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International Journal of Food Microbiology

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[https://doi.org/10.1016/0168-1605\(91\)90072-w](https://doi.org/10.1016/0168-1605(91)90072-w)

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FOOD 00380

Ecology of accelerated natural lactic fermentation of sorghum-based infant food formulas

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(Received 18 June 1990; accepted 25 October 1990)

Accelerated natural lactic fermentation of mixed sorghum-cowpea (Formula 1) and sorghum-milkpowder (Formula 2) infant food formulas was achieved by repetitive (60 fermentation cycles of 24 h each) use of the previous batch as an inoculum at a rate of 10% (w/w) and resulted in a gradual establishment of mixed populations of lactic acid bacteria and yeasts. In Formula 1, early fermentation stages (fermentation cycles 1–4) were dominated by *Leuconostoc* and *Lactococcus* spp. which caused conditions inhibitive to yeasts. Probably due to micronutrient deficiencies, the former lactic acid bacteria were succeeded by a combination of *Lactobacillus plantarum* and *Candida* spp. achieving pH values 4.2–4.3. In Formula 2, *Lactobacillus acidophilus*, *Lactococcus lactis* and *Lactobacillus brevis* consistently achieved pH 3.7–3.8, thereby allowing only a minority of yeasts, mainly *Candida* and *Trichosporon* spp. None of the isolated yeasts exhibited 'killer'-activity.

Key words: Infant food; Weaning food; Lactic fermentation; Succession; Lactic acid bacteria; Yeast; Killer activity

Introduction

In tropical regions, lack of refrigeration facilities at the household level enables rapid proliferation of microbial contaminants in foods. Especially with infant food like porridge which is often prepared only once or twice daily for use during the whole day, the growth of e.g. pathogenic bacteria may contribute to acute diarrhea, which is one of the predominant causes of childhood mortality (Snyder and Merson, 1982).

Lactic fermentation of foods of plant or animal origin is a widely accepted method of preservation which may as well impart desirable sensory and nutritional properties to the fermented product (Cooke et al., 1987). Whereas lactic fermentation in e.g. sauerkraut may be achieved by allowing a lengthy succession of naturally occurring microorganisms, the use of pure culture starters under aseptic conditions has found wide application in industrial-scale fermentations. However, the use of

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natural starters such as applied in e.g. the sourdough process (Sugihara, 1985), appears more appropriate in the household and small-scale industrial processes.

Accelerated natural lactic fermentation can be achieved by repetitive use of 5–10% (w/w) of a previously fermented batch as a starter. It was shown earlier (Nout et al., 1989a) that mixtures of cereals and pulses fermented in this way have a strong antimicrobial effect towards a range of pathogenic bacteria.

During the building-up of sourdough starters (Nout and Creemers-Molenaar, 1986) and accelerated natural lactic fermentation of soya beans for tempe manufacture (Nout et al., 1987b), it was observed that the process of acidification stabilised already after 3–5 consecutive fermentation cycles. However, it took much longer (30, resp. 60 cycles) for natural selection to result in a rather stable microbiological composition.

The present investigation was carried out to identify the predominant micro-organisms involved in the accelerated natural lactic fermentation of two sorghum-based infant food formulas, and the time-scale of the natural selection involved.

Materials and Methods

Ingredients

Ingredients included white sorghum (*Sorghum bicolor* ssp. *caffrorum*), red cowpeas (*Vigna unguiculata*), and low-fat medium-heated spray-dried milk powder (CCF, Leeuwarden, The Netherlands).

Preparation of formulas

Sorghum and cowpeas were cleaned dry and ground to whole-grain meals using a toothed disc mill (Condux type LV 15 M, Condux-Werk, Wolfgang bei Hanau, F.R.G.). Formulas were designed to contain approx 13% (w/w) of metabolisable protein (Nout et al., 1989b). Formula 1 consisted per 1000 g of 830 g of sorghum meal and 170 g of cowpea meal. Formula 2 consisted per 1000 g of 920 g of sorghum meal and 80 g of milk powder.

Accelerated natural lactic fermentation of formulas

The principle of starting a stable accelerated natural lactic fermentation was described earlier (Nout et al., 1987b, 1989b). For the first fermentation cycle, a mixture of 40 g dry formula and 60 ml tapwater was incubated in a 450-ml screw-capped glass jar at 30 °C for 24 h. Further fermentation cycles also lasted 24 h at 30 °C and the mixture consisted of 36 g dry formula, 54 ml tapwater and 10 g of the previous fermentation.

Measurement of pH and titratable acidity

Sampling and determinations were as described earlier (Nout et al., 1989a).

Microbiological analysis

Enterobacteriaceae were enumerated in pour-plates of Violet Red Bile Glucose (VRBG, Oxoid CM 485) medium with overlay after incubation at 37 °C for 24 h.

Enterobacteriaceae were isolated on VRBG (37°C, 24 h), purified on Plate Count Agar (PCA, Oxoid CM 325) (37°C, 24 h) and identified using the 'API 20 E' system (API System SA, Montalieu Vercieu, France).

Lactic acid bacteria were enumerated in pour-plates of de Man, Rogosa and Sharpe (MRS) medium (Merck No. 10661, Darmstadt, F.R.G.) to which 12 g agar and 1 g natamycin ('Delvocid', Gist-brocades, Delft, The Netherlands) were added per litre, with overlay, after incubation at 30°C for 3–5 days; they were isolated and purified on MRS agar and maintained in MRS broth. Initial confirmation and grouping of lactic acid bacteria was based on Gram stain, catalase activity, growth at 15°C and 45°C in MRS medium, gas from glucose and fermentation of lactose, sucrose, arabinose, sorbitol, cellobiose, fructose and mannitol added to glucose-free MRS medium. The square root of the number of colonies on the MRS agar plate dilution chosen for enumerations were isolated, purified and further characterised using the 'API 50 CHL' system (API System SA, Montalieu Vercieu, France).

Enumeration of yeasts and filamentous fungi was in pour-plates of Oxytetracycline Glucose Yeast Extract (OGYE) agar, incubated at 25°C for 5 days (Nout et al., 1987a). Isolations and purifications were carried out on Glucose Yeast Extract agar (Oxoid, CM545).

Filamentous fungi were identified after 10 days incubation at 25°C on Malt Extract agar (MEA, Oxoid CM 59) and Czapek Dox agar (Oxoid CM 97) according to Samson and Van Reenen-Hoekstra (1988). Yeasts were grouped according to their morphology, and ability to sporulate on either V-8 agar, MEA or Yeast-MEA (malt extract 10 g, yeast extract 4 g, glucose 4 g, agar 15 g, distilled water to 1000 ml, pH $\pm$ 7.3), their ability to ferment glucose, grow at 37°C and utilise KNO₃ or lysine as sole source of nitrogen (Campbell and Duffus, 1988). Further characterisation was carried out using the 'ATB 32 C' system (API System SA, Montalieu Vercieu, France).

All isolated yeast pure cultures were tested for 'killer' activity according to Somers and Bevan (1969) on YPG-K agar containing 10 g yeast extract (Oxoid), 10 g peptone (Difco), 20 g glucose, 20 g agar, 110 ml of a 1 M Na₂HPO₄-Na₃ citrate buffer of pH 4 and 0.03 g Methylene Blue per litre of medium. Reference strains included 'sensitive' strain FL100, 'killer-K1' strain K12 and 'killer-K2' strain Levuline killer K2, and were obtained from the Institut d'Oenologie, Université de Bordeaux II, France.

Results

The pH and counts of lactic acid bacteria, yeasts and Enterobacteriaceae at the end of consecutive fermentation cycles are presented in Table I.

In Formula 1, the final pH reached a minimum of 3.7 after 3 cycles whereupon it gradually stabilised at 4.2. Throughout the experiments, the number of lactic acid bacteria remained very high (log cfu/g 9.5–9.7). At first, yeast counts were quite low but they increased after some cycles to log cfu/g 6–7. This coincided with a

TABLE I

Acidification and content of lactic acid bacteria, yeasts and Enterobacteriaceae during accelerated natural lactic fermentation of sorghum-based infant food formulas

Number of completed fermentation cycles (24 h each)	pH of fermented product		Lactic acid bacteria (log cfu/g)		Yeasts (log cfu/g)		Enterobacteriaceae (log cfu/g)	
	Form. 1 ^a	Form. 2	Form. 1	Form. 2	Form. 1	Form. 2	Form. 1	Form. 2
0 (start)	6.0	6.3	2.8	2.2	3.6	3.0	2.5	4.6
1	4.5	4.5	9.5	9.6	3.7	4.2	8.2	<1.7
2	3.9	4.2	9.7	9.8	2.7	2.1	<1.7	<1.7
3	3.7	3.9	9.7	9.6	3.0	2.2	<1.7	<1.7
4	3.9	3.8	9.5	9.8	4.5	2.1	<1.7	<1.7
13	4.1	3.8	9.6	9.6	7.1	2.5	<1.7	<1.7
27	4.3	3.8	9.6	9.8	6.4	2.5	<1.7	<1.7
60	4.2	3.7	9.5	9.7	6.7	3.8	<1.7	<1.7

^a Form. 1: 830 g sorghum meal+170 g cowpea meal; Form. 2: 920 g sorghum meal+80 g non-fat milkpowder.

TABLE II

Lactic acid bacteria isolated during accelerated natural lactic fermentation of sorghum-based infant food formulas

Number of completed fermentation cycles (24 h each)	Formula 1	Formula 2
	n ^a Species	n Species
0 (start)	4 <i>Leuconostoc dextranicum</i>	3 <i>Leuconostoc dextranicum</i>
1	6 <i>Lactobacillus plantarum</i>	7 <i>Leuconostoc dextranicum</i>
2	5 <i>Leuconostoc dextranicum</i> 6 <i>Leuconostoc lactis</i> 2 <i>Lactococcus lactis</i>	10 <i>Lactobacillus acidophilus</i> 2 5 <i>Leuconostoc dextranicum</i>
3	2 <i>Lactobacillus acidophilus</i> 2 9 <i>Leuconostoc dextranicum</i> 5 <i>Lactobacillus acidophilus</i> 2	8 <i>Leuconostoc dextranicum</i> 4 <i>Lactobacillus acidophilus</i> 2
4	10 <i>Lactococcus lactis</i> 4 <i>Leuconostoc dextranicum</i>	8 <i>Leuconostoc dextranicum</i> 5 <i>Lactobacillus acidophilus</i> 2 3 <i>Lactobacillus brevis</i> 3
13	11 <i>Leuconostoc dextranicum</i>	9 <i>Lactobacillus acidophilus</i> 1
27	12 <i>Lactobacillus plantarum</i>	9 <i>Lactobacillus acidophilus</i> 1 5 <i>Lactococcus lactis</i>
60	12 <i>Lactobacillus plantarum</i>	11 <i>Lactobacillus acidophilus</i> 1 4 <i>Lactobacillus brevis</i> 3

^a n, number of isolates.

TABLE III

Yeasts isolated during accelerated natural lactic fermentation of sorghum-based infant food formulas

Number of completed fermentation cycles (24 h each)	Formula 1	Formula 2
	n ^a Species	n Species
0 (start)	3 <i>Rhodotorula glutinis</i> 3 <i>Rhodotorula rubra</i> 4 <i>Cryptococcus uniguttulatus</i> 3 <i>Cryptococcus albidus</i>	6 <i>Candida curvata</i> 7 <i>Candida curvata</i>
1	12 <i>Candida famata</i> 4 <i>Rhodotorula rubra</i>	8 <i>Candida curvata</i> 4 <i>Candida curvata</i>
2	9 <i>Candida famata</i> 3 <i>Rhodotorula rubra</i>	11 <i>Candida curvata</i>
3	8 <i>Candida famata</i>	
4		5 <i>Candida guilliermondii</i>
13	15 <i>Candida famata</i>	
27	9 <i>Candida famata</i>	8 <i>Candida famata</i>
60	9 <i>Candida famata</i>	5 <i>Candida famata</i> 4 <i>Trichosporon beigelii</i>

^a n, number of isolates.

slight increase of pH. Enterobacteriaceae were present during the first cycle after which they were eliminated.

In Formula 2, the final pH stabilised at 3.7–3.8. Whereas the counts of lactic acid bacteria and Enterobacteriaceae followed the same trend as in Formula 1, the yeast counts remained significantly lower with a slight increase after 27–60 cycles to log cfu/g 3.8. The pH of the fermented product remained constant. The Enterobacteriaceae isolated during the first fermentation cycle of Formula 1 included in order of decreasing occurrence: *Enterobacter agglomerans*, *Citrobacter freundii*, *Enterobacter sakazaki*, *Klebsiella ozaenae* and *Enterobacter cloacae*. From Formula 2, *Enterobacter sakazaki*, *Enterobacter agglomerans* and *Citrobacter freundii* were isolated during the first fermentation cycle.

In the raw materials and during the first fermentation cycle, filamentous fungi included *Aspergillus candidus*, *Aspergillus flavus*, *Aspergillus fumigatus*, *Aspergillus niger* and some *Penicillium* spp. During later stages, filamentous fungi could not be detected. The dominating groups of lactic acid bacteria during consecutive cycles of the fermentation of Formulas 1 and 2 are presented in Table II.

Similarly, Table III presents the yeasts, which were isolated during progressive cycles of fermenting Formulas 1 and 2. None of the isolated yeasts formed ascospores after 2 and 4 weeks incubation at 25°C, and none were of the 'killer' type. Towards the end of the experiment i.e. cycle 60, Formula 1 was dominated by *Lactobacillus plantarum* and *Candida famata*. Formula 2 contained *Lactobacillus acidophilus*, *Lactobacillus brevis*, *Candida famata* and *Trichosporon beigelii*.

Discussion

The Enterobacteriaceae occurring during the first fermentation cycle are common on plant material and were found also in natural fermentation of e.g. soya beans (Mulyowidarso et al., 1989) and early stages of the sourdough fermentation (Lönner et al., 1986). Their disappearance in the following cycles with $\text{pH} < 4.5$ is in accordance with the death kinetics of Enterobacteriaceae in similarly fermented material (Nout et al., 1989a).

Notwithstanding the 'non-sterile' character of the fermentation technique, naturally occurring filamentous fungi appear to be unable to compete in the combination of acidity and poor access of atmospheric oxygen prevailing in the fermenting dough-like mass. This implies that there is no risk that mycotoxins will be formed during the fermentation process. However, the risk of raw materials being contaminated with mycotoxins shall be mentioned.

From the results in Tables II and III it can be observed that the choice of raw materials influences the long-term succession and dominance of lactic acid bacteria and yeasts. In Formula 1, fermentation cycles 1–13 were dominated by *Leuconostoc dextranicum*, *Lactobacillus acidophilus* groups 1 and 2 and *Lactococcus lactis*, all having a limited assimilative spectrum (able to ferment 10–14 out of 48 tested sources of carbon) but achieving low final pH values. Depending on the presence of acetic acid (Nout et al., 1987b), the minimum pH allowing yeast growth in sourdoughs was 4.0 except for *Candida krusei* which still grew at pH 3.6 (Spicher and Schröder, 1980). The resulting low numbers of yeasts may have caused a deficiency of growth factors required for the lactic acid bacteria. This was shown earlier with lactic acid bacteria from sourdoughs which required vitamins (Spicher and Schröder, 1979a) and varying numbers of amino acids (Spicher and Schröder, 1979b), to be supplied either by fresh yeast extract (Kline and Sugihara, 1971), a peptide (Sugihara, 1985) or log cfu/g 6–7 live yeast cells (Spicher et al., 1981). As a result of such deficiencies, the lactic acid bacteria would produce less acid and in turn allow a significant increase of yeast counts after cycle 4. As long as the pH remains favourable (i.e. $\text{pH} > 4$) the yeasts can maintain their growth due to their broad assimilative spectrum (*Candida famata* being able to assimilate 21 out of 30 tested sources of carbon). An equilibrium is reached with *Lactobacillus plantarum* of broad assimilative spectrum (22 out of 48 carbon sources) which acidify to pH levels (4.2–4.3) at which *Candida famata* grows well.

In Formula 2, the presence of milk solids may satisfy the long-term nutritional requirements of lactic acid bacteria. Consequently, acid-tolerant lactose fermenting *Lactobacillus acidophilus* but also the lactose non-fermenting *Lactobacillus brevis* can co-exist with a minority of both lactose fermenting *Trichosporon beigeli* and lactose non-fermenting *Candida famata*. *Candida famata* isolated from maize silage was able to assimilate lactate, particularly under aerobic conditions (Middelhoven and Van Baalen, 1988). *Trichosporon beigeli* can also assimilate lactate; it is therefore important to maintain anaerobiosis.

Since no 'killer' yeasts were found among the dominating species, the 'killer' phenomenon does not appear to be involved in their succession. On the other hand,

it is interesting to note that *Rhodotorula glutinis*, *Rhodotorula rubra*, *Cryptococcus uniguttulatus* and *Cryptococcus albidus* which were present during the first fermentation cycle of Formula 1 were not resistant against 100 ppm cycloheximide. In later stages, only cycloheximide-resistant yeasts were isolated. This has also been found in equilibrated populations of yeasts and lactic acid bacteria in sourdoughs (Nout and Creemers-Molenaar, 1986).

In conclusion, it is suggested that the extent of acidification of long-term accelerated natural lactic fermentations of cereal-based mixtures will, inter alia, depend on the availability of micronutrients for lactic acid bacteria. If the latter are deficient in the raw material to be fermented, they will have to be supplied by yeast cells, and consequently an equilibrium situation will be established with final pH values and acetic acid concentrations enabling adequate yeast growth. Additional experiments with pure and mixed cultures in micronutrient deficient fermentations are needed to confirm this hypothesis.

Acknowledgements

The author gratefully acknowledges the collaboration of C.W.C. van Muijen and H.M. Bouwmeester, Department of Food Science, Agricultural University, Wageningen.

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