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Conclusions: The data demonstrate that soluble starch is substantially less cariogenic than glucose/sucrose.

Significance and Impact of the Study: By leading to the same scientific evidence as its in vivo counterpart, the described in vitro biofilm system provides an interesting and valuable tool in the quest to reduce experimentation with animals.
Cariogenicity of soluble starch in oral in vitro biofilm and experimental rat caries studies: a comparison

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Running title: Cariogenicity of starch

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Keywords: biofilms, starch, experimental caries, demineralization, quantitative light-induced fluorescence
**Introduction**

In 1890, Miller reported that acid produced after incubating a bread-saliva mixture with oral microorganisms was capable of demineralizing tooth enamel. Based on these *in vitro* studies Miller concluded that starch was more cariogenic than sugar (Miller, 1973 reprinted from the original work from 1890). Ever since, cariogenicity of starch is a matter of debate. Epidemiological studies indicated, for example, lower caries prevalence in a South African rural population consuming starch as the main carbohydrate source in comparison to other groups living in a more urban environment and eating a mixture of starch and sucrose (Staz, 1938; Retief et al., 1975; Cleaton-Jones et al., 1984). These findings suggested that caries incidence might be lower in populations consuming traditional starch-based foodstuffs than in urban populations with a sucrose-rich refined diet characteristic for many urban environments (Schmid et al., 1987), clearly contradicting Miller's original hypothesis. In the past decades a large number of studies addressed the cariogenicity of starch (reviewed by Rugg-Gunn, 1993; Grenby, 1997; Moynihan, 1998; Lingström et al., 2000; Moynihan, 2005). The inconsistent results obtained were in part explained by the fact that different textures or degrees of hydrolysation of starch were investigated and compared (Rugg-Gunn, 1993; Moynihan, 1998 for review). In animal experiments starch was found to lead to lower caries incidence compared to sucrose, glucose or fructose (Guggenheim et al., 1966; Moynihan, 1998). On the other hand, animal and *in situ* studies with mixtures of starch and sucrose revealed enhanced cariogenicity compared to starch alone (Shaw, 1980; Firestone et al., 1982; Ribeiro et al., 2005). *In vitro* studies by Brudevold *et al.* (1988) indicated that the cariogenicity of starch is related to both its digestion by salivary amylase and the presence of an
acidogenic plaque. The effect of low molecular weight degradation products of starch on both glucan synthesis produced from sucrose by *Streptococcus mutans* and microbial adherence has been discussed controversially on the basis of results from *in vitro* test systems (Gibbons and Nygaard, 1968; Newbrun et al., 1977; Balekjian et al., 1980; Vacca-Smith et al., 1996).

The objective of this study was to address the hypothesis that soluble starch is less cariogenic than sugars in biofilms growing in vitro or on rat teeth. We investigated the demineralization potential of starch, sucrose and glucose, *in vitro* and *in vivo*. In addition, in the *in vitro* biofilm tests carried out with the "Zürich biofilm model" (Guggenheim et al., 2001a; Guggenheim et al., 2001b; Shapiro et al., 2002; Gmür et al., 2006), the effect of these carbohydrates on the microbial composition of the biofilms were determined. In an animal experiment the effect of these carbohydrates on fissure, smooth surface caries, and dental plaque formation was examined. The application of an *in vitro* and *in vivo* assay allowed a comparison between the results of these two test systems.

**Materials and methods**

**In vitro biofilm experiments**

The procedures to produce six-species biofilms have been described in detail (Shapiro et al., 2002; Thurnheer et al., 2003). In brief, *Actinomyces naeslundii* OMZ 745, *Candida albicans* OMZ 110, *Fusobacterium nucleatum* KP-F2 (OMZ 596), *Streptococcus oralis* SK 248 (OMZ 607), *Streptococcus sobrinus* OMZ 176, and *Veillonella dispar* ATCC 17748^T^ (OMZ 493) were used for biofilm formation. Biofilms
were grown in 24-well polystyrene cell culture plates on bovine enamel disks of 6.8 mm Ø (Gmüör et al., 2006) that had been preconditioned for pellicle formation in whole unstimulated pooled saliva (in the following termed saliva). The processing of batches of saliva has been described in detail by Guggenheim et al. (2001a). To initiate a biofilm experiment disks were covered for the first 16.5 h with 1.6 ml of growth medium containing 70% saliva, 30% modified fluid universal medium (mFUM) (Guggenheim et al., 2001a) and 200 µl of a cell suspension prepared from equal volumes and densities of each strain.

During the initial culture period the carbohydrate component of the medium was 0.3% glucose. Thereafter, the growth medium contained besides 70% saliva either 30% strongly buffered mFUM for assays A-C, or weakly buffered FUM (Gmüör and Guggenheim, 1983) for assays D-F (see footnotes of Table 1). The growth medium was supplemented with glucose, sucrose and/or Zulkowsky’s soluble starch in various combinations amounting to 0.3% or 1% (Table 1). Soluble starch with an average molecular weight of 5000 and an average polymerization grade of 31 (Zulkowsky, 1880) was purchased from Merck (Darmstadt, Germany). Biofilm-covered disks were washed after 16.5, 20.5, 24.5, 40.5, 44.5 and 48.5 h by three consecutive dips in 2 ml of water (1 min per dip). They were incubated anaerobically at 37°C. At the end of the experiment (64.5 h), the pH of the culture supernatant was measured and biofilms were harvested at room atmosphere in 1 ml 0.9% NaCl by vigorous vortexing. Total CFU, streptococci and all taxa were assessed by anaerobic culture using selective and non-selective media (Guggenheim et al., 2001a; van der Ploeg and Guggenheim, 2004).

**Determination of amylase activity**
α-Amylase activity present in saliva and biofilm culture supernatants was determined with the Phadebas® test kit (Pharmacia, Dübendorf, Switzerland) according to the instructions of the manufacturer. Besides saliva, fractions of unprocessed saliva and freshly pasteurized saliva were tested for amylase activity. The amount of enzyme catalyzing the hydrolysis of 1 µmol glucosidic linkage per minute at 37°C is defined as 1 unit (U) of amylase activity.

Bacterial strains were screened for amylase activity as follows (Kilian and Nyvad, 1990): Single strains were grown anaerobically for 72 h at 37°C on mFUM-agar with 1% soluble starch and stained by covering the surface with Lugol's iodine. Strains that showed a cleared zone around colonies were scored amylase-positive.

**Demineralization of bovine enamel disks**

Enamel disks were harvested after 64.5 h and freed of any biofilm remnants by maximum vortexing. Demineralisation was measured *in vitro* by quantitative light-induced fluorescence (QLF) as described (Gmür et al., 2006) and expressed by ΔF, which is defined as the percent change in fluorescence radiance in each image point averaged over the entire analysis area of the disk.

**In vivo rat study**

The animal study was approved by the "Veterinäramt des Kantons Zürich" and conformed to the Swiss laws on animal protection. The experiment was conducted on 10 litters of Osborne-Mendel rats distributed at random among five treatments (Table 3). To avoid fissure impaction with food and bedding particles the pups and their dams were transferred on day 13 after birth to stainless-steel screen-bottom cages without bedding and fed a finely ground stock diet (diet No. 890, Nafag,
Gossau, Switzerland). Tap water was given *ad libitum*. On day 20 after birth, 50 pups were weaned and offered for three days drinking water with 2% glucose and 2% sucrose and the modified cariogenic diet 2000a (Guggenheim et al., 1966) containing 40% sucrose, 28% skim milk, 24% wheat flour, 5% brewer’s yeast, 2% Gevral protein (Whitehall-Robins, Zug, Switzerland), and 1% NaCl. On days 21 and 22, all rats were associated orally, twice daily, with the mixture of strains described above by inoculating 200 µl of a freshly prepared bacterial suspension using a 1 ml syringe without a needle. On day 23, the pups were distributed at random among five treatments (see Table 3), one animal per cage. Cages were connected to a programmed feeding machine (König et al., 1968; König-Hofer FAG 72 KT, Aathal-Seegräben, Switzerland) delivering 36 meals per day. Each meal consisted of 400 mg of the above-described basic diet (control group) or supplemented in the four test treatments with glucose, sucrose and/or soluble starch in various combinations (see Table 3). Drinking water, without glucose and sucrose, was available *ad libitum*.

To allow most of the care and feeding of the animals to take place during normal working hours, the circadian rhythm was reversed between days 16 and 19 by prolonging the active phase of the rats by 3 h each day. By day 19, the active phase for the animals was from 10.00 to 22.00 h. During this period the animals received their 36 meals, one every 20 min. This feeding regime continued for 40 days until the animals were 62 days old. On day 62 the animals were anesthetized with CO₂ and decapitated. The upper and lower jaws were dissected and immersed in fixative (10% phosphate-buffered formalin) for a minimum of 72 h. Erythrosin-stained maxillary molars were evaluated for plaque extent (Regolati and Hotz, 1972) and smooth-surface caries (Keyes, 1958). Mandibular molars were sectioned and scored for fissure caries (König et al., 1958).
**Statistical Analyses**

The null hypothesis that there was no microbiological difference between biofilms grown with different carbohydrate supplements was tested by paired t-tests. Data on enamel demineralization measured by QLF were tested analogously. Data from the rat study were compared by two-way analysis of variance and calculation of least significant differences. All statistical analyses were done with Statview 5.01 (SAS Institute, Cary, NC).

**Results**

**Biofilm experiments**

Only *Act. naeslundii* and *Fus. nucleatum* produced amylase activity when grown planktonically. Salivary amylase activity decreased slightly during saliva processing from 164'267±924, 143’200±8119, to 119’467±11’602 U for unprocessed, pasteurized, and processed saliva, respectively. In biofilm culture supernatants the amylase activity reached 184’267±128’430, 231’200±127’245, and 204’267±10’9601 U after 16.5, 40.5, and 64.5 h of growth (mean values and standard deviations from triplicates).

The effects of the different concentrations of glucose, sucrose and starch (see footnotes of Table 1) on the microbial composition of the biofilms were studied with a strongly and a weakly buffered medium (assays A-C and D-F, respectively). Among strongly buffered biofilms exposed to a total carbohydrate concentration of 0.3%, no
major microbial shifts occurred (Fig. 1), although the differences between assays A and C in total CFU, CFU of total streptococci, CFU of *Act. naeslundii*, *Fus. nucleatum* and *Strep. oralis*, but notably not *Strep. sobrinus*, were significant (Table 1, Table 2). With biofilms grown in weakly buffered saliva/FUM and a total carbohydrate supplement of 1% (composed of glucose/sucrose, glucose/sucrose/starch, or starch; assays D to F) microbial biofilm populations differed between treatments (Fig. 1, Table 1). Differences in all bacteria, *Act. naeslundii* and *V. dispers* were significant due to increased CFU numbers (assays D vs F), whereas *Strep. oralis* and *C. albicans* CFU were decreased (*P* = 0.06 and 0.08, respectively; Table 2). Apart from this carbohydrate effect all biofilms formed in weakly buffered medium revealed a smaller total count of bacteria than those propagated in strongly buffered saliva/medium (Fig. 1). *Act. naeslundii* and *Strep. oralis* were affected by several log-steps, and *Fus. nucleatum* dropped to or below the detection limit under these weakly buffered conditions. Exceptional and remarkable was the large variation of *Strep. sobrinus* CFU among individual biofilms exposed to 1% starch under weakly buffered conditions.

Independent of the carbohydrate supplement, no demineralization was observed when biofilms were grown for 64.5 h in strongly buffered medium (Fig. 2). In contrast, differential demineralization was noted underneath biofilms grown in weakly buffered medium. The least demineralization was found with biofilms grown in the presence of starch alone (Fig. 2, Table 1). Greatest mineral loss occurred when biofilms were propagated in the presence of 0.5% glucose and 0.5% sucrose (assay D), whereas significantly less mineral loss was observed in assay E (Table 1, Table 2). Medium harvested after 64.5 h from assays A–F showed respective average pH
values of 6.47, 6.56, 6.65, 4.56, 4.67, and 4.84 with narrow scattering, the latter three values being well below the “critical pH” of enamel demineralization.

Animal experiment
At the end of the experimental period, all animals were in good health. With few, randomly distributed exceptions, the animals consumed all meals offered. With respect to smooth surface plaque formation there were no significant differences amongst the treatments. The incidence of initial dentinal fissure lesions was significantly less in treatment group 5 (15% starch) in comparison to groups 2 (sucrose; \( P<0.001 \)), 3 (sucrose + glucose; \( P<0.01 \)), and 4 (sucrose, glucose and starch; \( P<0.01 \)). Treatments 1 (flour diet) and 5 did not differ (Table 3). The data for initial and advanced dentinal fissure lesion incidence were similar. However, in treatment groups 1 and 5 the advanced dentinal fissure lesion incidence was significantly lower (\( P<0.001 \)) in comparison to the three treatments receiving sucrose (Table 3). Smooth surface caries incidence followed a similar pattern but inter-treatment differences remained insignificant.

Discussion

Variations in types of starch or starchy food are reasons for the controversial results regarding starch cariogenicity (reviewed by Moynihan, 1998). Cariogenicity of raw starch is very low (Rugg-Gunn, 1993; Grenby, 1997). Therefore, and because raw starch is not consumed by men, soluble starch was tested in the present study and since starch-mediated effects will likely be modulated by amylase present in the test-system, amylase activity was investigated. In plaque microbial amylase activity was
shown to be low compared to that of salivary origin (Birkhed and Skude, 1978). Plaque and *in vitro* biofilms are not directly comparable, but our tests proved that amylase activity was present in biofilms as well as in the applied processed saliva. The portion of processed saliva present in the medium contained around 70% of the amylase activity of fresh saliva. Considering the amylase activity in biofilm supernatants after 64.5 h, it follows that at least 40% of the activity was attributable to biofilm-generated amylase. These findings imply that the applied starch was hydrolyzed by the biofilm.

Some studies suggested that initial adherence of *Strep. mutans* and *Strep. sobrinus* is in fact inhibited by hydrolysation products of starch such as maltose (Gibbons and Nygaard, 1968; Newbrun et al., 1977; Balekjian et al., 1980), whereas, using a resting cell *in vitro* test system, it was shown that glucosyltransferases and amylase increase initial adherence (Vacca-Smith et al., 1996). However, results of modulation of initial adherence by such compounds in static adherence test systems cannot be compared with growing biofilms as *in vitro* biofilms the growth conditions determine the microbial composition in these consortia. The effect of partial adherence inhibition is annulated rapidly during the following growth phase. In the present *in vitro* biofilm experiments, *Strep. sobrinus* colonized equally well with all three carbohydrate medium supplements (glucose + sucrose, glucose/sucrose + starch, or starch alone) concentrated at 0.3%. These findings clearly fail to provide evidence for an inhibition of mutans streptococci colonization by starch breakdown products. On the other hand, *Act. naeslundii*, *Fus. nucleatum*, and *Strep. oralis* CFU were significantly reduced with starch as the lone carbohydrate supplement. Such differences are possibly explained by reduced co-adherence in the absence of extracellular polysaccharides produced from sucrose or may reflect less favorable
growth conditions in the absence of these sugars. Effects on enamel
demineralization were not observed under these strongly buffered experimental
conditions.

In biofilms grown in unbuffered medium with 1% carbohydrate of any sort, Act. naeslundii, Fus. nucleatum, Strep. oralis (and to a lesser extent C. albicans and Strep. sobrinus), but not V. dispar, were reduced substantially compared to biofilms grown in buffered medium. This drop is explained by lower acid tolerance. In addition, the presence of starch affected the microbial ecology in a subtle species-specific manner and resulted in highly significantly less enamel demineralization. Under unbuffered conditions, Act. naeslundii and V. dispar reached significantly higher cell numbers in the presence of starch instead of glucose + sucrose, whereas Strep. oralis and C. albicans CFU dropped. The production of comparatively less acid in the presence of starch may be an important factor (pH 4.84 with starch opposed to 4.56 with glucose + sucrose), but can explain hardly the entire phenomena observed. Their causes are most likely multifactorial and must be clarified in future experiments.

To validate the in vitro biofilm analysis and to verify our observation that soluble starch is less cariogenic than sugars, we studied the effects of glucose, sucrose, and soluble starch also in an animal experiment. The basic experimental design was similar to the one used by Firestone et al. (1982), who reported that raw starch had little cariogenic potential, cooked starch was more cariogenic than raw starch, and a 1:1 mixture of sucrose and starch was equally or more cariogenic than sucrose alone. Our findings show that the mixture of dietary starch and sugars resulted in more dentinal fissure caries than starch alone confirming the previous results (Firestone et al., 1982). However, they do not corroborate studies showing that foods containing 1% or more hydrolysable starch in combination with sucrose or other
sugars resulted in enhanced cariogenicity (Mundorff et al., 1990), and that a small amount of added starch increases sucrose cariogenicity (Ribeiro et al., 2005). Importantly, the results from the present in vivo study with rats correspond well with those gained with unbuffered in vitro biofilms where starch alone was barely cariogenic, whereas mixtures of glucose and sucrose with or without the presence of starch caused significantly more enamel demineralization. The correspondence between the described in vivo and in vitro results should contribute towards the elimination of the use of animal caries models, thus implementing 3R (refinement, replacement and reduction) principles as defined by Russell and Burch (1959).

Acknowledgments

We are grateful to André Meier, Martin Gander, and Hilary Holmes for excellent assistance and the University of Zürich for funding.

References


**Legends to Figures**

**Figure 1** Box plot diagram showing the effect of starch on the microbial composition of biofilms. Concentrations of glucose (G), sucrose (S) and starch (ST): 0.15% G + 0.15% S (□); 0.075% G + 0.075% S + 0.15% ST (■); 0.3% ST (■); 0.5% G + 0.5% S (□); 0.25% G + 0.25% S + 0.5% ST (■); 1% ST (□). The last three assays contained no additional buffer in the medium (see footnote to Table 1). Horizontal bars within boxes are median values, whiskers indicate 25 and 75% percentiles. Data originate from three independent experiments run in triplicate.

**Figure 2** Box plot diagram showing the effect of different carbohydrate composition and concentration on the extent of demineralization of bovine enamel disks underneath biofilms. Measurements were made using QLF and are expressed by ΔF. Horizontal bars within boxes are median values, whiskers indicate 25 and 75% percentiles. Data originate from three independent experiments run in triplicates.
Table 1  Biofilm composition (mean CFU ± SD, x 10^6) and enamel mineralization (mean ΔF ± SD) after growth in medium containing glucose and sucrose (assays A and D), glucose, sucrose and starch (B and E), or starch alone (C and F)

<table>
<thead>
<tr>
<th>Assays*,†</th>
<th>investigated microbiota</th>
<th>demineralization</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>all bacteria</td>
<td>streptococci</td>
</tr>
<tr>
<td>A</td>
<td>598.9 ± 137.8§</td>
<td>176.0 ± 53.6</td>
</tr>
<tr>
<td>B</td>
<td>554.4 ± 206.8</td>
<td>180.0 ± 55.0</td>
</tr>
<tr>
<td>C</td>
<td>174.3 ± 92.5</td>
<td>67.8 ± 34.9</td>
</tr>
<tr>
<td>D</td>
<td>45.3 ± 44.7</td>
<td>21.4 ± 34.6</td>
</tr>
<tr>
<td>E</td>
<td>87.6 ± 74.4</td>
<td>24.7 ± 20.5</td>
</tr>
<tr>
<td>F</td>
<td>104.6 ± 59.5</td>
<td>12.7 ± 14.5</td>
</tr>
</tbody>
</table>
Carbohydrate concentrations used in assays: A, 0.15% glucose (g) + 0.15% sucrose (s); B, 0.075% g + 0.075% s + 0.15% starch (st); C, 0.3% st; D, 0.5% g + 0.5% s; E, 0.25% g + 0.25% s + 0.5% st; F, 1.0% st.

† Assays A-C were done with strongly buffered saliva/medium, assays D-F with weakly buffered saliva/medium as growth medium.

‡ Abbreviation: QLF, quantitative light-induced fluorescence.

§ Data originated from three independent experiments run in triplicate (n = 9).
Table 2 Statistical comparison of biofilm composition and demineralization after growth in medium containing glucose and sucrose (assays A and D), glucose, sucrose and starch (B and E), or starch alone (C and F)

<table>
<thead>
<tr>
<th>Compared assays</th>
<th>P-values from paired t-tests for investigated microbiota (CFU) and enamel mineralization (ΔF)†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>all streptococci An‡ Fn So Ss Vd Ca QLF</td>
</tr>
<tr>
<td>A (g/s) vs B (g/s/st)</td>
<td>0.5989  0.8777  0.9353  0.1616  0.4224  0.1467  0.9248  <strong>0.0025</strong>  0.5244</td>
</tr>
<tr>
<td>B (g/s/st) vs C (st)</td>
<td><strong>0.0001</strong>  <strong>&lt;0.0001</strong>  0.0007  <strong>0.0400</strong>  <strong>&lt;0.0001</strong>  <strong>0.0319</strong>  0.3594  <strong>0.0002</strong>  0.8287</td>
</tr>
<tr>
<td>A (g/s) vs C (st)</td>
<td><strong>&lt;0.0001</strong>  <strong>&lt;0.0001</strong>  <strong>0.0001</strong>  <strong>0.0082</strong>  <strong>&lt;0.0001</strong>  0.3891  0.3838  0.1083  0.6406</td>
</tr>
<tr>
<td>D (g/s) vs E (g/s/st)</td>
<td>0.1633  0.8102  <strong>0.0498</strong>  0.3516  0.1826  0.9245  0.0574  0.3196  <strong>0.0028</strong></td>
</tr>
<tr>
<td>E (g/s/st) vs F (st)</td>
<td>0.5988  0.1899  0.3357  <strong>0.4534</strong>  <strong>0.0060</strong>  0.2106  0.9317  0.0726  <strong>0.0003</strong></td>
</tr>
<tr>
<td>D (g/s) vs F (st)</td>
<td><strong>0.0295</strong>  0.5193  <strong>0.0050</strong>  <strong>0.4327</strong>  0.0622  <strong>0.4622</strong>  <strong>0.0471</strong>  0.0812  <strong>&lt;0.0001</strong></td>
</tr>
</tbody>
</table>
* see footnotes to Table 1

† Data for the calculation of p-values originated from three independent experiments run in triplicate (n = 9).

‡ Abbreviations: An, Act. naeslundii; Fn, Fus. nucleatum; So, Strep. oralis; Ss, Strep. sobrinus; Vd, V. dispar; Ca, C. albicans; QLF, quantitative light-induced fluorescence.

§ Bold font indicates statistical significance at P<0.05.
Table 3  Mean of plaque extent and caries incidence in 10 rats receiving as sole source of nourishment 36 daily meals containing glucose, sucrose and/or soluble starch in various combinations over a 40-day experimental period

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Plaque extent (Δ)*</th>
<th>Initial dentinal fissure lesions (ΔΔ)</th>
<th>Advanced dentinal fissure lesions (ΔΔΔ)</th>
<th>Smooth surface caries (ΔΔΔΔ)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1  Diet†</td>
<td>2.1 ± 0.74</td>
<td>5.6 ± 2.99</td>
<td>2.0 ± 2.11</td>
<td>1.0 ± 1.831</td>
</tr>
<tr>
<td>2  15% sucrose + diet‡</td>
<td>2.0 ± 0.67</td>
<td>9.4 ± 0.52</td>
<td>7.5 ± 1.18</td>
<td>2.5 ± 2.682</td>
</tr>
<tr>
<td>3  7.5% sucrose + 7.5% glucose + diet‡</td>
<td>2.1 ± 0.74</td>
<td>8.7 ± 0.95</td>
<td>6.7 ± 1.77</td>
<td>1.8 ± 1.480</td>
</tr>
<tr>
<td>4  3.75 % sucrose + 3.75 % glucose +</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5  7.5% starch + diet‡</td>
<td>2.1 ± 0.57</td>
<td>9.1 ± 1.45</td>
<td>6.7 ± 1.89</td>
<td>1.2 ± 0.79</td>
</tr>
<tr>
<td>6  15% starch + diet‡</td>
<td>2.4 ± 0.52</td>
<td>6.3 ± 2.31</td>
<td>3.1 ± 2.51</td>
<td>0.4 ± 0.97</td>
</tr>
</tbody>
</table>

Statistical analyses

Significance level of variance ratio, F: pF

| ns§ | <0.001 | <0.001 | ns |

Least significant differences at P <0.05

| - | 1.69 | 1.75 | - |

| - | 2.95 | 3.06 | - |

* Symbols: Δ, 4 units at risk; ΔΔ, 12 fissures at risk; ΔΔΔ, 20 units at risk.
† Basic diet with 64% wheat flour, 28% skim milk, 5% brewer’s yeast, 2% Gevral protein, 1% NaCl.
‡ Adjusted basic diet with 49% wheat flour, 28% skim milk, 5% brewer’s yeast, 2% Gevral protein, 1% NaCl.
§ ns, not significant
Fig. 1.