Significance of Lactobacilli in Cheddar Cheese

Armis No. 4209

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Summary and Conclusions

Despite the availability of excellent mature and extra-mature Cheddar cheeses, flavour tends to be inconsistent and is sometimes described as bland. This is observed even in cheeses produced in the same plants and under apparently identical manufacturing conditions. The underlying reasons for this phenomenon are not well understood, but it is suspected that non-starter lactobacilli bacteria, as a major unknown and uncontrolled factor in modern cheesemaking, play a critical role. It is known that these organisms typically reach high numbers in the cheese within the first 2 months of ripening which is likely to effect flavour development, since several strains of lactobacilli metabolise citrate to diacetyl and acetate - key compounds in flavour production in cheese. However, neither the numbers, nor types of strains which play a significant role in flavour development during cheese ripening is known. In addition, the ability of those lactobacilli strains, to metabolise citrate in the physiological environment of cheese ripening (e.g. pH, energy source, etc.) has not yet been elucidated.

Hence the objectives of this project were to isolate and identify the non-starter lactobacilli in mature Cheddar cheese, identify strains which impart mature flavours to cheese and determine their role in developing cheese flavour.

The main conclusions were as follows:

- Based on an analysis of 18 mature Cheddar cheeses, selected from 7 commercial manufacturers, non-starter lactic acid bacteria typically numbered, as expected, 10^6-10^8 per gram and were dominated (97 percent) by Lactobacillus paracasei.
- Although a small number of strains (typically 1 to 4) was found in each cheese there was considerable strain diversity in cheeses within as well as between manufacturing plants.

- When selected strains were investigated for survival and flavour enhancement when added (as starter adjuncts) with the normal starter cultures in Cheddar cheese manufacture, it was found that they remained dominant for up to 3 months of ripening. Commercial grading of these cheeses at 3 and 6 months confirmed that the added strains did modify flavour development and one (DPC 4103), in particular, had a beneficial effect.
- It was confirmed that two selected strains of non-starter lactobacilli were capable of metabolising citrate under the conditions of Cheddar cheese ripening and, consequently, if present in sufficient numbers, would influence flavour development.
- The work was greatly facilitated by the successful and novel adaptation of a modern rapid molecular technique (RAPD) for species and strain classification.

In summary these studies found that one species of lactobacilli *(Lb. paracasei)* was the dominant non-starter lactic acid bacteria in mature Cheddar cheese. Although a wide variety of strains were identified, only a few were found in any particular cheese, suggesting their likely role in cheese flavour diversity even within the same manufacturing plant. This suggests the potential for flavour control or enhancement through the selective and controlled use of non-starter lactic acid bacteria.

Preliminary investigations of the metabolism of those organisms supports this view and a follow-up study now in progress should provide greater clarity on this matter.

Research and Results:

Isolation of Strains

Because mature and extra-mature Cheddar cheese were considered a potent source of non-starter lactic acid bacteria, likely to impart positive effects on cheese flavour, 18 mature Cheddar cheeses, from 7 commercial companies were selected for analysis. The gross composition of these cheeses (pH salt and moisture) were generally within the range expected for such cheeses. Total free amino acids, which are considered a useful index of cheese maturity, were in the range 10 - 36 mg/g. The numbers of non-starter lactic and bacteria in the cheeses varied from $2.4x10^6$ to $1.1x10^8$ cfu/g which is typical of what one would expect in mature and extra-mature cheeses. A total of 360 isolates (20/cheese) were selected for further study.

All isolates were catalase negative and rod-shaped, and therefore, members of the genus Lactobacillus. Fermentation of 8 sugars showed that 96.8% of the 320 strains were *Lb. paracasei*, 2.7% *Lb. plantarum* and 0.4% *Lb. curvatus*. (Fig. 1)

Therefore Lb. paracasei was by far the dominant non-starter lactic acid bacteria found in these cheeses.

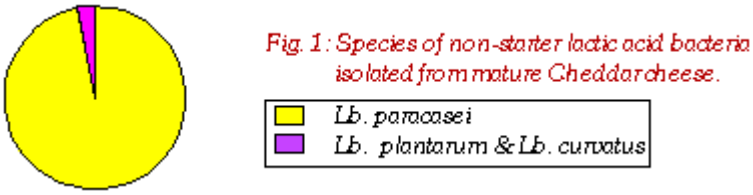

Fig. 1: Species of non-starter lactic acid bacteria isolated from mature Cheddar cheese.

Methodology for Strain Identification

Since the above information did not distinguish between the strains in each species, a technique which could accurately and rapidly identify strains of these organisms had to be developed. Hence Randomly Amplified Polymorphic DNA (RAPD) PCR was investigated as

a suitable method to determine the number of strains of lactobacilli isolated from the commercial Cheddar cheeses. A rapid method for the isolation of DNA was optimised and proved to be a very efficient method for extraction of total DNA from large numbers of lactobacilli. A number of random 10mer and 8mer primers were selected based on the Tm and GC content of the DNA. These were initially tested for their capacity to amplify genomic DNA from known strains of lactobacilli under low stringency conditions. Four primers which demonstrated the capacity to distinguish between these strains were selected for further study. RAPD PCR conditions, which gave the maximum number of randomly amplified genomic fragments for each of the 4 primers, were determined by adjusting the annealing temperature used in the PCR with each of the primers. DNA concentrations in the range 1-100ng gave optimum and highly reproducible results.

To validate this new methodology, strains of well-characterised lactobacilli were obtained, the inter-strain relationships of which had previously been demonstrated. These strains were subjected to RAPD in an effort to determine the capacity of RAPD to distinguish between closely related strains. The results demonstrated that a number of common bands were obtained in the RAPD profiles from strains of a particular species and that RAPD has the potential to rapidly classify isolates to species level. The banding patterns obtained between strains of the same species were not always identical, indicating that RAPD could be used to distinguish between strains *(Fig. 2)*.

Hence, the RAPD method was proven to have sufficient resolving power to distinguish between the main groups of strains of non-starter lactic acid bacteria and was both accurate and rapid in handling large numbers of isolates.

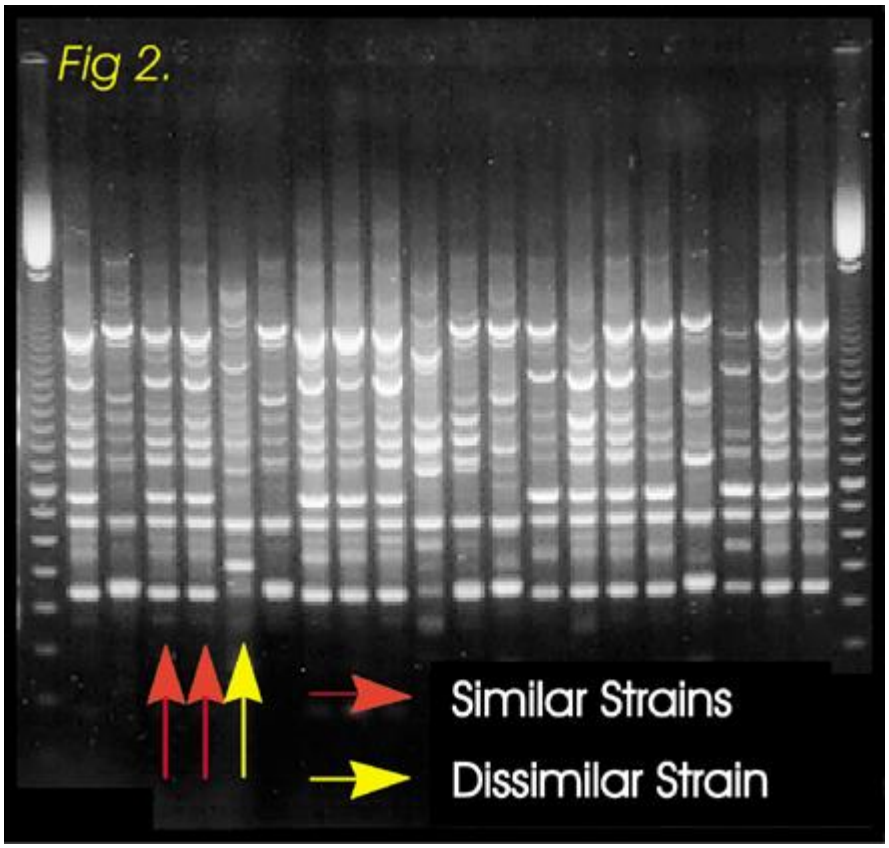

Fig. 2: RAPD profiles of 20 cheese isolates. Examples of similar and dissimilar strain are indicated.

Strain Analysis

Comparative analysis indicated that while the non-starter lactic acid bacteria population of all the cheeses was dominated by *Lb. paracasei*, there was considerable variation in the strains present in cheeses from the different factories. No strain was identified which was common to all cheese plants. Analysis of the isolates from individual cheeses within a cheese plant also demonstrated considerable variation with the exception of one factory where 1 strain was found at relatively high levels in each of the 3 cheeses examined.

This indicates that there is considerable diversity in the strains between particular cheeses, which may also explain why there is such variation in the flavour of Cheddar cheese, even from the same plant.

Twenty-two strains were selected for further study on the basis that they composed 20% or more of the non-starter lactic acid bacteria population in their particular cheese of origin, or were identified in more than 1 cheese. The strains formed 6 clusters on the basis of growth on lactose, proteolytic ability and acidification of milk - potentially important technological characteristics. To investigate the survival and flavouring capacity of the selected strains Cheddar cheese was manufactured using 3 (randomly selected) strains as starter adjuncts. Adjuncts were added such that they were present at 10^6 cfu/g at day 1 of ripening. *Analysis of the non-starter lactic acid bacteria populations of these cheeses by RAPD demonstrated that the adjuncts remained the dominant member of the NSLAB population for up to 3 months of ripening and evolution of adventitious strains was not observed. Commercial grading of these cheeses at 3 and 6 months indicated that the addition of the adjuncts modified flavour development and that one strain (DPC 4103) in particular, had a beneficial effect.*

Citrate Metabolism

Information on the factors influencing citrate metabolism in lactobacilli is limited and could be useful in understanding the growth of lactobacilli in ripening cheese. Hence two non-starter lactobacilli (*Lb. casei* ATCC 393 and *Lb. plantarium* 1919), known to metabolise citrate, were studied and growth rate was not affected when citrate was co-metabolised with glucose or galactose (Fig. 3).

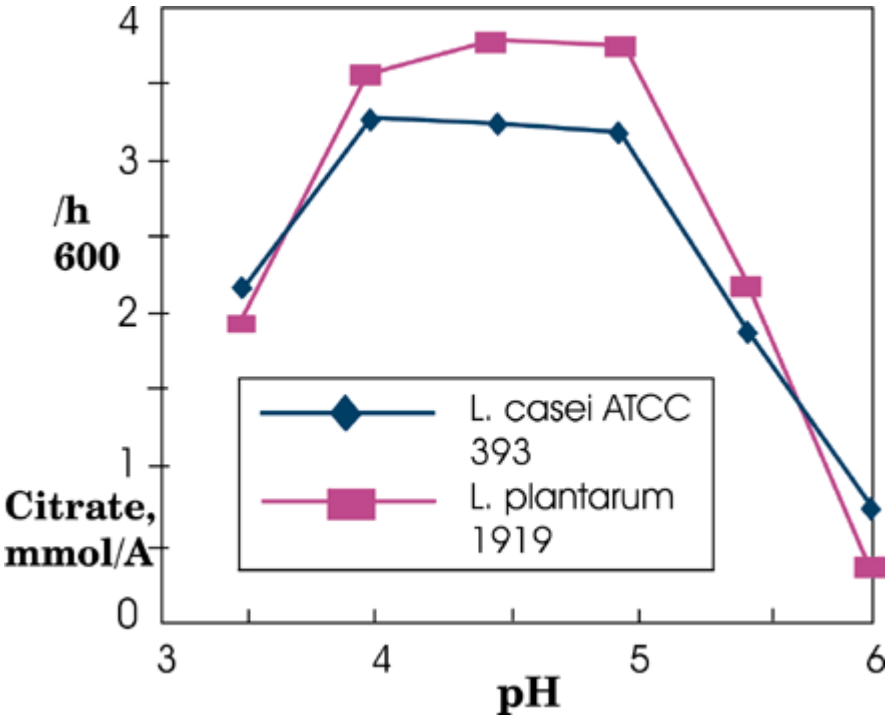

Fig. 3: Effect of pH on Citrate utilisation; by two strains of lactobacilli, in the absence of sugar.

In growing cells, metabolism of citrate was minimal at pH 6 but significant at pH 4.5 and was greater in cells co-metabolising galactose than in those co-metabolising glucose or lactose. In non-growing cells, optimum utilisation of citrate also occurred at pH 4.5 and was not increased substantially by the presence of fermentable sugars. In both growing and non-growing cells, acetate and acetoin were the major products of citrate metabolism; pyruvate was also produced by non-growing cells and was transformed to acetoin once the citrate was exhausted. Citrate was metabolised more rapidly than sugar by non-growing cells; the reverse was true of growing cells. Citrate metabolism by *Lb. plantarum* 1919 and *Lb. casei* ATCC 393 increases 6- and 22- respectively, when the cells were pre-grown on galactose plus citrate compared to when they were pre-grown on galactose only. This was shown to be due to induction of citrate lyase by growth on citrate plus sugar.

These results confirm that non-starter lactobacilli, if present in large enough numbers, can metabolise citrate in ripening cheese in the absence of an energy source.