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ORIGINAL ARTICLE

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The effect of liquorice extract-containing starch gel on the amount and microbial composition of plaque

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Abstract The aim of this study was to find out whether liquorice-containing starch gel could affect plaque accumulation and its microbial composition. Sixteen healthy volunteers (mean age: 30.4±6.9 years) used 6 g of either control [8% acid-hydrolyzed corn starch, 25% maltitol syrup, water (*w*/*w*)] or liquorice gel (control + 2.5% liquorice extract), three times a day for 2 weeks. The gels were used in a random order with a 2-week washout period in between. At the end of each fortnight, plaque was allowed to accumulate for 2 days and all available plaque from the right side of the mouth was collected, weighed, and transferred to transport medium. The plaque on the left side was dyed and photographed in a standardized manner. Mutans streptococci, total streptococci, and facultative bacteria were assessed from the plaque using plate culturing. Plaque index (0−5) of incisors and canines on the left side was evaluated from the photographs. The clinical study was preceded by an in vivo acid production test. The acid production from gels containing 2.5–10% liquorice extract was monitored with a microelectrode. The in vivo acid production potential of the maltitol-containing starch gel was about 50% compared to the sucrose control. Liquorice inhibited acid production from the gel. In the clinical study, the weight of plaque after consumption of the liquorice gel did not differ from that of the control gel. No differences were found in the microbial counts nor in the plaque index between the two gels. In addition, the liquorice gel had no effect on the stability of the predominant bacterial populations of the plaque samples of 16 individuals as detected by PCR-denaturing gradient gel electrophoresis. In conclusion, an addition of liquorice extract to starch-containing gel with a low acid production potential had no effect on the plaque formed during a 2-week gel consumption period.

Keywords Liquorice · Glycyrrhizin · Starch · Plaque · Mutans streptococci · Acid production

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Introduction

Starch is the main constituent of edible plants and an important source of energy. It provides 70–80% of the calories consumed by humans worldwide. Starch extracted from plants, cereals, and roots are used in foods as texture modifiers. The amount of starch used in food manufacturing exceeds by far the amount of all other food hydrocolloids combined, mainly due to its fair supply and low cost [23].

In the mouth, food is disrupted by chewing and salivary α-amylase. The degree of hydrolysis of starchy foods, and thus the acid production in plaque, is highly dependent on the food product. Starch in its natural state is not cariogenic or of very low cariogenicity [2], but it is seldom consumed in this form. Processing such as heating induces gelatinization of starch and makes the product more susceptible to hydrolysis. Processed starch causes rapid acid production in plaque and is actually considered as cariogenic as sucrose [6]. Retrogradation (recrystallization) of starch upon cooling and storage of the food product may, on the contrary, render the product more resistant to amylolysis [14].

In tooth-friendly, sugar-free lozenges, like throat pastilles and tablets, gum arabic is often used as texturizing agent. It is not fermented by oral microorganisms and thus does not give rise to a pH fall in human dental plaque. Due to the increasing cost and frequent shortage of gum arabic on the market, some modified starches have been developed to be used instead of gum arabic. A similar texture than obtained with gum arabic is achieved with these modified starches, but the cariogenic properties of the products can be negatively affected [5]. The harmful effects of starch may be inhibited by adding plant extracts which

have shown to have "anticaries activity" to these kinds of products.

Some plant extracts, often rich in polyphenolic compounds, show properties useful for prevention and treatment of oral diseases [15]. The tea extracts in particular have been connected with anticaries activity. Tea extracts were shown to inhibit adhesion of *Streptococcus mutans* [4, 7]. Oolong and green tea polyphenols showed cariostatic effects in rat experiments [11, 12] and oolong tea extracts inhibited plaque deposition in humans [10]. Also, other plant extracts like those of liquorice [6] and perilla seed [24] have showed antimicrobial effects against oral pathogens. Recently, extracts from grape fruit seed, green tea, and liquorice were shown to inhibit acid production from *S. mutans* in vitro and plaque in vivo [19]. However, it is often the taste of these extracts that limits their use in products designed for consumption.

The aim of this research was to study whether the addition of liquorice extract to a starch gel had an effect on plaque acid production and the amount and microbial composition of plaque formed after consumption of the gel.

Materials and methods

Experimental and control gels

The control starch gel consisted of 8% acid-hydrolyzed corn starch, 25% maltitol syrup, and water (*w*/*w*). The pH of the gel was adjusted close to 6.5. The experimental gel contained 2.5–10% liquorice extract (Mafco Worldwide, Camden, NJ, USA). The liquorice extract contained 22% glycyrrhizin and 10% sucrose (maximum percentage of dry weight). The gels were prepared by mixing starch and water in a kettle heated on a hot plate. The viscosity of the mixture first increased upon heating, but during further heating, the mixture turned fluid again and transparent. At this point, maltitol syrup, NaOH, and liquorice extract (to the experimental gel) were added to the mixture. The hot mixture was poured into plastic containers (50 g/container). The gel was formed upon cooling. The gels were stored, refrigerated, and consumed within 1 week of preparation. The liquorice extract addition affected the texture of the gels: the control gels were harder than the liquorice-containing gels.

The starch-maltitol gel used in the present study causes some acid production in plaque but is slowly hydrolyzed to acids [19, 22]. Thus, it was considered to be a good bulk agent for the present study.

In vivo acid production tests

The in vivo acid production test was performed using one female subject (42 years old). The test was repeated twice to ensure that the result was reliable. The subject accumulated plaque for 3 days. After a single intake of the test product, it was swished around for 2 min and swallowed. Remnants of the product will remain in contact with the plaque during the whole measurement period. Changes in plaque pH in three interdental sites were measured with a microelectrode (Beetrode, WPI, Hertfordshire, UK) for 30 min. The acid production potential of the control gel was measured before the experimental gel. The experimental gels contained 2.5, 5, or 10% liquorice. Before and between the tests, the subject rinsed her mouth twice with 10 ml water and chewed paraffin to elevate the pH to neutral starting levels. After testing the gels, the subject rinsed her mouth with 10 ml 10% sucrose, and the pH was followed up for 30 min (positive control).

Clinical study

Subjects

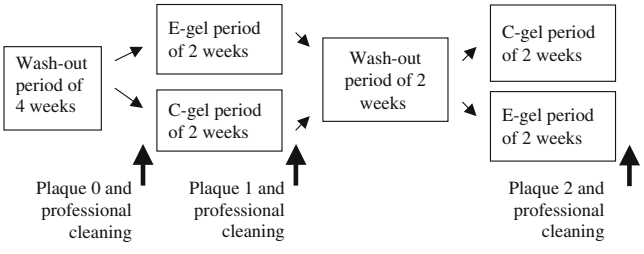
Sixteen healthy volunteers (two males, 14 females) with a mean age of 30.4±6.9 years (23−50), whose salivary flow rate of paraffin that stimulated whole saliva was normal (0.8±0.4 ml/min), and who had a moderate or a high density level of salivary mutans streptococci (MS≥10^5 cfu/ml) and a mean decayed, missing, and filled teeth level of 8.0±6.4, were recruited for the present study.

The Ethics Committee of the Turku University and the Turku University Hospital approved the study. Informed consent was obtained by all subjects.

Study design

For 1 month before and during the study, the subjects were instructed not to use antimicrobial medications or mouthwashes, not to use xylitol products, consume a normal, habitual diet, and continue habitual toothbrushing. Compliance of given instructions was checked at the beginning of each plaque sampling visit. Throughout the study, the subjects used the same brand of fluoridated toothpaste provided to them free of charge.

The study had a double-blind, cross-over design as shown in Fig. 1. The subjects were randomly allocated into



E = experimental gel, C = control gel

Fig. 1 The design of the crossover study. Before starting the use of the gels, professional cleaning was provided to all subjects. After the first 2 weeks period of gel use, plaque samples were collected and the professional cleaning repeated. After a 2-week washout period, subjects who first used experimental gel switched to a 2-week period of control gel use and vice versa. At the end of the experiment, plaque samples were collected and professional cleaning was repeated for all subjects

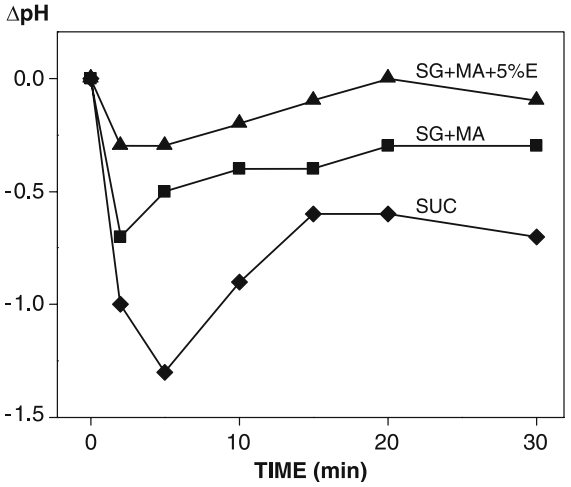


Fig. 2 In vivo acid production from the starch-maltitol (SG+MA) gel containing 5% liquorice extract (E) (▲), the control gel (SG+MA) (■), and the sucrose solution (♦) used as a control. The curves represent measurements from one interdental site

two groups: half of the subjects consumed 6 g of control gel first, and then 6 g of the experimental gel containing 2.5% liquorice for 2 weeks three times a day, while the other half used the gels in the reverse order. The subjects were instructed to chew thoroughly the gels to disrupt the structure of the gel before swallowing it. There was a 2-week washout period in between the use of the gels. Professional cleaning was carried out before the onset of each gel period.

Plaque sampling, dying, photographing, and scoring

Plaque was allowed to grow during two consecutive days with no oral hygiene at baseline, and at the end of both gel periods. Plaque sampling was performed between 8 and 12 a.m. Cotton rolls were used to isolate the areas to be harvested and the teeth were gently air-dried before collection. All available plaque was collected with dental curettes from the right side of the upper and lower jaw. All plaque collections were performed by one and the same examiner. The harvested plaque was weighed without delay with a laboratory scale. The plaque was divided into two aliquots and thereafter transferred to transport tubes containing tryptic soy broth (Difco, Detroit, MI, USA) and stored at −70°C before analyses.

Dying of plaque on the left side of the dentition was carried out with a piece of sponge impregnated with concentrated, edible dye, Rondell Blue (LIC Dental, Upplands Väsby, Sweden). The subject was asked to rinse for 30 s to remove excesses of dye. Lip retractor was placed to expose the stained plaque for photographing. The subject was then asked to hold the upper and lower incisors together in edge-to-edge position. The exposure was carried out with a constant view frame always including both the right and left canines. A set of three photographs of the stained plaque was taken of each subject, first at baseline and then after the use of both gels.

Professional cleaning with prophylaxis cups, cleaning paste, and careful interdental flossing was performed thereafter.

The photographs were used for scoring the amount of dyed plaque from 0 to 5 as described by Quigley and Hein [13]. This was performed in a standardized manner by an experienced laboratory technician blinded to the gels and their order of use.

Microbiological analyses

For the microbiological analyses, the transport tubes were thawed and vortexed for 1 min. To disrupt bacterial aggregates, the samples were treated with mild sonication (Dr. Hielscher, Berlin, Germany). After serial tenfold dilutions, the bacteria were plated on Mitis salivarius (Difco, Detroit, MI, USA) agar containing bacitracin. The plates were incubated for 2 days in a 7% CO_2 atmosphere at 37°C. Mutans streptococci were identified based on colony morphology. Total streptococci were cultured on Mitis salivarius agar (Difco) and total facultative bacteria on blood agar (Orion Diagnostica, Espoo, Finland). The results were expressed as colony-forming units (CFU).

Analysis of the dominant bacterial population with PCR-denaturing gradient gel electrophoresis (DGGE)

DNA was isolated from thawed plaque samples with FastDNA Spin Kit for Soil (Bio 101, Carlsbad, CA, USA) according to manufacturer's instructions with a modification; the bacterial cells were broken with a Fast Prep

Table 1 Mean (SD) plaque index scores of the upper and lower left incisors and canines of the 16 subjects assessed from the dyed 2-day-old plaque as described in the "Materials and methods" section

The WHO number of the tooth	21, Mean (SD)	22, Mean (SD)	23, Mean (SD)	31, Mean (SD)	32, Mean (SD)	33, Mean (SD)
Experimental gel	2.4 (0.8)	2.6 (0.9)	3.1 (0.8)	2.4 (1.2)	2.9 (0.9)	2.8 (0.8)
Range	1–4	1–4	1–4	0–5	1–5	1–4
Control gel	2.7 (0.8)	3.1 (0.9)	3.2 (0.7)	2.4 (1.1)	2.8 (1.0)	2.5 (0.6)
Range	2–4	2–4	2–4	1–5	2–5	2–4
Sig.* (two-tailed)	0.157	0.023	0.317	0.655	0.317	0.096

*Wilcoxon signed ranks

Table 2 The effect of 2 weeks use of experimental and control gels on the number of mutans streptococci, facultatives, and total streptococci in 16 healthy volunteers

	After 2 weeks use of experimental gel mean (SD)	After 2 weeks use of control gel mean (SD)	p*
Mutans streptococci (log CFU)	6.3 (1.1)	6.2 (1.3)	0.438
Facultatives (log CFU)	8.9 (0.3)	8.8 (0.4)	0.272
Total number of streptococci (log CFU)	8.3 (0.5)	8.1 (0.4)	0.301

*Paired t test

instrument (Bio 101 Savant, Holbrook, NY, USA) at 6.0 m/s for 60 s three times.

PCR amplification for predominant bacterial population was performed from V6–V8 hypervariable region of the 16S rRNA gene [9]. The PCR mixture (50 μl) contained 10 pmol of each of the primers: forward primer U968f + GC and reverse primer U1401, 0.4 μl of the four deoxynucleoside trisphosphates (25 mM), 3 U Dynazyme II enzyme (Finnzymes, Espoo, Finland), 5 μl of 10 X PCR buffer (Finnzymes), and 1 μl of template DNA. Thermocycling consisted of 35 cycles of 94°C for 30 s, 50°C for 20 s, and 72°C for 40 s in the UNOII Thermocycler system (Biometra, Göttingen, Germany). The amplified DNA fragments were visualized with 1% agarose gel electrophoresis, which also allowed the size determination of the PCR product.

DGGE analysis was performed using the Dcode Universal Mutation Detection System (Bio–Rad, Hercules, CA, USA) maintained at 60°C and 85 V for 16 h in 0.5× TAE buffer (20 mM Tris-acetate, 0.5 mM EDTA, pH 8.0). Samples were loaded onto 8% acrylamide–bisacrylamide (37.5:1) gels with linear denaturing gradients from 38 to 60% [where 100% is 7 M urea and 40% (vol/vol) deionized formamide]. The gels were stained with SYBR Green I (FMC BioProducts Europe, Vallensbaek Strand, Denmark) for 20 min at room temperature and the images were captured with a Gel Doc 2000 Gel Documentation System (Bio–Rad).

Statistical analysis

The t test for paired samples was used to study differences in plaque wet weight and microbial results after the test gel on one hand and control gel on the other. Wilcoxon signed ranks test was used to study differences between plaque scores after the test and the control gels. The program package SPSS 9.0 for Windows was used. The level of statistical significance was set at $p<0.05$.

Table 3 Mean (SD) plaque index scores of the upper and lower left incisors and canines of the light (n=7) and heavy plaque-formers (n=9) assessed from the photographs of the dyed 2-day-old plaque as described in the "Materials and methods" section

	Experimental gel mean (SD)	Control gel mean (SD)	p*
Light-plaque formers	2.6 (0.5)	2.7 (0.9)	0.486
Heavy-plaque formers	2.8 (0.6)	2.8 (0.7)	0.836

*Wilcoxon signed ranks
The scores are means of the six incisors and canines of each subject in each group

Results

The in vivo acid production potential of the control gel was about 50% compared with the sucrose control (Fig. 2). Liquorice inhibited effectively acid production from the gel: the inhibition with 2.5, 5 (Fig. 2), and 10% was of similar magnitude.

At baseline, the two groups participating in the clinical study did not differ from each other with respect to the amount of plaque, the plaque indices, or any microbiological variable. Thus, the groups were compared with each other after the use of the experimental vs control gels.

No consistent statistically significant differences were detected when the amounts of plaque or plaque indices were compared before and after the use of the experimental or control gel (Fig. 3 and Table 1). The microbiological analyses also failed to differentiate between the experimental and control gels (Table 2).

The subjects were then divided into light- and heavy-plaque formers according to the weight of plaque harvested at baseline. Subjects who formed less plaque than the average (<16.3 mg) were the light-plaque formers (n=7), and those producing the mean or more were the heavy-plaque formers (n=9). This comparison revealed no significant differences between the two gels (Table 3). Figure 4 shows an example of the dyed plaque after using the two gels by light and heavy plaque-forming subjects.

In the DGGE analyses, baseline plaque was compared to the plaque collected after the use of the liquorice gel. According to the results, the DGGE profiles of predominant bacterial population showed intraindividual biodiversity as well as uniqueness of the plaque microbiota (Fig. 5). The differences between the two samples of each individual were minor. The DGGE profiles of individuals 14, 15, and 16 were stable. The DGGE profiles of individuals 2, 4, 5, 6, 8, and 12 were rather stable. The amplicons from two samplings migrated similarly, but there were differences in the intensity of the bands. The DGGE profiles of individuals 1, 3, 9, 10, 11, and 13 were also rather stable. The two samplings differed with 1–2 amplicons. The DGGE profile of the individual 7 was unstable.

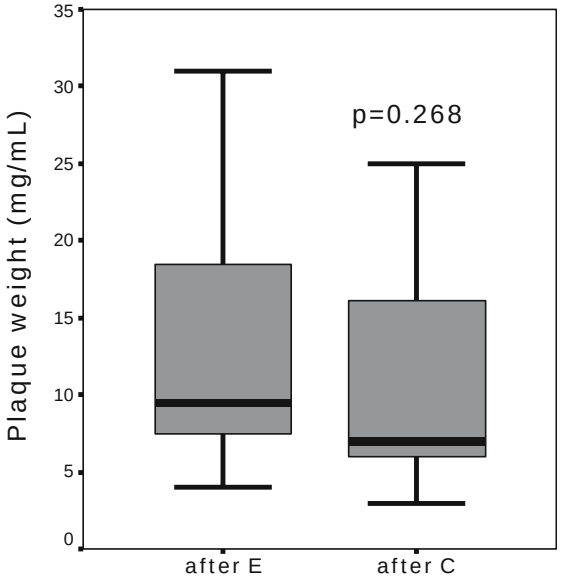


Fig. 3 The effect of 2 weeks use of experimental (E) and control (C) gels on the plaque wet weight: 12.9 (7.7) mg and 10.9 (6.7) mg, respectively. Paired *t* test

Discussion

The study failed to reveal any differences between the effects of the liquorice-containing vs control starch gel on the amount or microbial composition of plaque. The baseline plaque accumulation was not preceded by professional cleaning; thus, most variables obtained only after the consumption of the gels can be compared with each other. However, the DGGE analyses can be compared at baseline and after the consumption of the experimental gel. In DGGE, PCR-amplified DNA fragments of the same length but with different DNA sequences can be differentiated. DGGE analysis combines a direct visualization of bacterial diversity and the opportunity to subsequently identify community members by DNA fragment sequence analysis or hybridization with specific probes [8]. The performed analyses support the idea that the liquorice gel consumption had little or no effect on the stability of the predominant bacterial populations of the plaque samples of the 16 subjects (Fig. 5).

This study design has been used in earlier studies and it is applicable to studies of diet-induced changes in the plaque [20]. Liquorice candies generally contain components like sucrose or starch causing acid production in plaque [21]. Liquorice extracts, however, contain components which are supposed to have a positive impact on dental health. Though liquorice extracts contain phenolic compounds [3], the most important liquorice ingredient having antimicrobial activity is glycyrrhizin. Glycyrrhizin is a nontoxic saponin, the concentration of which in liquorice extracts can be high. Liquorice extracts and glycyrrhizin did not inhibit the growth of *S. mutans* but they both inhibited adhesion of *S. mutans* [16]. This effect was attributed to inhibition of glucosyltransferase by glycyrrhizin [17]. The liquorice extract used in the present study inhibited effectively acid production in vivo with concentrations ranging from 2.5 to 10%. Earlier, it was shown that also in vitro acid production from *S. mutans* was inhibited by 2.5–10% liquorice in the maltitol-containing gel [19]. To our knowledge, only two studies on the effects of liquorice extracts or glycyrrhizin on in vivo plaque formation have been published. In a pilot study conducted with 21 dental students, glycyrrhizin inhibited plaque formation [18]. In a study where the subjects brushed their teeth twice a day for 42 days with toothpastes containing low concentrations (0.25% and 0.50%) of glycyrrhizin, no inhibition of plaque formation could be seen [1]. Our results are in line with the toothpaste study. Why the liquorice extract failed to affect plaque formation can only be discussed. Possibly, the positive plaque effects of glycyrrhizin may have been masked by the negative effects of sucrose, which the liquorice preparations of natural origin contain even up to 10%.

In conclusion, an addition of liquorice extract to starch-containing gel with a low acid production potential had no effect on the plaque formed during a 2-week gel consumption period.

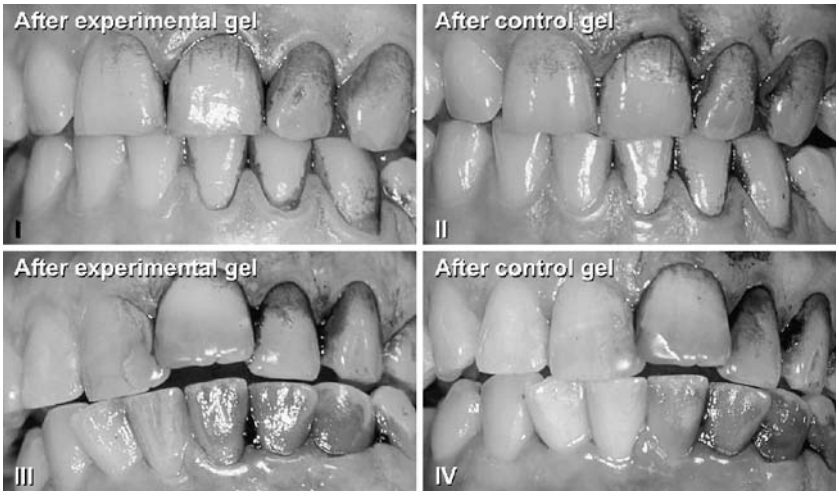


Fig. 4 Photographs I and II describe a low-plaque former (<16.3 mg), and photographs III and IV a high-plaque former (≥16.3 mg) after 2 weeks use of experimental and control gels and after 2 days of plaque accumulation and plaque staining

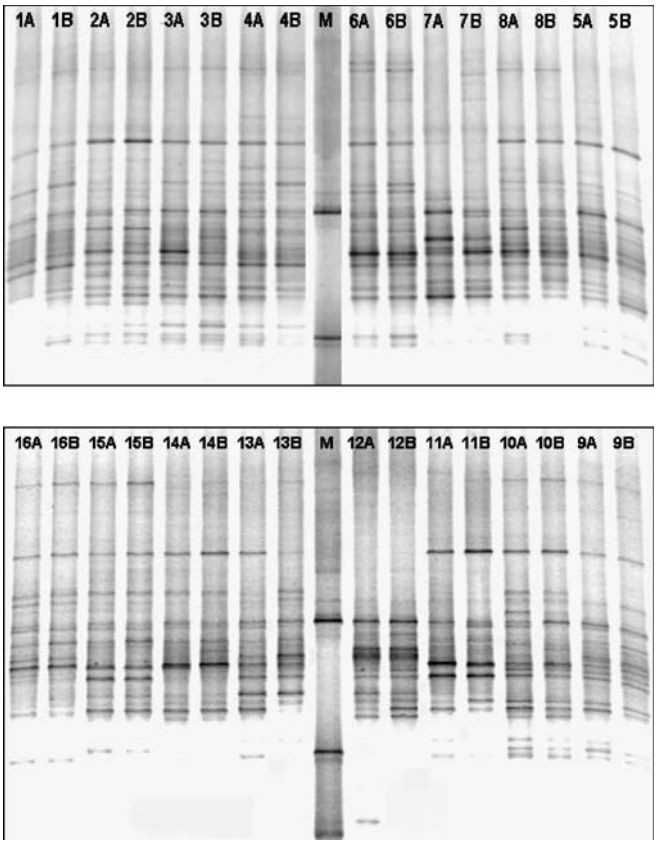


Fig. 5 DGGE profiles of the predominant bacterial population of plaque samples of 16 subjects before (**A**) and after (**B**) the 2-week consumption of the liquorice-containing starch gel. Subject number 1–16; *M* marker

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