 0126* □———□, *Yersinia enterocolitica* ×———×.

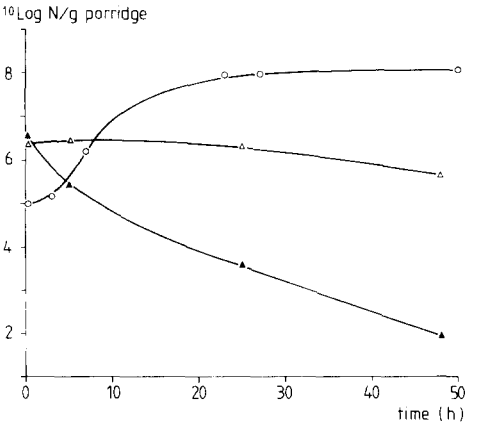

Fig. 4. Fate of *Candida albicans* and phage MS-2 inoculated into porridge (formula 2, pH 3.80) made from fermented sorghum/pigeon pea and incubated at 30 °C. *Candida albicans* ○———○, Phage MS-2 ▲———▲, Phage MS-2 in control (pH 6.00) porridge △———△.

acid conditions could be achieved. This was done by culturing the tested strains in successive porridges in which increasing proportions of fermented ingredients were used. Since the strains died in porridges of pH $\leq$ 4.0 at repeated attempts (data not included), we concluded that there is little risk of the development of acid-resistant adaptations. Our results show that porridges of pH $\leq$ 4.0 made with lactic fermented ingredients have significant antimicrobial properties through which they might contribute to the reduction of the foodborne infection pressure at the household level.

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