Bacteriological Safety of a Fermented Weaning Food Containing L-lactate and Nisin

ROKIAH M. YUSOF, JANE B. MORGAN, and MARTIN R. ADAMS*

1Department of Nutrition and Community Health, Faculty of Human Ecology, University Pertanian Malaysia, 43400 UPM, Serdang, Selangor, Malaysia and 2School of Biological Sciences, University of Surrey, Guildford, Surrey GU2 5XH, United Kingdom.

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ABSTRACT

Using a rice-based model weaning food, the effect of Lactococcus lactis on the growth and survival of a range of enteric pathogens has been investigated. The starter organism used produces the bacteriocin nisin and the physiological L-lactate isomer, thus avoiding the risk of D-lactate acidosis when consumed by infants. L. lactis was a less effective antagonist than stronger acid producers such as the DL lactate producer, Lactobacillus plantarum, and only produced a potentially useful inhibition of pathogens when present in a large numerical superiority (>10^5:1).

Prefermentation of the weaning food with L. lactis for 24 h when present in a large numerical superiority (>10^5:1). Prefermentation of the weaning food with L. lactis for 24 h produced a product with a pH of 3.7-3.8 containing ~ 0.25% lactate (>96% L-lactate). The prefermented product was bactericidal for pathogens introduced subsequently. Despite the production of 100-150 international units nisin per g during fermentation, the inhibition of pathogens could be ascribed to acid production alone.

Weaning diarrhea is recognized as a major cause of infant morbidity and mortality in developing countries. Precise data on the extent to which this is foodborne are not available and published figures, which estimate that between 15 and 70% of all diarrhea episodes in young children are food associated, reflect this uncertainty (9).

Most of the pathogens implicated in weaning diarrhea are those spread by the fecal-oral route such as enterotoxigenic and enteropathogenic Escherichia coli, Campylobacter jejuni, Shigella, and rotavirus (3,7). Foods used to replace or supplement maternal breast milk at weaning are a potential source of such organisms, and a number of studies have demonstrated the presence of enteric indicator bacteria and pathogens in weaning foods (4,6,8,14,15,21).

Lactic acid fermentation has attracted interest as a simple technique which can inhibit pathogens and improve safety in weaning foods. Using both prefermented model weaning foods and traditional fermented products, significant lethal effects on a range of enteric bacterial pathogens have been demonstrated (18-20,22-24), although rotavirus is markedly less sensitive (27).

The principal lactic bacteria involved in cereal fermentations produce a mixture of D- and L-lactate isomers. For example, studies on traditional African products have reported Lactobacillus fermentum, Lactobacillus brevis, and Lactobacillus plantarum as major components of the microflora (1,2), and in one fermented weaning food, D-lactate comprised 42% of the total acidity (22). D-lactate is a nonphysiological isomer in mammals and is poorly metabolized (13). Excessive consumption of D-lactate or overproduction in vivo as a result of gut dysfunction can result in D-lactate acidosis (3,12,17,26). As a result, the World Health Organization has recommended that neither D- nor DL-lactic acid should be added to food for very young infants and that, for adults, an acceptable daily intake of D-lactate is 100 mg per kg body weight (11). Although it is clear that fermented weaning foods should contain only L-lactate, this problem has not yet been addressed.

Here we report a study into the use of L-lactate producing bacteria to ferment weaning foods and the possible role of bacteriocins in pathogen inhibition.

MATERIALS AND METHODS

Bacteria

Escherichia coli ATCC 25922, Salmonella typhimurium USCC 305, Shigella sonnei USCC 2006, Staphylococcus aureus ATCC 25923, Lactococcus lactis NCIB 497, and L. plantarum were provided by the Microbiology Department Culture Collection. Lactobacillus salivarius USCC 2253, Lactobacillus alimentarius ATCC 29643, Lactobacillus havalicus DSM 20269, and Lactobacillus casei subsp. casei ATCC 393 were obtained from the National Collection of Food Bacteria, Reading, United Kingdom.

Stock cultures of pathogens were maintained on nutrient agar (Oxoid) slopes incubated at 37°C for 2 d and stored at 4°C with weekly subculturing. Lactic acid bacteria were maintained under similar conditions after growing for 2 d at 30°C on MRS agar (Oxoid) under microaerophilic conditions.

For experiments, a single colony from a fresh streak dilution plate on the appropriate growth medium was inoculated into the corresponding broth medium and incubated for 18-22 h at 37°C (pathogens) or 30°C (lactic acid bacteria) to reach late exponential phase. These cultures were inoculated into the model weaning food after decimal serial dilution in maximum recovery diluent (MRD) (Oxoid) according to the experimental requirement at a level of 1 ml in 100 g weaning food.

Nisin, when added, was the commercial preparation Nisaplin (Apin & Barrett, Beaumins, United Kingdom).

Preparation of model weaning food

Rice (100 g) (American Long Grain, Sainsbury) was cooked with stirring in 1 L of distilled water for 40 min to produce a
viscous porridge. The highest temperature reached during cooking was 98°C. The hot product (100 g portions) was dispensed into sterile beakers and covered with sterilized aluminium foil and allowed to cool to 30°C. Diastatic malt extract (Diamalt 400L, British Diamalt) diluted with an equal volume of sterile distilled water was added to the porridge at a level of 1%.

The resulting product was inoculated with bacteria from broth cultures (see above) to give the level required for that particular experiment (as indicated in the Results section) and incubated at 30°C for 24 to 30 h in a hot water bath with periodic sampling. Prefermented weaning food was made by inoculating the freshly prepared product with *L. lactis* to give an initial level of 10^6/g and incubating at 30°C for 24 h.

All trials were performed a minimum of two times.

**Bacterial enumeration**

Sample (10 g) was mixed with MRD (90 ml) and homogenized for 1 min in a Colworth Stomacher 400. A decimal dilution series was prepared using MRD. *E. coli*, *S. typhimurium*, and *Shigella sonnei* were enumerated by spreading dilutions on to tryptone soy agar (Oxoid) followed after 1 h by an overlay of violet red bile glucose agar (Oxoid). The plates were incubated at 37°C for 24 h. *S. aureus* were enumerated as spread plates on Baird-Parker agar (Oxoid) at 37°C for 24 to 48 h. Lactic acid bacteria were enumerated as spread plates on MRS agar incubated under microaerophilic conditions in a gas jar at 30°C for 48 h.

**Chemical analyses**

Sample pH was measured on a 10% slurry in distilled water using a pH meter with combination electrode (PTI-55, Aqua Scientific).

Total acidity was determined by titration of 5 g of sample mixed with 50 ml of distilled water against 0.1 M NaOH to an end point of pH 8.5, while stirring continuously.

D- and L-lactate were measured using an enzyme assay (Boehringer, Mannheim). Fermented weaning food (5 g) in 10 ml of hot water tube was heated in a boiling water bath for 10 min, made up to a volume of 50 ml, mixed and filtered through a Whatman No. 1 filter paper. The clear filtrate was used for the assay according to the manufacturer’s instructions.

For the determination of nisin content, samples of weaning food (5 g ± 0.1 g) were weighed out and dispersed evenly in 5 ml of 0.02 M HCl. The pH was adjusted to 2.0 ± 0.01, and the sample was heated at 98°C for 10 min. After cooling to 20°C, the sample was centrifuged for 15 min at 4,000 x g. The supernatant was removed after standing for 30 min. The nisin content of the extract was determined using the enzyme-linked immunosorbent assay (ELISA) protocol and reagents of Falahee et al. (10). Samples were assayed in triplicate using a calibration curve prepared at the same time on the same microtiter plate.

**RESULTS**

Five L-lactate producing species gave similar acidity (0.20-0.28%) and pH values (3.44-3.71) in the model weaning food over 24 h fermentation at 30°C. In this respect, they were inferior to the DL-lactate producer *L. plantarum* which was also nonamylolytic but produced 0.41% acidity and a pH of 3.35 under the same conditions (Table 1). Among the L-lactate producers, *L. lactis* gave the most rapid pH decrease over the first 8 h of fermentation and was a known nisin-producing strain.

When *L. lactis* and an enteric pathogen (*E. coli*, *S. typhimurium, S. sonnei*, or *S. aureus*) were inoculated simultaneously into weaning food at the same concentration (10^5 CFU/g), inhibition of the pathogen was slight. In the presence of Lactococcus, the *E. coli* population after 9 h was more than 10^7 CFU/ml, only 0.6 log cycles lower than the control.

Inhibition of *E. coli* growth increased as the inoculum ratio of Lactococcus to *E. coli* increased (Fig. 1). Reduction in the stationary phase population of *E. coli* by more than 1 log cycle occurred only when the inoculum level of *L. lactis* exceeded *E. coli* by a factor of more than 10^2. When the inoculum ratio was 10^2:1, *E. coli* still grew for 5 h, its numbers increasing by almost 2 log cycles. For each inoculum ratio tried, no appreciable slowing of growth was apparent until the pH of the weaning food had dropped below 5.0.

Similar results were obtained with other pathogens (*S. sonnei, S. typhimurium or S. aureus*) (Fig. 2); an inoculum of

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**Table 1. Growth and acid production by lactic acid bacteria in model weaning food at 30°C. Lactate isomer in parentheses.**

<table>
<thead>
<tr>
<th>Time (h)</th>
<th><em>L. lactis</em> (L)</th>
<th><em>L. plantarum</em> (DL)</th>
<th><em>L. salivarius</em> (L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>5.95 (L)</td>
<td>5.08 (DL)</td>
<td>5.08 (L)</td>
</tr>
<tr>
<td>4</td>
<td>7.47 (L)</td>
<td>6.10 (DL)</td>
<td>6.10 (L)</td>
</tr>
<tr>
<td>8</td>
<td>7.38 (L)</td>
<td>6.95 (DL)</td>
<td>6.95 (L)</td>
</tr>
<tr>
<td>24</td>
<td>7.26 (L)</td>
<td>6.77 (DL)</td>
<td>6.77 (L)</td>
</tr>
</tbody>
</table>

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**Figure 1. Growth of *E. coli* at 30°C in model weaning food in coculture with *L. lactis* (counts not shown). Inoculum level of *L. lactis*: ●, 10^2 CFU/g; ▽, 10^5 CFU/g; ▼, 10^6 CFU/g; ▲, 10^7 CFU/g; ■, 10^8 CFU/g; ◇, 10^9 CFU/g; ○, control.
ratio of \textit{L. lactis} to pathogen of 10^4:1 decreasing the pathogen stationary phase population by between 2 and 4 log cycles.

By comparison, the stronger acid producer \textit{L. plantarum} was more effective; with an inoculum ratio of 10^5, \textit{E. coli} growth stopped after only 3 h and numbers fell after 6 h (data not shown). The lactate produced by \textit{L. plantarum} was, however, an almost equimolar mixture of D- and L-isomers compared with >96% L-lactate produced by \textit{L. lactis}.

Pathogens were unable to grow when added to weaning food which had been prefermented with \textit{L. lactis} for 24 h. Surviving numbers decreased over a subsequent 24 h storage at 30°C to below detectable levels (< 10^2 CFU/g) in the cases of the gram-negative pathogens (Fig. 3). The gram-positive \textit{S. aureus} was still detectable, although this may reflect the higher inoculum level used.

Prefermented weaning food had a pH of 3.7-3.8, a titratable acidity of 0.23-0.27% (96% L-lactate), and contained 103-150 international units (IU) nisin per g. Addition of the same quantity of lactic acid to an unfermented weaning food prior to addition of the pathogens, produced a similar decline in pathogen numbers. Simultaneous addition of nisin, at a level (110 IU/g) similar to that found in the prefermented weaning food, and lactic acid to the unfermented product did not increase the unfermented product's lethality toward any of the pathogens tested (results for \textit{S. aureus} shown in Fig. 4). In separate experiments, nisin was shown to have no effect on the gram-negative pathogens used and its minimum inhibitory concentration for the \textit{S. aureus} strain used was shown to be 300 IU/ml.

Prefermented weaning food could be used as an inoculum for subsequent batches. Using an inoculum level of 10%

DISCUSSION

A number of factors have been identified as potential contributors to the antibacterial activity of lactic acid bacteria (16). The results reported here indicate that the observed inhibition can be attributed solely to lactic acid production.
The effect could be reproduced by addition of lactic acid to unfermented weaning food and a stronger acid producer such as _L. plantarum_ was a more effective inhibitor.

There was no evidence of appreciable pathogen inhibition by _L. lactis_ when the two organisms were present at the same low level. Inhibition only occurred under conditions when the _L. lactis_ was able to reach numbers capable of affecting the pH while pathogen numbers were still relatively low.

Although there has been considerable interest recently in bacteriocins from lactic acid bacteria, only nisin is approved by regulatory authorities and widely used commercially. It differs from many bacteriocins in having a wider spectrum of activity and is bactericidal to many gram-positive bacteria. More recently it has been shown to be effective against gram-negative bacteria when used in conjunction with a chelating agent (25). Significant concentrations of nisin accumulated during fermentation with _L. lactis_ but did not have any detectable effect on the pathogens tested. Nisin levels were below the measured minimal inhibitory concentration for _S. aureus_, and there was no evidence of any contribution from nisin to overall pathogen inhibition. Lactic acid bacteria are also sensitive to nisin, and its production may help maintain the dominance of the added L-lactate-producing starter under nonaseptic conditions.

Prefementation with an L-lactate producer provides useful protection against the bacterial pathogens tested and, unlike natural fermentations, produces a product acceptable to regulatory authorities. Successful application of this technique in developing countries could pose problems, although the fermentation appears stable and reproducible over an extended period. The evidence presented here suggests that further trials are warranted to test the feasibility of this under field conditions.

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REFERENCES


