ACTA CHEMICA SCANDINAVICA 6 (1952) 1258–1264

Chemical Factors Affecting Associations of Lactic Acid Bacteria

VEIKKO NURMIKKO

Laboratory of Valio, Biochemical Institute, Helsinki, Finland

It is known that various micro-organisms are able to form associations in which one organism may stimulate or inhibit the growth of another. In particular, lactic acid bacteria have the ability to enter into associative inter-relationships in mixed cultures [Cf. e.g. 1–4]. However, although such phenomena were early recognised, the associative, and especially symbiotic, relationships of lactic acid bacteria in mixed populations are very little explored, and the chemical mechanism of associative action is mostly not definitely known.

In the present investigation the associations of lactic acid bacteria in a medium of known chemical composition have been studied. Attention has been focussed upon chemical factors affecting these associations.

EXPERIMENTAL

Cultures and inocula

The organisms used in this study were *Lactobacillus arabinosus* 17—5, *Streptococcus faecalis* R, and *Leuconostoc mesenteroides* P-60. The cultures were handled in the manner usually employed in microbiological techniques [5]. These were carried by bi-weekly transfer as stab cultures in a medium containing 1% glucose, 1% sodium citrate, 0.5% Bacto-tryptone, 1.5 % agar and 20 per cent by volume yeast extract. (The yeast extract was prepared as follows. 1 kg fresh yeast was suspended in 1 litre water, kept for approximately 24 hours at 42° C. It was then centrifuged and the cell-free solution used.) The inoculum used for the experiments was prepared from a 12—18 hour culture grown at 37° C in a medium above mentioned, without agar. The cells were washed twice by centrifugation and resuspension in sterile 0.9% saline, and one drop of the barely visible suspension (96—98% transmission, Klett-Summerson photoelectric colorimeter with filter no. 66) was used to inoculate each experimental tube.

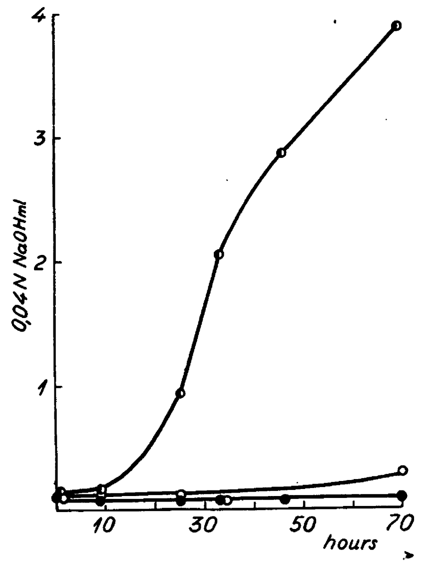


Fig. 1. The growth of Lactobacillus arabinosus 17—5 and Streptococcus faecalis R in symbiosis. Basal medium without phenylalanine and folic acid.

● *L. arabinosus 17—5 (phenylalanine-requiring strain).*
○ *Str. faecalis R (folic acid-requiring strain).*
◐ *L. arabinosus 17—5 and Str. faecalis R together.*

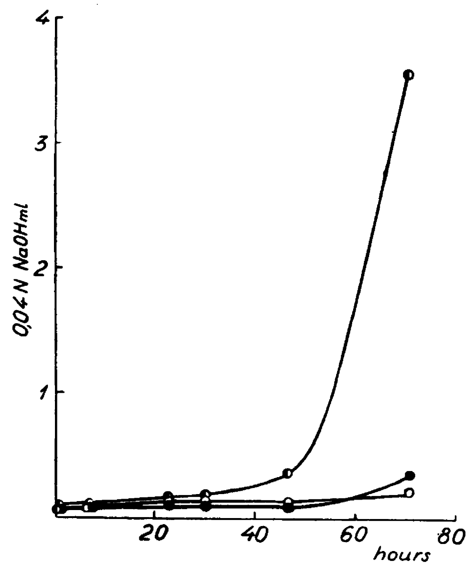


Fig. 2. The growth of Lactobacillus arabinosus 17—5 and Streptococcus faecalis R in symbiosis. Basal medium without threonine, phenylalanine and folic acid.

● *L. arabinosus 17—5 (phenylalanine-requiring strain).*
○ *Str. faecalis R (threonine and folic acid-requiring strain).*
◐ *L. arabinosus 17—5 and Str. faecalis R together.*

Basal medium and methods

The microbiological techniques used in these experiments are essentially the same as those described by Henderson and Snell [5]. The uniform basal medium used contained 0.5 times the concentration of all the ingredients in the medium used by these authors, with the exception of glucose, which was kept at the original concentration. All media were prepared at twice their final concentrations, adjusted to pH 6.8, added to test tubes in 1 ml quantities, diluted with water or supplements (vitamin or amino acid) to 2 ml, plugged with cotton wool, and autoclaved at 112° C for 5 minutes. After cooling, the tubes were inoculated, then incubated at 37° C in a covered water bath. Growth response was followed titrimetrically, and turbidimetrically. The lactic acid produced was titrated electrometrically directly in the test tubes with 0.04 *N* NaOH. Turbidity was measured with the Klett-Summerson photoelectric colorimeter with filter no. 66.

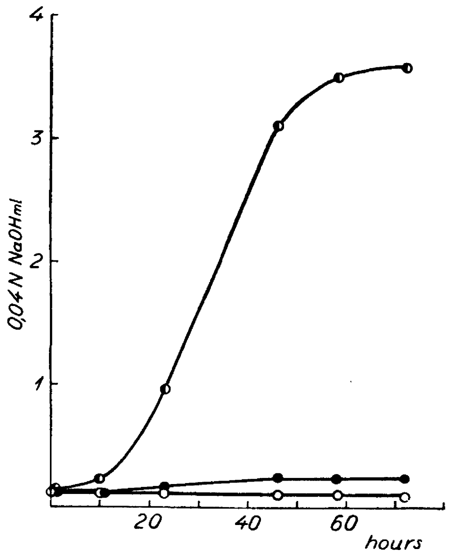


Fig. 3. The growth of Leuconostoc mesenteroides P-60 and Streptococcus faecalis R in symbiosis. Basal medium without proline and folic acid.

- ○ *Leuconostoc mesenteroides P-60 (proline-requiring strain).*
- ● *Str. faecalis R (folic acid-requiring strain).*
- ◐ *Leuconostoc mesenteroides P-60 and Str. faecalis R together.*

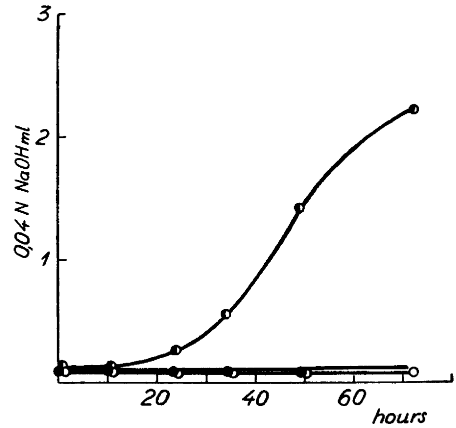


Fig. 4. The growth of Leuconostoc mesenteroides P-60 and Streptococcus faecalis R in symbiosis. Basal medium without proline, phenylalanine, glycine and folic acid.

- ○ *Leuconostoc mesenteroides P-60 (proline, phenylalanine, and glycine-requiring strain).*
- ● *Str. faecalis R (folic acid-requiring strain).*
- ◐ *Leuconostoc mesenteroides P-60 and Str. faecalis R together.*

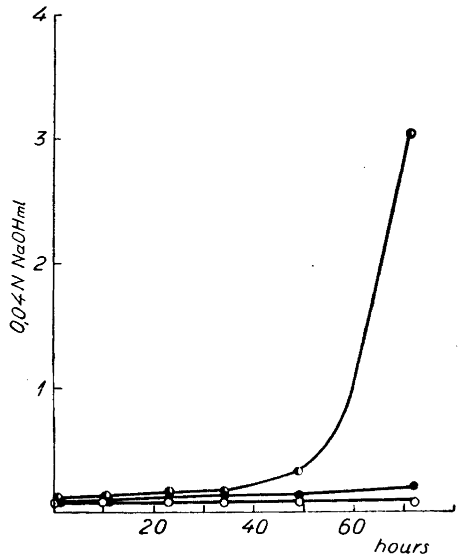
RESULTS

In all experiments either one vitamin and one or more amino acids, or amino acids only, which were essential for the growth of bacteria, were omitted from the basal medium. Amino acid and vitamin requirements of organisms employed, had previously been investigated separately for each experiment.

It can be seen from Fig. 1 that *Str. faecalis* R and *L. arabinosus* 17—5 do not grow alone, but are able to grow together in a medium lacking folic acid and phenylalanine, although folic acid is required by *Str. faecalis* R and phenylalanine by *L. arabinosus* 17—5. If threonine, which has been found to be an essential amino acid for the growth of *Str. faecalis* R, was also omitted

Fig. 5. The growth of Lactobacillus arabinosus 17—5 and Streptococcus faecalis R in symbiosis. Basal medium without phenylalanine and threonine.

● *L. arabinosus 17—5 (phenylalanine-requiring strain).*
○ *Str. faecalis R (threonine-requiring strain).*
◐ *L. arabinosus 17—5 and Str. faecalis R together.*



from this medium (already free of folic acid and phenylalanine), the above mentioned bacteria can still be grown together, as shown in Fig. 2. In this case, however, the rate of growth in the association was slower. When serine was absent from this medium instead of threonine, the bacteria still grew in association, but it was shown microscopically that *L. arabinosus* 17—5 was grown more rapidly than *Str. faecalis* R.

As shown in Fig. 3, the proline-requiring strain *Leuconostoc mesenteroides* P-60 and the folic acid-requiring strain *Str. faecalis* R could be grown together, but not alone, in a medium from which proline and folic acid had been omitted. If, in addition to these compounds, phenylalanine and glycine, which are essential amino acids for *Leuconostoc mesenteroides* P-60 (but not for *Str. faecalis* R) were absent from this medium, both bacteria still grew together, as can be seen from Fig. 4.

From Fig. 5 it appears that *Str. faecalis* R and *L. arabinosus* 17—5 grew together, but not alone, in a medium lacking the two amino acids phenylalanine (required by *L. arabinosus* 17-5 alone) and threonine (required by *Str. faecalis* R alone). The lag phase in this association was relatively long. —However, when one of the missing amino acids was essential for both strains together (in this case methionine), they could not be grown in association, as can be seen from Fig. 6.

In the experiment illustrated by Fig. 7. *Str. faecalis* R was cultured first with growth-limiting amounts (0.005 γ per test tube) of folic acid in a medium

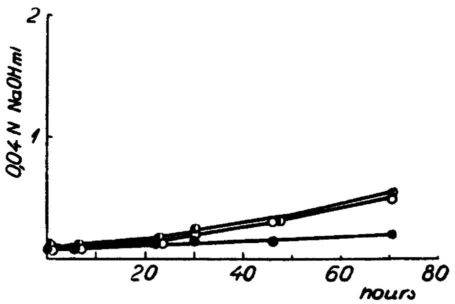


Fig. 6. *Lactobacillus arabinosus* *17—5 and Streptococcus faecalis R in a basal medium lacking methionine and phenylalanine.*

- ● *L. arabinosus 17—5 (methionine and phenylalanine-requiring strain).*
- ○ *Str. faecalis R (methionine-requiring strain).*
- ◐ *L. arabinosus 17—5 and Str. faecalis R together.*

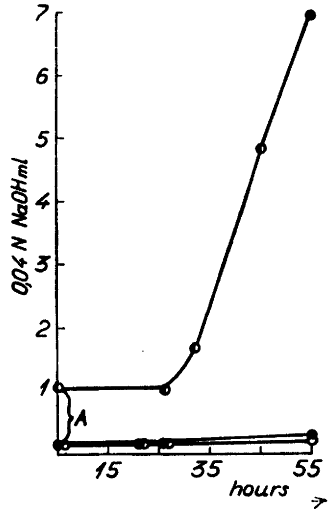


Fig. 7. The growth of Lactobacillus arabinosus 17—5 after Streptococcus faecalis R. Basal medium without phenylalanine and folic acid. Str. faecalis R was first cultured with growth-limiting amounts of folic acid. A indicates the amount of acid produced by Str. faecalis R, corresponding to a weak growth.

- ● *L. arabinosus 17—5 (phenylalanine-requiring strain).*
- ○ *Str. faecalis R (folic acid-requiring strain).*
- ◐ *L. arabinosus 17—5 grown after Str. faecalis R.*

lacking phenylalanine. After 25 hours the cultures were centrifuged, and the clear solutions were each added to one ml of a basal medium lacking folic acid and phenylalanine. The test tubes were then kept for 3 minutes at 100° C, after cooling, inoculated with *L. arabinosus* 17-5. This was able to grow in this medium after *Str. faecalis* R. As mentioned above, phenylalanine is required for growth of *L. arabinosus* 17-5.

Fig. 8 shows that *Leuconostoc mesenteroides* P-60 was not grown after *L. arabinosus* 17-5 in a medium deficient in phenylalanine, because this amino acid was a requirement common to both bacteria. (*L. arabinosus* 17-5 was cultured first with growth-limiting amounts (2 γ per tube) of L-phenylalanine, and was then handled in the manner described in the preceding experiment.)

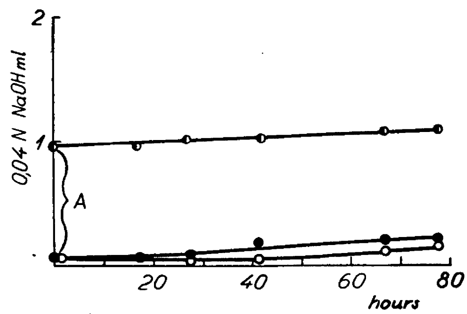


Fig. 8. Leuconostoc mesenteroides P-60 in a phenylalanine-deficient medium, in which Lactobacillus arabinosus 17—5 was first cultured with growth-limiting amounts of phenylalanine. A indicates the amount of acid produced by L. arabinosus 17—5, corresponding to a weak growth.

● *Leuconostoc mesenteroides P-60 (phenylalanine-requiring strain).*
○ *L. arabinosus 17—5 (phenylalanine-requiring strain).*
◐ *Leuconostoc mesenteroides P-60 inoculated after L. arabinosus 17—5.*

Finally, it has been found that a dialysing membrane (Cellophane) could not prevent the growth of *L. arabinosus* 17-5 and *Str. faecalis* R in association, in a medium from which phenylalanine and folic acid was omitted. (See also the experiment illustrated by Fig. 1.)

DISCUSSION

The results obtained in this preliminary investigation show that the two different strains of lactic acid bacteria can be grown together in symbiosis in a synthetic medium, each producing growth factors needed by the other. As a rule, this seems to be possible only in such cases where none of the vitamins or amino acids omitted is a requirement common to both organisms (Figs. 1—5 and 7). In cases in which a common amino acid requirement is omitted, bacteria cannot be grown in symbiosis (Figs. 6 and 8).

Although it seemed probable that the growth factors affecting the associations of lactic acid bacteria are these vitamins and amino acids which are required for the growth of organisms in a deficient synthetic medium, direct evidence has not been obtained in the present study about the type of these compounds. In view of the observation obtained using a dialysing membrane, the molecules in question are comparatively small and may be rapidly dialysed. In any case, the results obtained suggest that the chemical factors produced by bacteria in symbiosis under the conditions used, have the activities of those amino acids and vitamins (in some experiments these factors seem to have activities which are greater than those of the corresponding amino acid or vitamin), which are absent from the synthetic basal medium, but which have been found to be essential metabolites for the growth of lactic acid bacteria which form these associations.

In view of these findings, it appears probable that the above mentioned growth factors also play an important part in the associations of lactic acid bacteria in milk and dairy products. For this reason, and especially because of the special theoretical interest of the symbiotic relationships among lactic acid bacteria, further work on their associations is in progress.

SUMMARY

Symbiotic inter-relationships have been shown to exist between different strains of lactic acid bacteria in a medium of known chemical composition. When certain vitamin and amino acid(s), which are essential substances for the growth of two lactic acid bacteria, were omitted from the synthetic medium, both organisms were able to grow in symbiosis together, but not alone, each producing the chemical factors needed by the other.

The results are discussed briefly.

REFERENCES

1. Sherman, J. M., and Stark, P. *J. Bact.* **36** (1938) 77.
2. Hassouna, M. M., and Allen, L. A. *J. Dairy Research* **10** (1939) 7.
3. Peppler, H. J., and Frazier, W. C. *J. Bact.* **43** (1942) 181.
4. Pette, J. W., and Lolkema, H. *Neth. Milk Dairy J.* **4** (1950) 197.
5. Henderson, L. M., and Snell, E. E. *J. Biol. Chem.* **172** (1948) 15.

Received September 25, 1952.