# Secretory and serum antibodies against *Streptococcus lactis*, *Streptococcus thermophilus*, and *Lactobacillus bulgaricus* in relation to ingestion of fermented milk products

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Serum, saliva, and urine were analyzed for the presence of IgA, IgG, and IgM antibodies reactive with the yoghurt bacteria *Streptococcus thermophilus* and *Lactobacillus bulgaricus*. A comparison was made between four subjects who frequently ate yoghurt and four subjects who never ate yoghurt. Salivary IgA and serum IgG activity against the milk-fermenting bacterium *S. lactis* was studied in five other subjects before, during, and after a period of ingestion of a fermented milk product, *filmjölk*. All analyses were carried out by an enzyme-linked immunosorbent assay method. Antibody activity against the yoghurt bacteria was found in saliva, serum, and urine. No difference in antibody activity between yoghurt eaters and non-yoghurt eaters was measured for salivary IgA, but for serum IgG a lower activity against *S. thermophilus* was present among the yoghurt eaters. Antibody activity against *S. lactis* was present already before the ingestion of *filmjölk* began, and the activity was not altered during the period of ingestion. It is concluded that in adult subjects, the ingestion of milk-fermenting bacteria does not result in a significant change in the antibody activity against these bacteria. □ *IgA; oral immunization; salivary antibodies; yoghurt*

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On the assumption that *Streptococcus mutans* is a cariogenic microorganism and that salivary anti-*S. mutans* antibodies may prevent its colonization of the teeth, several successful experiments have been performed aiming at raising the levels of such antibodies by swallowing large amounts of the bacteria (1, 2). In these studies it has been observed that antibody activity can be demonstrated in saliva before immunization, an activity possibly induced by the *S. mutans* colonizing the teeth.

It has, however, been noted that salivary IgA antibodies against *S. mutans* can also be found in subjects who apparently do not harbor this organism (3). Furthermore, *S. mutans*-reactive antibodies were demonstrated in predentate infants, a fact that is rather surprising, since *S. mutans* colonizes the oral cavity only after the eruption of the teeth (4). One explanation for these findings is that the production of the antibodies may be stimulated by organisms with cross-reacting antigens. Another approach to stimulate the production of anti-*S. mutans* antibodies would therefore be to find a relevant cross-reacting bacterial strain that could be used for immunization. In the search for potential antigens cross-reacting with *S. mutans*, Kilian (5) has shown *Escherichia coli* and *Klebsiella* to lack such antigens. Bammann & Gibbons found that 'mixed yoghurt culture', most likely consisting of the yoghurt bacteria *S. thermophilus* and *Lactobacillus bulgaricus*, and salivary antibodies reactive with *S. mutans* serotype c cross-reacted (3). We believe that this manner of immunization would have several advantages from a practical point of view, if the appropriate microorganisms could be found. A first step should be the investigation of the immunological reactions in body fluids to the bacteria ingested in fermented milk products like yoghurt.

## Materials and methods

### *Study I*

Samples of whole saliva, parotid saliva, serum, and urine were collected from four subjects with daily consumption of yoghurt containing viable bacteria and from four subjects with no consumption of yoghurt during the last 4–5 years. Samples were collected and analyzed as described below.

### *Study II*

This longitudinal study was carried out with one female and four male healthy subjects. The subjects had not eaten *filmjölk* for at least 6 months before the experiment but had earlier eaten it occasionally. Five samples of whole saliva and serum were collected—samples I to IV on days 1, 7, 14, and 28 of the experiment, respectively, and sample V after day 60. After the sampling on day 7, the subjects began a regular daily ingestion of 1–5 dl commercially available *filmjölk* produced by the regional dairy (Skånemejerier Eslöv, Sweden). After day 28, the subjects discontinued the ingestion of *filmjölk* until after sample V. Fresh milk was consumed by all subjects throughout the experiment.

### *Bacterial strains*

*S. thermophilus* and *L. bulgaricus* used for yoghurt production were kindly provided by Skånemejerier in mixed culture. The *Streptococcus* and *Lactobacillus* were isolated.

During the experimental period an isolate of *S. lactis* was obtained from commercially available *filmjölk* (Skånemejerier) by culturing on SS agar (Merck, Darmstadt, FRG). The identity of the isolate was confirmed by biochemical and serological methods. *S. mutans* KPSK2, serotype c, was obtained from the culture collection at the Department of Cariology, University of Lund.

*E. coli*, strains 45/41, 49/41, 44/41, Bi7458/41, Bi7509/41, G3404/41, F10018, DM3219/54, and E36 were kindly provided by Dr. Frits Örskov, International Escherichia and Klebsiella Centre (Statens Serum Institute, Copenhagen, Denmark).

### *Antigen preparation*

*S. mutans* KPSK2, *S. thermophilus*, and *S. lactis* were inoculated in dialyzed tryptose–yeast medium (6) and incubated anaerobically at 37°C (*S. mutans, S. lactis*) or 40°C (*S. thermophilus*) for 24 h. *L. bulgaricus* was inoculated in Todd–Hewitt broth and incubated anaerobically at 37°C for 24 h. The bacteria were harvested by centrifugation, washed twice in phosphate-buffered (0.01 M) saline (0.15 M NaCl) (pH 7.2) (PBS) and resuspended in 0.75% formaldehyde–PBS solution. After incubation at 4°C for 24 h the bacteria were washed twice and resuspended in PBS to an optical density of 1.0 (*S. mutans, S. thermophilus*, and *L. bulgaricus*) or 0.4 (*S. lactis*) at 650 nm. The *E. coli* strains were inoculated in dialyzed tryptose–yeast medium (6) and incubated aerobically at 37°C for 24 h and harvested. Antigen extracts were prepared from equal amounts of suspension with OD 1.0 (650 nm) in accordance with Rantz & Randall (7). The antigen extracts were mixed, thus forming a pool of *E. coli* antigens containing the somatic antigens 01, 02, 04, 06, 07, 08, 018 abc, and 075. The *E. coli* antigen pool was diluted to 1:250 before use.

### *Collection of body fluids*

Whole saliva was collected during 5 min of stimulation by paraffin chewing and immediately frozen. Approximately 5 ml parotid saliva was collected by means of Curby cups during 10–15 min of citric acid stimulation and was then immediately frozen. Serum was prepared from venous blood clotted at room temperature and subsequently frozen. Urine was collected in the morning and frozen within 2 h. The samples were stored at −20°C.

### *Antibody determination by ELISA*

The antibody activity was determined by a previously described enzyme-linked immunosorbent assay (ELISA) technique (8, 9) with some modifications. The assay was carried out in Microelisa M129A microtiter plates (Dynatech Produkt AG, Kloten, Switzerland), in which all samples were made in

quadruplicate. The plates were incubated at 37°C overnight with 200 µl of antigen preparation in each well. The saliva samples were centrifuged at 2000 *g* for 20 min.

In study I, serum was diluted in PBS + 0.05% Tween 20 (PBST) to 1:500, and whole saliva, parotid saliva, and urine to 1:2 in the same medium. In study II, serum was diluted to 1:500 and whole saliva to 1:4 in PBST.

After incubation with antigen as mentioned above, the microtiter plates were washed twice with PBS and incubated with 150 µl of the diluted samples at 37°C for 3 h. The plates were then washed three times for 3 min with saline (0.15 M NaCl) + 0.05% Tween 20 (ST), and 150 µl of rabbit anti-human IgA, rabbit anti-human IgG, or rabbit anti-human IgM (Behringwerke AG, Marburg, FRG) diluted to 1:1000 in PBST were applied. After 18 h at room temperature, the plates were washed three times for 3 min in ST, and 150 µl swine anti-rabbit IgG alkaline phosphatase conjugate (Orion Diagnostica, Helsinki, Finland) diluted 1:200 in PBST were added. The plates were incubated at 37°C for 5 h and washed as previously described. The phosphatase activity was determined by adding 150 µl *p*-nitrophenylphosphate solution (p-NPP (Sigma, St. Louis, Mo., USA) dissolved in 1.0 M diethanolamine, 0.00024 M $MgCl_2$ buffer, pH 9.8; final concentration, 1 mg/ml) to each well and incubating the plates at 37°C in darkness.

After approximately 50 min the absorbance at 405 nm was read by means of a spectrophotometer (Titertek Multiscan, Eflab Oy, Helsinki, Finland). The results were expressed as mean absorbance of the quadruplicate multiplied by 100/t, where t is the time (in minutes) after which the absorbance was read.

### *Inhibition test of cross-reactivity between* S. lactis *and* E. coli *antigen preparations*

Cross-reactivity between *S. lactis* and *E. coli* antigens was tested by an inhibition test. Aliquots of pooled whole saliva, diluted 1:2, and the *E. coli* antigen preparation, diluted 1:20 to 1:2560, were mixed and incubated at 37°C for 3 h. Saliva incubated with PBS served as positive control. After incubation the samples were analyzed by ELISA for IgA activity against *S. lactis* and *E. coli* as described above.

### *Determination of total amount of IgA in saliva*

In study II the amount of IgA in each saliva sample was determined by an immunobead ELISA method (10) adapted to saliva samples by Bratthall & Ellen (11). The assay was carried out with anti-human IgA ($\alpha$-chain-specific)-coated immunobeads obtained from Bio-Rad Laboratories (Richmond, Calif., USA) and alkaline phosphatase-conjugated anti-human IgA ($\alpha$-chain-specific) from Orion Diagnostica (Helsinki, Finland). A standard was prepared from standard human serum (Behringwerke AG, Marburg, FRG) containing 2.39 mg IgA/ml.

### *Statistical methods*

Differences between distributions were compared by Wilcoxon's rank sum test. The level of significance was set at $p < 0.05$ on the basis of two-tailed tests.

## Results

### *Study I*

Antibody activity against *S. thermophilus* and *L. bulgaricus* was found in the body fluids from both yoghurt eaters and non-yoghurt eaters (Fig. 1). Antibodies against *S. thermophilus* were present in most samples with the exception of urine, in which IgA and IgM against this bacterium were not detectable above background control levels.

All classes of antibodies against *L. bulgaricus* were present in all body fluids. Significantly lower IgG activity in serum and IgM activity in whole saliva against *S. thermophilus* were found among the yoghurt eaters than the non-yoghurt eaters. Serum IgM against *L. bulgaricus* was also lower among the yoghurt eaters. In the other Ig classes and body fluids no statistically significant differences were found between the

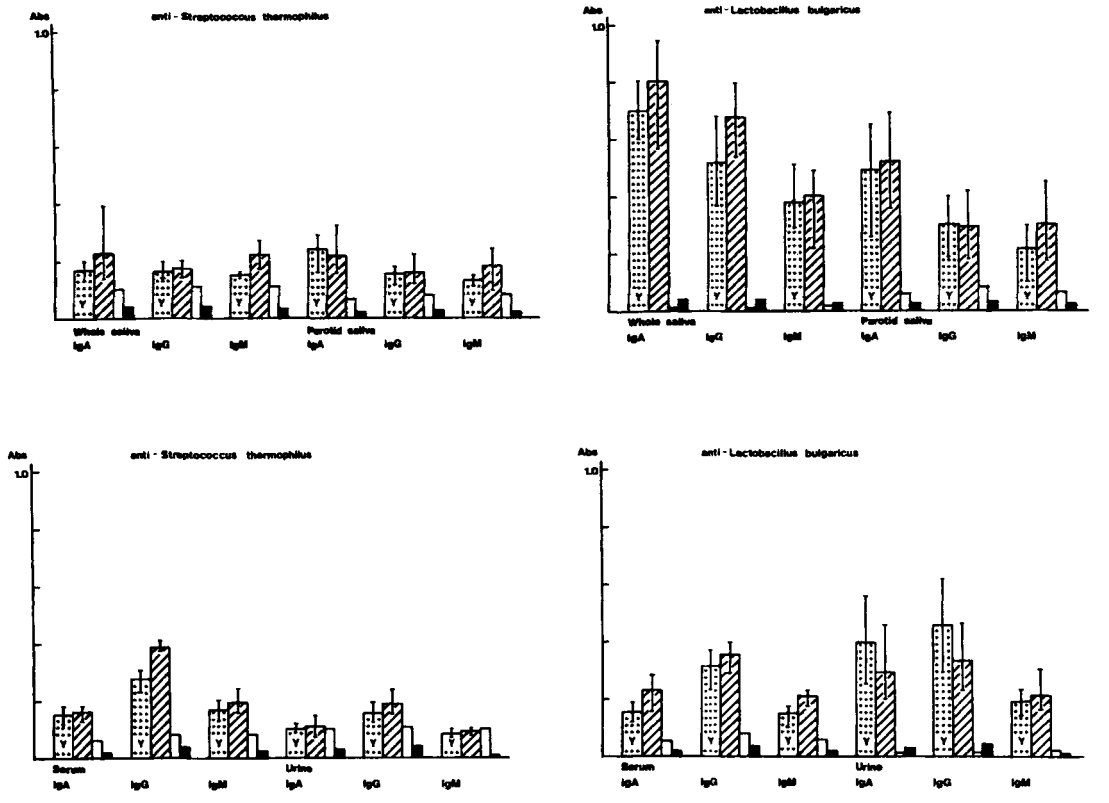


Fig. 1. Antibody activity, expressed as ELISA absorbance values, against *S. thermophilus* (left) and *L. bulgaricus* (right) in body fluids from yoghurt eaters and non-yoghurt eaters.

two groups. This was also true for antibodies against *S. mutans* KPSK2.

*Study II*

Anti-*S. lactis* IgA activity could be demonstrated in all saliva samples. The changes in anti-*S. lactis* IgA activity relative to anti-*E. coli* IgA activity per amount of IgA in saliva during the experiment are shown in Fig. 2. In serum, IgG reactive with *S. lactis* was also present in all samples. The variation in serum anti-*S. lactis* IgG activity during the experiment is shown in Fig. 3. The differences in saliva IgA activity and in serum IgG activity between the different sampling occasions were not statistically significant.

The inhibition test for cross-reactivity between the *E. coli* antigen prepration and *S. lactis* showed that the anti-*S. lactis* activity in saliva was not affected by addition of *E. coli* antigen, whereas the anti-*E. coli* activity was reduced (Fig. 4).

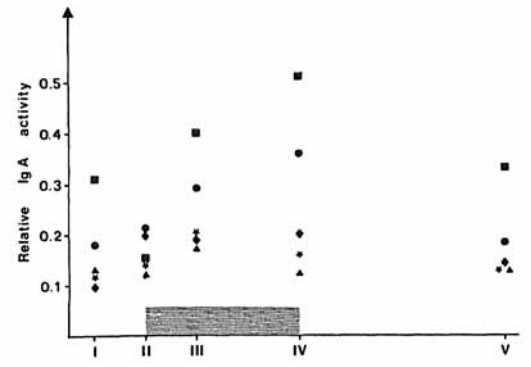


Fig. 2. Salivary IgA activity against *S. lactis* for the five subjects at the different sampling occasions. Ingestion of *filmjölk* between samples II and IV. IgA activity expressed as IgA anti-*S. lactis* activity/anti-*E. coli* activity per mg IgA/l saliva.

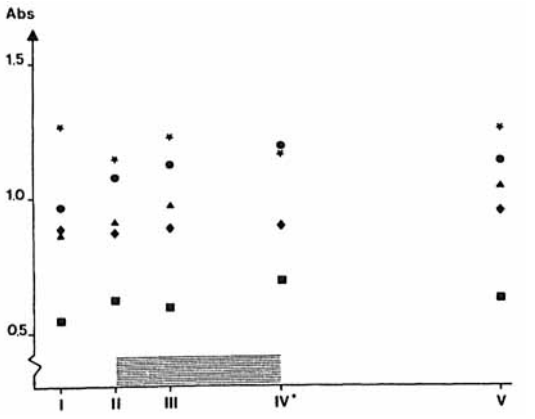


Fig. 3. Serum IgG activity against *S. lactis* for the five subjects. Ingestion of *filmjölk* between samples II and IV. IgG activity is expressed as ELISA absorbance. IV* One sample lost at sample IV.

## Discussion

Secretory IgA antibody activity can be induced by various organisms such as viruses (12), *E. coli* (13), and *S. mutans* (2) administered orally. The mode of administration of the bacteria therefore does not seem to be the most likely explanation for the lack of immune response to *S. thermophilus, S. lactis*, and *L. bulgaricus* in the present study.

Antibody activity against the yoghurt bacteria was found in secretions from both yoghurt and non-yoghurt eaters. This may indicate a certain degree of cross-reactivity between the antigens of the yoghurt bacteria and other antigens to which the two groups had been exposed. Since whole cells were used in the analysis of antibody activity, activities against both common and strain-specific antigens were measured. Differences between yoghurt and non-yoghurt eaters in antibody activity against antigens specific for the yoghurt bacteria could then be masked by activity against cross-reacting antigens that the two groups were equally exposed to.

A difference was observed in serum IgG against *S. thermophilus*, with a lower activity

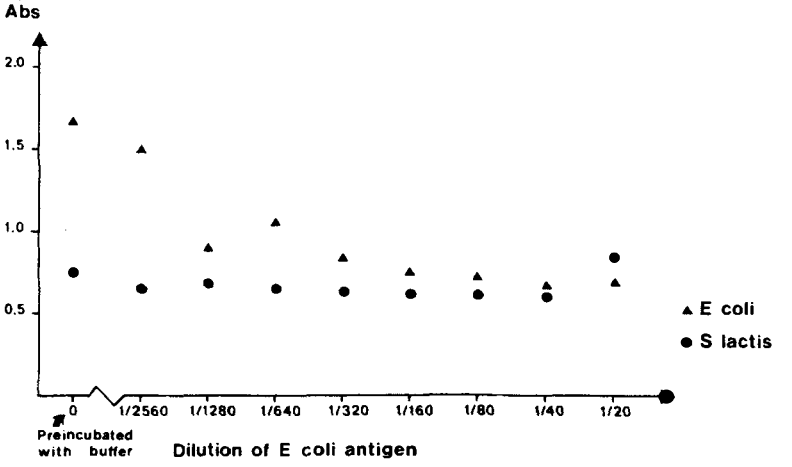


Fig. 4. Inhibition test for cross-reactivity between *S. lactis* antigens and *E. coli* antigens. Anti-*S. lactis* and anti-*E. coli* IgA activity in saliva after pretreatment with different dilutions of *E. coli* antigen. IgA activity expressed as ELISA absorbance.

against the bacterium among the yoghurt eaters. The nature of the depressed IgG activity is not clear. If not a coincidental finding, it can be interpreted as the result of an induced systemic tolerance to the bacterium, a common result after peroral exposure to an antigen (17).

No significantly increased immune response to *S. lactis* was elicited, although the increased IgA activity in some subjects may suggest a stimulation to have taken place. The findings may be explained in different ways.

Antibody activity was demonstrated already at the first two sampling occasions. This could be a response to an earlier exposure to *S. lactis* to a level at which further increase would be difficult to obtain. This is, however, not supported by the findings of Mestecky et al. (2), who demonstrated a pronounced secondary IgA response induced by an orally administered booster dose given 2 months after the primary immunization.

A critical point is the dose of antigen used to raise a secretory immune response (14). In animal models, doses below a certain level did not elicit a secretory antibody response, whereas a tenfold antigen dose did. However, if a dose hundredfold to the optimal was given, no response could be demonstrated. If these conditions apply to humans, the lack of response in this study could be explained by either sub- or supra-optimal doses of antigen. In the present study each subject can be estimated to have ingested $10^9$ cells daily. For *S. mutans* $10^{11}$ cells have been reported to give a salivary anti-*S. mutans* IgA response (2).

In two studies showing an induction of salivary IgA response after oral administration of *S. mutans* (2, 16), the cells were given in enteric-coated capsules. In a similar experiment in which *S. mutans* cells were given in a suspension, Gahnberg & Krasse (15) found no increase in the salivary IgA activity against *S. mutans*. This may indicate that exposure to saliva and gastric juice can interfere with the immunogenicity of the antigen. In the present study the bacterial cells were not only exposed to saliva and gastric juice but also to compounds in the milk in which they have grown. This may account for the lack of response to the antigens found in this study.

In conclusion, this study does not provide support to the idea of increasing the salivary IgA antibody activity against milk-fermenting bacteria by ingestion of fermented milk, even if the possibility cannot be excluded that in some subjects antibody production was stimulated. However, since antibody activity against these bacteria was demonstrated in saliva, an influence on oral bacteria via antigens present in the diet is still possible.

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