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GROWTH OF SOME LACTIC ACID BACTERIA IN MILK CONTAINING SULFADIAZINE

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SOBACK, STEFAN: *Growth of some lactic acid bacteria in milk containing sulfadiazine.* Acta vet. scand. 1981, 22, 493—500. — A method for testing the effect of sulfonamides on lactic acid bacteria was developed. Sulfadiazine was used as the inhibitory substance. In the study 3 lactic acid bacteria, *Lactobacillus lactis*, *Lactobacillus helveticus* and *Streptococcus thermophilus*, were used. Lactic acid production, pH and plate count were used as parameters when monitoring the effect of Sulfadiazine on the test organisms under anaerobic conditions in milk medium.

A depressing effects due to Sulfadiazine on each of the test microbes could be demonstrated by all of the parameters used. Measurement of the inhibition in lactic acid production using *Streptococcus thermophilus* as test organism should be used when rapid determination of Sulfadiazine residues in milk is preferred.

sulfadiazine; lactic acid bacteria; residues.

Sulphonamides are widely used in bovine practice to control infectious diseases. Although there is a great variation on the mammary excretion of different sulphonamide compounds (*Rasmussen* 1958), the ratio between ultrafiltrates milk/serum range from 0.1 to 1.0, the concentrations give rise to residues in milk. *Reinbold & Reddy* (1974) found dairy starter bacteria, except *Streptococcus thermophilus*, resistant to 8 different sulphonamides. In the case of *S. thermophilus* they pointed out that more appropriate methods should be used.

The purpose of this study was to develop a method able to detect effects of sulphonamide (or any inhibitory substance) on lactic acid producing bacteria in conditions closely related to those of a cheese factory. This led to a continuous growth model using milk as medium. Thus the problems arising from sulphonamide inhibitors containing mediums described by *Reinbold &*

Reddy could be avoided. It was also of interest to study whether the partial loss of antibacterial activity of sulphonamides in milk (Ziv 1980) would influence the inhibition in cheese factory conditions.

MATERIALS AND METHODS

Two lactobacilli, *Lactobacillus lactis* ATCC 12315 and *Lactobacillus helveticus* ATCC 15009, and *Streptococcus thermophilus* ATCC 19258 were used in the studies. The strains were grown in 10 ml test tubes of MRS-broth (Oxoid LTD., London, England) at 30°C for 24 h in an anaerobic jar (GasKit, Oxoid LTD., London, England). After the incubation the cultures were tested by the API 50 Lactobacilli (API System S.A., La Balme Les Grottes, France) for the purity and divided into 2 ml test tubes which were restored at -70°C to be used as inoculums in the study.

In the test model the strains were cultured in commercial skimmilk. The skimmilk was tested for inhibitory substances by the modified Thermocult method (Orion Diagnostica, Helsinki, Finland) described by Soback & Lamminsivu (1979) before it was sterilized by boiling for 20 min 3 times during 3 consecutive days. The residual fat was removed after the first boiling.

The incubation took place in an apparatus (Fig. 1) especially designed for this purpose. The waterbath was equipped with a thermostat (Thermo-Boy Type B 1, MGW Lauda, Federal Republic of Germany). Two anaerobic jars (6.5 l) each containing a 1.5 l Erlenmeyer flask were put in the waterbath. Magnetic stirrs (Heidolph MR-80, Federal Republic of Germany) were placed under the waterbath and the magnetic bars in the flasks. Palladium coated aluminium oxide catalysts (Baltimore Biological Laboratories, Maryland, U.S.A.) were used.

Each experiment began by heating 1.0 l of milk in each of the flasks to boiling point in order to remove oxygen from the milk. Before the flasks were placed in the anaerobic jars 4 ml Resazurin (British Drug House LTD. No. 20101, Poole, England) solution containing 1.0 mg Resazurin were added as redox potential indicator and 1.0 ml of Sulfadiazine 1 µg/ml solution was added to one of the flasks hereafter called the test flask. Before closing, two gas producing GasKit envelopes (Oxoid LTD., London, England) were put in each jar to produce anaerobiosis. The temperature in the waterbath was adjusted to 50°C which re-

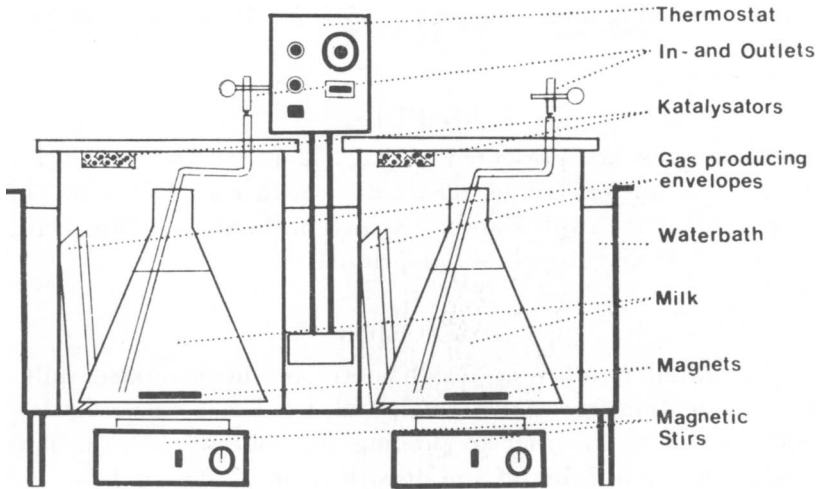


Figure 1. The incubation apparatus.

sulted in a temperature of 46°C in the milk. The rotation speed of the magnetic stirs were adjusted to 275 r.p.m. After 18–20 h from the onset of each experiment a sample of 10 ml was taken from both flasks. From this sample an aliquot was taken to check the sterility of the milk before the inoculation. This was done by culturing the samples on blood agar aerobically and anaerobically and on Rogosa agar (Oxoid LTD., London, England) or Lee's agar (Lee *et al.* 1974) anaerobically. The initial pH and lactic acid levels were measured from the same samples. For pH determinations an IM-555 pH & mV meter (Datex LTD., Helsinki, Finland) was used. The pH was always determined immediately after sampling. Lactic acid concentrations were measured according to Pryce (1969) using a Beckman-DB spectrophotometer (Fullerton, Ca., U.S.A.). Before each experiment an aliquot of the inoculum was taken for plate count determination and 0.1 ml was mixed with 9.9 ml of sterile milk and pipetted in each flask through an inlet pipe. The inoculation was done only if the milk in the flasks was free of the pink colour of Resazurin indicating that no leakage of oxygen had taken place. Samples of 10 ml were taken from both flasks 0, 1, 3, 5, 7, 9, 11 and 24 h after the inoculation. Each sample was investigated for plate count, pH and lactic acid concentration. Plate counts for lactobacilli were done from Rogosa pour plates and for *S. thermophilus* from spread plates on Lee's agar. The plates

were incubated in anaerobic jars at 35°C for 48 and 24 h, respectively.

RESULTS

The results are presented in Figs. 2 to 4. The values are means \pm s.e.m. for 3 experiments done with each bacteria. The s.e.m. values for the pH's are not shown in the figures thus being less than 0.01 and therefore not printed.

DISCUSSION

The initial number of test bacteria in the skimmed milks after inoculation were intentionally adjusted to a relatively low level (10^3 — 10^4) in order to prolong the time of growth. This enabled the monitoring of the growth in more detail. The temperature was chosen to be close to 50°C thus resembling the temperature present in the beginning of the pressing process in Emmental cheese making (Gehrig *et al.* 1975). The concentration of Sulfadiazine was adjusted to 1 $\mu\text{g}/\text{ml}$ because it is the lowest detection level of the Thermocult method for this compound.

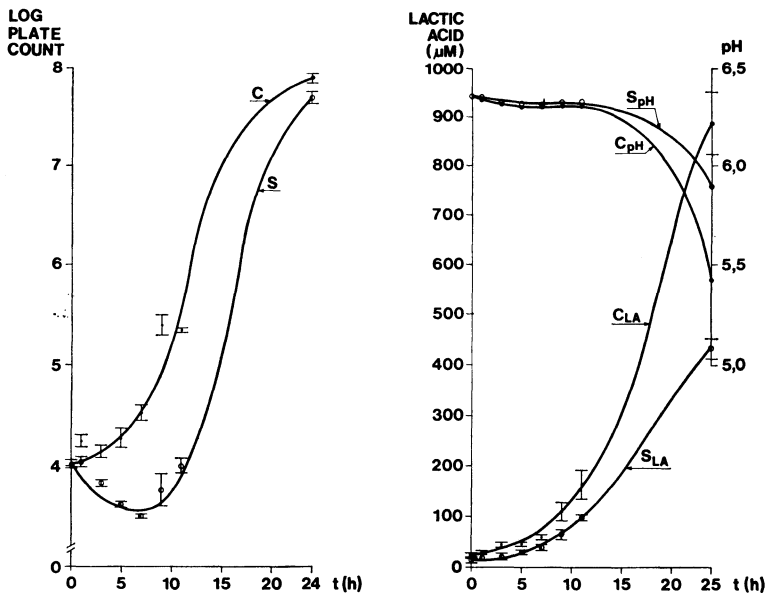


Figure 2. The plate count, pH and lactic acid concentration of *Lactobacillus lactis* (C = control and S = test).

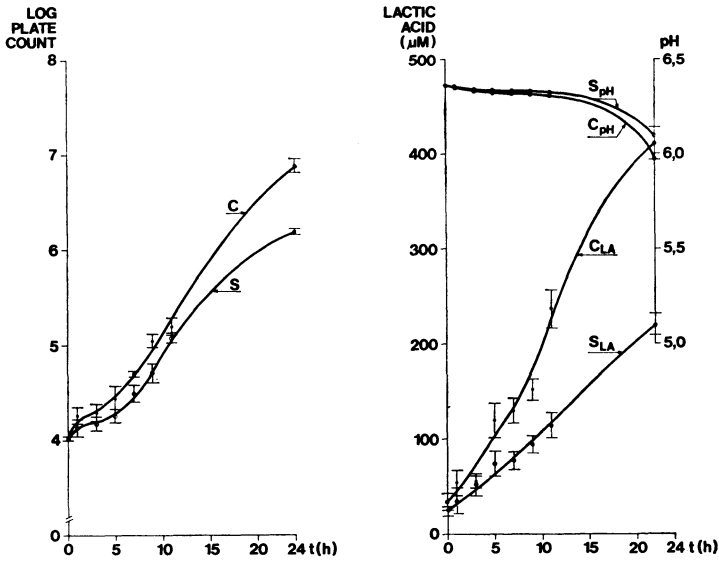


Figure 3. The plate count, pH and lactic acid concentration of *Lactobacillus helveticus* (C = control and S = test).

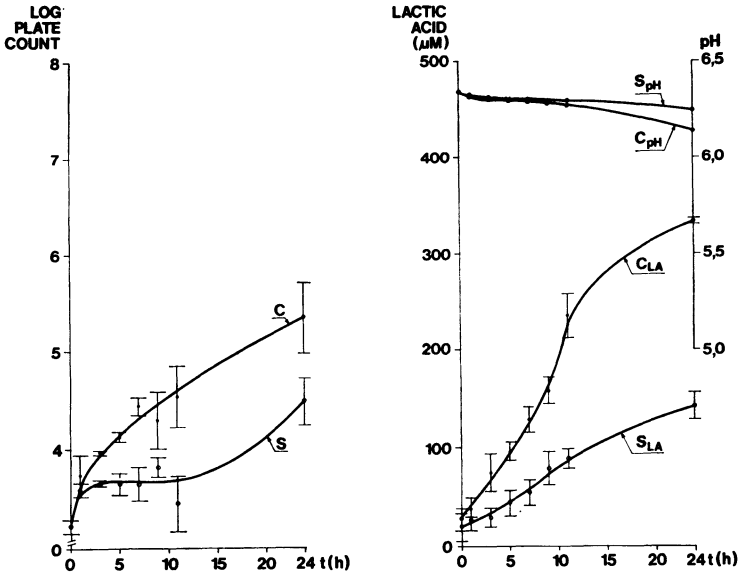


Figure 4. The plate count, pH and lactic acid concentration of *Streptococcus thermophilus* (C = control and S = test).

According to the results Sulfadiazine was found to inhibit the growth of all the bacteria studied but to a different degree with reference to time. In the case of *L. lactis* (Fig. 2) there is a clear decrease in the growth in the test flask during the first 7 h of incubation. *S. thermophilus* reaches a plateau of growth in the test flask after 1 h which remains stable during the next 10 h (Fig. 4). The growth rate of *L. helveticus* is least affected by Sulfadiazine during the first 11 h after incubation. The growth rate is practically the same as in the control medium but on a lower level (Fig. 3). After 24 h from inoculation the difference between the plate counts determined from samples taken from the control flask and the test flask is smallest in the case of *L. lactis*. The difference in plate counts is largest for *S. thermophilus* but only for *L. helveticus* the difference seems to be growing at this stadium. The growth kinetics in the control flasks are in agreement with earlier studies reviewed by *Mocquot* (1979).

For each of the tested bacteria the effect on the pH decrease due to Sulfadiazine was hardly detectable during the first 11 h of incubation (Figs. 2 to 4).

The difference in the mean pH's after 24 h of incubation was only 0.1 for *L. helveticus* or *S. thermophilus* (Figs. 3 and 4). Correspondingly the difference of the mean pH's for *L. lactis* was found to be 0.5. The Sulfadiazine thus markedly affects the measured pH lowering ability of *L. lactis* at 24 h in the milk as shown in Fig. 2. The difference in the plate count at the same time was only about log 0.2/ml.

The inhibition of lactic acid production due to Sulfadiazine is consistent throughout the experiment for each bacteria studied (Figs. 2 to 4). The rate of the inhibition is longer for *L. helveticus* and *S. thermophilus* compared to that for *L. lactis* during the 11 h from incubation. This appears slightly controversial when the results are compared to the inhibition rates for the plate counts for the respective bacteria. At 24 h after inoculation the production of lactic acid was reduced to half due to Sulfadiazine inhibition for each bacteria studied. At this stage the inhibition in lactic acid production for *L. lactis* seems to be poorly correlated to the difference in plate count at this time.

All the parameters measured in the test model were able to detect the inhibitory effect due to Sulfadiazine on the bacteria used in this study. The effect of the decrease of pH was difficult

to read during 11 h after inoculation obviously due to the buffering capacity of milk. Measurement of inhibition in lactic acid production seems to be a better indicator than plate count for Sulfadiazine effect due to its consistent behaviour.

CONCLUSION

Changes in each of the 3 parameters, growth rate, decrease of the pH and production of lactic acid, used in this study indicated a depressing effect due to Sulfadiazine on the test microbes, *Lactobacillus lactis*, *Lactobacillus helveticus* and *Streptococcus thermophilus*, in skimmed milk medium.

The measurement of the inhibition in lactic acid production seems to be best suited for testing milk for Sulfadiazine residues.

Streptococcus thermophilus should be preferred as test organism because of its fast and continuing response to Sulfadiazine inhibition in lactic acid production and growth.

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SAMMANFATTNING

Växt av några mjölksyrabakterier i mjölk med Sulfadiatsinhalten.

En metod för testning av sulfonamidens effekt på mjölksyrabakterier utvecklades. Sulfadiatsin användes som inhiberande substans. I undersökningen användes tre mjölksyrabakterier, *Lactobacillus lactis*, *Lactobacillus helveticus* och *Streptococcus thermophilus*. Mjölksyraproduktion, pH och plate count användes som parametrar, när Sulfadiatsins effekt på testorganismer undersöktes under anaerobiska förhållanden i mjölk medium.

Sulfadiatsin förorsakade en nedsättande effekt på varje testmikrob, vilket kunde påvisas med alla använda parametrar. Mätning av inhibition i mjölksyraproduktion med *Streptococcus thermophilus* borde användas, när snabb undersökning av Sulfadiatsin restkoncentrationer i mjölk förutsättes.

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