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(54) Title: USE OF EXOGENOUS LACTIC BACTERIA STRAIN AGAINST ACTINOMYCES NAESLUNDII RELATED DISEASES

(57) Abrégé/Abstract:
The use of a lactic bacteria strain that is exogenous to the oral microflora, which has been selected for its ability to adhere the pellicle of the teeth and to produce a growth inhibition factor, for the preparation of a composition intended for reducing dental plaque and for treating or preventing root caries and other diseases related to Actinomyces naeslundii in mammals.

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(54) Title: USE OF EXOGENOUS LACTIC BACTERIA STRAIN AGAINST *ACTINOMYCES NAESLUNDII* RELATED DISEASES

(57) Abstract: The use of a lactic bacteria strain that is exogenous to the oral microflora, which has been selected for its ability to adhere the pellicle of the teeth and to produce a growth inhibition factor, for the preparation of a composition intended for reducing dental plaque and for treating or preventing root caries and other diseases related to *Actinomyces naeslundii* in mammals.

Use of exogenous lactic bacteria strain against *Actinomyces naeslundii*-related diseases

The present invention relates to the incorporation in the oral microflora of exogenous lactic bacteria which are able to modulate the colonization of *A. naeslundii* and to reduce the severity of *A. naeslundii*-related diseases.

Background of the invention

The mouth (oral cavity) contains a resident and a non-resident microflora. The first includes microorganisms that are able to establish a more or less permanent residence on the oral surfaces. These bacteria are mainly localised on the tongue, the buccal mucosa and the teeth while the gingiva, lips, cheeks, palate and floor of the mouth only support a very sparse microflora.

The dental plaque is a film that forms on the surface of teeth consisting of bacterial cells in a matrix of extracellular polysaccharides and salivary products. Immediately after eruption, the teeth are covered with an amorphous layer of saliva, the acquired enamel pellicle (AEP) that is about 1.3 μm thick and cannot be removed by normal tooth brushing. The deposition of bacteria on teeth follows immediately the formation of the AEP and plaque becomes evident in 8-12 hours as a multi-layered structure. The first layer consists of bacteria (earliest colonisers) that attach to teeth mainly via specific adhesin-receptor recognition; it forms a substratum for the second colonisers that adhere one to the other via analogous specific binding or via simple juxtaposition.

On the tongue and the buccal mucosa, the natural resident microflora includes microorganisms selected from Streptococcus, Veillonella, Bacteroides and Haemophilus. On the teeth, Streptococci and Actinomyces predominate but a variety of Gram positive and negative cocci and rods can be found.

Many of these microorganisms are innocuous commensal, but a lot of them have been recognised as the etiologic agent of quite a few diseases (Hill, M. J. and Marsh, P. D. eds. Human Microbial Ecology, 1990, CRC Press, Boca Raton Florida, USA).

In particular, *Actinomyces naeslundii* genospecies 1 (formerly *A. naeslundii*) and 2 (formerly *A. viscosus*) are common members of human dental plaque. They are among the strongest plaque forming oral strains, because of their capacity to firmly adhere to the teeth and to coaggregate with many other bacterial species, thus fostering their establishment in the mouth. Moreover, in the elderly, they are commonly isolated at root caries sites, and they are believed to be the major aetiological agent of this disease (Bowden, G.H., et al. 1999, The diversity and distribution of the predominant ribotypes of *Actinomyces naeslundii* genospecies 1 and 2 in samples from enamel and from healthy and carious root surfaces of teeth. *J.Dent.Res.* **78**, 1800-1809).

The transient microflora comprises exogenous bacteria that can be occasionally present in the mouth, but that do not establish a permanent residence (even if repeated oral administrations of these bacteria are carried out). All the food bacteria, and in particular lactic acid bacteria, can be part of this transient microflora.

Some of these exogenous lactic bacteria have been shown to be capable of adhering to the pellicle of teeth. For example, WO 00/09080 (Société des Produits Nestlé) discloses lactic bacteria strains, that are not part of the resident microflora of the mouth, that are low acidifying and that are capable of adhering directly to the pellicle of the teeth. These bacteria are particularly used for treating or preventing dental caries and periodontal infection that are caused by cariogenic microorganism such as *Streptococcus mutans and Streptococcus sobrinus*.

Exogenous bacteria can also produce factors that inhibit the growth of the resident microflora in the mouth. For example, EP759469 (Société des Produits Nestlé) described the use of a bacteriocin produced by *Micrococcus varians* for inhibiting the development of the oral pathogens *S. sobrinus, S. sanguis, S. mutans* and *A. viscosus*. The application of bacteriocins is also one of the investigated strategies which have been set up to reduce tooth caries. These molecules have attracted interest as prospective anticaries agents and as factors important in modulating colonisation of the oral cavity.

It is to note that the prior art does not provide any information concerning strains that can establish in the oral cavity by directly adhering to the pellicle of the teeth and also produce factors such as growth inhibition factors, which can modulate the colonization of *A. naeslundii* so as to reduce the severity of *A. naeslundii*-related diseases.

Summary of the invention

Consequently the present invention aims to provide the use of lactic bacteria that are exogenous to the oral microflora, which have been selected for their ability to adhere to the tooh surface and to produce a growth inhibition factor, for the preparation of a composition intended for reducing dental plaque and for treating or preventing root caries or other disease related to *Actinomyces naeslundii* in mammals.

The lactic bacteria may be selected from the group consisting of *Streptococcus thermophilus*, *Lactococcus lactis subsp. lactis*, and *Lactococcus lactis subsp. lactis biovar diacetylactis* and particularly from the group consisting of the strains CNCM I-1984, CNCM I-1985, CNCM I-1986, CNCM I-1987.

Thus, by colonising the surface of teeth and producing growth inhibition factors, such lactic bacteria can exert an significant reduction of the extent of *Actinomyces naeslundii*, thus reducing dental plaque, root caries and other *Actinomyces naeslundii* related infections.

Another object is to provide a composition for maintaining the health of the mouth by reducing the colonization of *Actinomyces naeslundi*, said composition comprises an exogenous lactic bacteria that has been selected for its ability to adhere to the tooh surface and to produce a growth inhibition factor.

Such a composition may contain at least 10^4-10^9 cfu /g of lactic bacteria.

The invention also provides a method for the prevention or the treatment of *Actinomyces naeslundi* related infections, particularly dental plaque extent and root caries in a mammal, comprising the step of feeding a mammal a composition containing at least one lactic bacteria strain selected for its ability to adhere to the tooh surface and to produce a growth inhibition factor, said composition reduces the colonization of *Actinomyces naeslundi*.

Detailed description of the invention

Within the following description, the mouth defines the oral cavity of humans or animals such as pets, composed by the oral mucosa (gums, lips, cheeks, palate and floor of the mouth), the tongue and the teeth (including artificial structures).

The terms "inhibition growth factor" defines any extracellular substance produced by the adherent exogenous lactic bacteria that enables it to inhibit the growth of *A.naeslundi.*

With respect to the first object of the present invention, the use of an exogenous lactic bacteria that has been selected for its ability to adhere to the tooh surface and to produce a growth inhibition factor, for the preparation of a composition intended for reducing dental plaque and for treating or preventing root caries or other disease related to *Actinomyces naeslundi*, is concerned.

The lactic bacteria may be selected from the group consisting of *Streptococcus thermophilus*, *Lactococcus lactis subsp. lactis*, and *Lactococcus lactis subsp. lactis biovar diacetylactis* and particularly from the group consisting of the strains *Sreptococcus thermophilus* (NCC 1529) (CNCM I-1984), *Sreptococcus thermophilus* (NCC 1561) (CNCM I-1985), *Lactococcus. lactis subsp. lactis* (NCC 2211) (CNCM I-1986), *Lactococcus. lactis subsp. lactis biovar dioacetylactis* (NCC 2225) (CNCM I-1987).

The lactic bacteria is preferably from dairy origin (i.e. originating from milk or cheese, for example).

The lactic bacteria according to the invention is "low acidifying", which means that it is less acidifying than pathogenic strains. Accordingly, it can contribute to a pH in the oral cavity of about 5.5-7.

These strains have been selected among latic bacteria strains for their capacity of adherence to the pellicle of the teeth, their optimal growth temperature is about 37°C, which is the temperature in the oral cavity. They are also capable of producing a growth inhibition factor, which combined to their adhesion properties allow them to significantly decrease the colonization extent of *A. naeslundii* genospecies 1 and 2.

Moreover they are capable of fermenting glucose and sucrose and do not synthesise glucans, which are factors of pathogenicity of the cariogenic strains.

It is also possible to use at least one lactic bacteria strain in combination with a bacteriocin, for example.

The lactic bacteria strains may be included in a food, pet food, cosmetic or pharmaceutical composition, for example. Accordingly, these compositions are preferably toothpaste, mouth rinse, gum, spray, beverage, candies, infant formula, ice cream, frozen dessert, sweet salad dressing, milk preparations, cheese, quark, yogurt, acidified milk, coffee cream or whipped cream, for example.

The exogenous lactic bacteria may be used in an amount of at least 10^4-10^9 cfu /g of lactic bacteria.

The effect of incorporating the above-mentioned bacteria in the oral microflora was tested in a rat model. The strains CNCM I-1985 and CNCM-1986 were able to modulate the oral microbial ecology, significatively reducing the number of total CFU. More specifically, the strains were able to significantly decrease the colonization extent of *A. naeslundii* genospecies 2, with which the rats had been infected (see examples).

BIOCHEMICAL CHARACTERIZATION OF THE SELECTED STRAINS

Fermentation patterns: 49 simple sugars were tested with the api 50 CH bioMerieux strip test (bioMérieux SA, 69280 Marcy-l'Etoile, France) and the results are given in Table 1.

Table 1. Sugar fermentation of *L. lactis* CNCM I- 1987 (A), *L. lactis* CNCM I-1986 (B), *S. thermophilus* CNCM I-1984 (C), *S. thermophilus* CNCM I-1985 (D).

Sugar	A	B	C	D	Sugar	A	B	C	D
Adonitol	+++				Inulin				
Aesculin	++	++++			Lactose	+	++++	+++	++++
Amygdalin	++++				D-Lyxose				
D-Arabinose					Maltose	++			
L-Arabinose					Mannitol	+++	++		
D-Arabitol					D-Mannose	+	++++		
L-Arabitol	+++				Melezitose				
Arbutin	+++	+++			Melibiose				
Cellobiose	+++	++++			α-Methyl-D-glucoside				
Dulcitol					α-Methyl-D-mannoside				
Erythritol					D-Raffinose				
D-Fructose	+	++++			Rhamnose				
D-Fucose					Ribose	++	++		
L-Fucose					Salicin	+++	+++		
Galactose	++	++++			Sorbitol				
β-Gentiobiose	+++				L-Sorbose				
Gluconate					Starch				
2-ketoGluconate					Sucrose			+++	++++
5-ketoGluconate					D-Tagatose				
GlcNAc	+	++++			Trehalose	++			
D-Glucose	+	++++	+	++	D-Turanose	++			
Glycerol					Xylitol	+++			
Glycogen					D-Xylose				
Inositol					L-Xylose				
					β-methil-xyloside				

+, ++, +++, ++++ show if the fermentation beguns after 3, 6, 24 or 48 hours.

The strains *Sreptococcus thermophilus* (NCC 1529), *Sreptococcus thermophilus* (NCC 1561), *Lactococcus. lactis subsp. lactis* (NCC 2211), *Lactococcus. lactis subsp. lactis biovar dioacetylactis* (NCC 2225) were deposited under the Budapest Treaty, at the Collection Nationale de Culture de Microorganismes (CNCM I-1984, CNCM I-1985, CNCM I-1986 and CNCM I-1987 respectively), 25 rue du docteur Roux, 75724 Paris, France, on March 3rd, 1998.

The second main object of the present invention relates to a composition for maintaining the health of the mouth by reducing the colonization of *A.*

naeslundii in mammals, said composition comprises an exogenous lactic bacteria, which has been selected for its ability to adhere to the tooh surface and to produce a growth inhibition factor.

These compositions are particularly intended for the prophylaxis or the treatment of dental plaque and infection related to *A. naeslundii* disease such as root caries, for example.

The lactic bacteria strain according to the present invention is selected from the group consisting of *Streptococcus thermophilus*, *Lactococcus lactis subsp. lactis*, and *Lactococcus lactis subsp. lactis biovar diacetylactis* and preferably from the group consisting of the strains CNCM I-1984, CNCM I-1985, CNCM I-1986 and CNCM I-1987.

Such a composition may contain at least 10^4-10^9 cfu /g of lactic bacteria.

Synergistic compositions may also be prepared, adding at least one bacteriocin, which is active against Gram-positive oral bacteria. In that case, the oral hygiene compositions may comprise 0.00001 to 50%, and preferably from 0.00001 to 15% of purified bacteriocin, by weight of the composition. The bacteriocin is preferably variacin (EP 0 759 469).

In order to protect the composition from degradation, an oil-soluble antioxidant may also be included. Suitable antioxidants include the "tocopherols", butyl-hydroxyanisole (BHA), butyl-hydrxytoluene (BHT), and ascorbyl palmitate. The oil soluble antioxidant is present in amounts of from 0.005% to 0.5%, preferably 0.005% to 0.01% by weight of the composition.

Suitable abrasives for use in dentifrice compositions of the present invention include calcium carbonate, calcium aluminosilicate, alumina, hydrates alumina, zinc orthophosphate, plastic particles, and silica, of which silica is the preferred abrasive.

Compositions according to the invention will have a pH which is orally acceptable and within which the activity of the said lactic bacteria is not compromised. The pH may be in the range 3.0-9.5, preferably in the range 3.5 to 6.5.

These compositions may be prepared by conventional processes comprising admixing the ingredients together in the appropriate relative amounts and finally, and if necessary, adjusting the pH to desired value.

Actinomyces naeslundii genospecies 1 (formerly *A. naeslundii*) and 2 (formerly *A. viscosus*) are among the strongest plaque forming oral strains. They are commonly isolated at root caries sites, in particular in humans over 40 years, and they are believed to be the major aetiological agent of this disease.

Thus, the invention also provides a method for the prevention or the treatment of *Actinomyces naeslundi*-related infections in mammals, particularly dental plaque extent and root caries, comprising the step of feeding the mammal a composition containing at least one lactic bacteria strain selected for its ability to adhere to the tooh surface and to produce a growth inhibition factor.

The amount to be administred may be of at least about 10^4-10^9 cfu /g of lactic bacteria.

The present invention is not to be limited in scope by the specific embodiments described herein. Indeed, various modifications of the invention, in addition to those described herein, will become apparent to those skilled in the art from the foregoing description. Such modifications are intended to fall within the scope of the claims. Various publications are cited herein, the disclosures of which are incorporated by reference in their entireties to the extent necessary for understanding the present invention.

Example 1: in-vitro trials

The strains *S. thermophilus* NCC 1561 (CNCM I-1985) and *L. lactis subsp. lactis* NCC2211 (CNCM I-1986) (hereinafter *L. lactis* NCC2211) were incorporated in vitro in a biofilm mimicking dental plaque *in vitro*.

The oral strain *A. naeslundii* genospecies 1 (formerly *A. naeslundii*) OMZ745 and *A. naeslundii* genospecies 2 (formerly *A. viscosus)* OMZ105 were obtained from the Institute für Orale Mikrobiologie und Allgemeine

Immunologie, University of Zürich and they were cultured in FUM medium in anaerobiosis (GasPackSystem, BBL) at 37°C.

All the strains were stored in glycerol at –20°C and precultured for 14 hours prior to use at their specific optimal temperature;

The two selected strains *L. lactis* NCC2211 and *S. thermophilus* NCC1561 were inoculated in an *in vitro* system in which a biofilm, composed by bacteria commonly found in the human mouth after 40 years, developed on saliva coated-hydroxyapatite discs. Fluid Universal Medium (FUM), the growth medium used, was especially formulated to buffer the acidity produced by the test strains and to have therefore a continued growth (plaque development), like it is in the mouth (Gmur and Guggenheim, 1983). The assays were done in triplicate and the mixtures with and without the dairy strains were tested in parallel. The strains listed in the Table 2 were used.

Table 2. Bacterial strains used and culture conditions applied in the *in vitro* dental plaque experiments.

Strain	Relevant properties	Growth conditions
S. thermophilus NCC1561	S-HA adherent	FUM, Belliker; 37 °C
L. lactis subsp. lactis NCC2211	S-HA adherent	FUM, M17-lactose; 37 °C
S.sobrinus OMZ176	Cariogenic	FUM; 37 °C
S.oralis OMZ607	Plaque forming	FUM; 37 °C
A. naeslundii OMZ745	Plaque forming root caries causative agent	FUM; 37 °C
V.dispar OMZ493	Plaque forming	FUM; 37 °C
F. nucleatum OMZ596	Plaque forming	FUM; 37 °C

Procedure

- ***Saliva pellicle formation***: cover synthetic hydroxyapatite discs of 10 mm diameter (HY-APATITE®, Euro-Crystals, Landgraaf, The Netherlands) with 800 µl of human saliva and incubate for 4 h at room temperature under shaking (1 disc/well in a 24 holes sterile Nunclon plate).

- ***Bacterial consortium preparation***: grow *S. thermophilus* NCC1561, *L. lactis subsp. lactis* NCC2211, *S. sobrinus* OMZ176, *S. oralis* OMZ607, *A. naeslundii* OMZ745, *V. dispar* OMZ493 *and F. nucleatum* OMZ596 overnight at 37°C in anaerobiosis in FUM-glucose (*S. thermophilus* NCC1561 in FUM-lactose), adjust the OD_{550} to 1 with FUM and pool 2 ml of each oral bacterial suspension with 2 ml of either *S. thermophilus* NCC1561 or *L. lactis subsp. lactis* NCC2211. The control mixture contains the five oral strains only.

- ***Biofilm formation and recovery***: the procedure is as described in Guggenheim et al., 1998, Validation of a new biofilm model. J.Dent.Res. **77**, (Spec Iss A): 110 (Abstract #38).

- ***Cultural analysis of the biofilm***: spiral plate the suspension on Columbia Blood Agar (5% sheep blood, Becton Dickinson, Meylan Cedex, France) for the total count and for the differentiation of *A. naeslundii*. Incubate the plates at 37°C in anaerobiosis for 48 h.

Growth antagonism between the oral and the dairy strains under study

Strains and culture conditions used are listed in the Table 3.

Table 3 Bacterial strains used and culture conditions applied in the growth antagonism experiments.

Strain	Relevant properties	Growth conditions
S. thermophilus NCC1561	S-HA adherent	Belliker; 42 °C
L. lactis subsp. lactis NCC2211	S-HA adherent	M17-lactose; 37 °C
S. thermophilus NCC1536	non-adherent	Belliker; 42 °C
A. naeslundii OMZ745	plaque forming	BHI, 37 °C; anaerobiosis
A. viscosus OMZ105	plaque forming	BHI; 37 °C

S. thermophilus NCC1561, *S. thermophilus* NCC 1536 and *L. lactis* NCC2211 (killer strains) were tested for growth antagonism against *A. naeslundii* OMZ745 and *A. viscosus* OMZ105 (target strains).

Procedure

- Grow the killer strains overnight on agar plates in anaerobiosis and the target strains in BHI until middle stationary phase
- Dilute 20 µl of the target strains suspension in 3 ml of BHI soft agar (agar 7 g/l) containing glucose and lactose, vortex and pour immediately on a BHI agar plate
- Solidify for 1 h at room temperature, then streak the killer strain from the M17 plate in the form of a cross. Streak in parallel the killer and the target strains alone as a control
- Incubate at 37 °C in anaerobiosis for 24 hours

Growth antagonism is revealed by an inhibition halo around the cross.

Statisticals

Differences between the control and test consortia were determined by Student's *t* test.

RESULTS AND DISCUSSION

S. thermophilus NCC1561 and *L. lactis* NCC2211 could be incorporated and grown in the plaque-like biofilm on the S-HA discs, and their total CFU/disc after 40.5 h are given in the Table 4.

Table 4. Level of incorporation of the two dairy strains in the biofilm (CFU/disc). The values are the mean of three experiments with their standard deviations.

Method of inoculation	***S. thermophilus* NCC1561 ($x10^6$)**	***L. lactis* NCC2211 ($x10^6$)**
Together with the oral strains	4.08 +/-1.78	5.76 +/-3.64
Before the oral strains	5.03 +/-2.21	3.87 +/-4.01

The effect of incorporating the dairy strains in the biofilm on the oral species is indicated in the Tables 5 and 6. When *S. thermophilus* NCC1561 was included (Table 5), a general decrease of the total flora, that is represented by the counts on Columbia blood agar plates (CBA), and of 4 of the oral species was noticed.

When *L. lactis* NCC2211 was introduced in the oral strains consortium (Table 6) the total flora counts notably diminished (CFU on CBA). The decrease was significant in the case of *A. naeslundii* OMZ745 that significantly diminished (p = 0.021) The decrease was even stronger if the strain was inoculated on the discs before the oral bacteria.

Table 5 Modulation of the oral strains consortium by *S. thermophilus* NCC1561 (CFU/disc).

Treatment	CBA (x 10^8)	*A. naeslundii* OMZ745 (x 10^6)	MS (x 10^8)
Control	2.86 +/- 2.14	5.29 +/- 2.58	2.02 +/- 1.68
+ NCC1561	1.63 +/- 0.55	4.75 +/- 1.45	1.21 +/- 0.81
Pre-incubation with NCC1561	2.32 +/- 0.38 °°	4.78 +/- 2.29	1.48 +/- 0.29

N = 3. *: p-values are calculated in respect to the control (*: p<0.05; **: p<0.01); °: p-values are calculated in respect to the "+NCC1561" treatment (°: p<0.05; °°: p<0.01).

Table 6. Modulation of the oral strains consortium by *L. lactis* NCC2211 (CFU/disc).

Treatment	CBA (x 10^8)	*A. naeslundii* OMZ745 (x 10^6)	MS (x 10^8)
Control	2.77 +/- 2.16	6.07 +/- 2.70	3.04 +/- 2.88
+ NCC2211	0.65 +/- 0.33	4.59 +/- 2.81	0.65 +/- 0.33 *
Pre-incubation with NCC2211	0.27 +/- 0.11 **°	3.91 +/- 3.29 *°	0.27 +/- 0.11 **°°

N = 3. *: p-values are calculated in respect to the control (*: p<0.05; **: p<0.01); °: p-values are calculated in respect to the "+NCC2211" treatment (°: p<0.05; °°: p<0.01).

Some assays were carried out to verify if the reduction of the oral strains was due to growth antagonism of the dairy strains towards them (Table 7). The strains *A. viscosus* OMZ105 and *S. thermophilus* NCC1536 were also included in the test since they are part of the *in vivo* model (example2)

All the four dairy strains inhibited the growth of the Gram-negative strain *A. viscosus* OMZ105. This inhibition cannot be attributed to lactic acid production. *A. viscosus* is able to metabolize lactate only under aerobic conditions (van der Hoeven et al. (1990) *Oral Microbiol. Immunol.* **5**, 223-225) and it is very aciduric. These findings have been confirmed by plating *A. viscosus* in presence of 1% lactic acid: no inhibition was observed.

Table 7. Oral strains growth inhibition by *S. thermophilus* NCC1561, *S. thermophilus* NCC1536 and *L. lactis* NCC211.

	KILLER STRAINS			Lactic
Target	**NCC1561**	**NCC1536**	**NCC2211**	**acid 1%**
***A. naeslundii* OMZ745**	+	+	+	-
***A. viscosus* OMZ105**	+	+	+	-

CONCLUSIONS

S. thermophilus NCC1561 and *L. lactis* NCC2211 could be incorporated in a biofilm mimicking dental plaque and were able to modulate the oral microflora, significantly reducing the number total of cfu, and more specifically, these strains were able to significantly decrease the colonization extent of *A. naeslundii* genospecies 2. In addition the strains could inhibit the growth of *A. naeslundii* genospecies 1 and 2 in co-cultures.

Example 2: in-vivo trials.

An in-vivo study was performed on a rat model. In this study, the association of the selected strains was continued during the whole experimental period on a daily basis, by the way of a chilled dairy product feeding.

The study was carried out in 58 days. In order to perform the experiment during the day, the active period of the animals had to be advanced of 7 hours totally; this was done in three steps on day 16, 17 and 18 as further described in detail. The cariogenic strains were associated on days 21 and 22, while association of the dairy strains started on day 23 and lasted until day 57. The animals were fed the dairy strains as supplement in a yogurt base that was included in the normal diet, as explained in the section. The rats teeth were swabbed at the end of the study, on day 58.

Animals and diet

10 litters consisting of 4 Osborne-Mendel rats pups each (animal production section of the Institute für Orale Mikrobiologie und Allgemeine Mikrobiologie, University of Zurich, Zurich, Switzerland) were used in the experiment. All animals were weighed at the beginning and at the end of the experimental period. When 13 days old, the dams and the pups were transferred to screen-bottom stainless-steel cages without bedding and nourished with low-fluoride powdered (0.2 μm) Nafag diet to avoid fissure impaction (Rat Checkers No. 184, NAFAG, Gossau SG, Switzerland), and tap water *ad libitum*. The active phase during which the rats eat is during the night, i. e. 18:00 – 06:00.

In order to allow refilling of the food cups during normal working hours, the circadian biorhythm was stepwise reversed between days 16 and 18 by advancing the active phase of the rats each day on three occasions by adjusting the automatic light controls.

On day 16/17 the beginning of the active period was brought forward from 18:00 h to 15:00 i. .e. it was night from 15:00 – 03:00 and it was day onwards. On day 17/18 the beginning of the active period was brought forward from 15:00 to 12:00 h, i. e. it was night from 12:00 h – 00:00 h and day from 00:00 h onwards.

Finally on day 18/19 the beginning of the active period was brought forward from 12:00 to 10:00 i. e. it was night from 10:00 h to 22:00 h and day from 22:00 h onwards.

Therefore by day 19 the shift of the active phase for the rats from the hours of darkness to normal working hours (10:00 – 22:00 h) was completed.

On day 20 the dams were removed, and the rats started to be fed *ad libitum* with the modified diet 2000a containing 40% sucrose, 28% substitute for skim milk (soya protein extract SVPRO-PP 1611 39.4%, lactose 49.3%, 0.6%, L-Methionine 0.3%, L-Lysine HCl 0.1%), 24% wheat flour, 5% brewer's yeast, 2% Gevral® Instant Protein (Whitehall-Robins SA, 6301 Zug, Switzerland) and 1% NaCl.

During the association period (days 21 and 22) the drinking water was supplemented with 2% glucose and 2% sucrose to support the implantation of the associated bacteria. On day 23 the litters were distributed among the 3 treatments, 1 animal per cage, in a programmed feeder machine and began to receive the test diet as indicated in the table 10. The test diet consisted of 18 yogurt meals containing the test strains alternating with 18 meals of the modified diet 2000a previously described.

Drinking water was supplied *ad libitum.* Following the swabbing procedure on day 58 the animals were overdosed with Thiopental sodium (100 mg/Kg of body weight) given by intra-peritoneal injection and decapitated when comatose.

Bacterial strains : The strains listed in the Table 8 were used.

Table 8 Bacterial strains that were used in this study

Strain	Relevant properties	Growth conditions
S. thermophilus NCC1561	S-HA adherent	Belliker; 42°C
S. thermophilus NCC1536	Non-adherent control	Belliker; 42°C
L. lactis NCC2211	S-HA adherent	M17-lactose; 37°C
A. viscosus OMZ105	Plaque forming, S-HA adherent	BHI; 37°C

Preparation of the tested LAB strains for the association.

A preliminary study was done to assess the growth parameters, especially the hours required to reach the stationary phase in the specific conditions further described. It was therefore established to grow the *S. thermophilus* strains for 7 h and the *L. lactis* one for 6 h. Also a study of the viability after freezing of the dairy strains was performed by plating the same cell suspension before and after freezing. In order to be associated to the animals, the dairy strains were treated according to the following procedure.

Procedure

- Inoculate the strains 1% overnight in their proper medium from a glycerol stock
- Inoculate them 5% from this culture into 10 batches of 4 liters of their proper medium pre-heated at the optimal growth temperature, and grow them until the end of the log phase/beginning stationary phase

- Determine the final CFU/ml by plating on M17-lactose agar from two randomly chosen batches for each of the 4 strains. Incubate the plates overnight in anaerobiosis.
- Centrifuge the cultures from each batch at 6000 rpm for 10 min and re-suspend the pellets in 150 ml of fresh medium; keep overnight at 4°C
- Centrifuge again and re-suspend in the freezing medium (15% glycerol in Belliker or M17).
- Split in aliquots in order to have $2x10^{11}$ viable cells/vial, taking into account the viability loss due to freezing, and store at –20°C until needed.

Association of the animals with the bacterial strains

The animals were arranged in 3 treatments (Table 9). Each treatment consisted of 10 pups that were distributed in 1 per cage.

Table 9. Arrangement of the treatments.

Treatment	Associated bacteria
1	*A. viscosus* OMZ105; *S. thermophilus* NCC1536
2	*A. viscosus* OMZ105; *S. thermophilus* NCC1561
3	*A. viscosus* OMZ105; *L. lactis* NCC2211

All the rats were first infected on days 21 and 22 with *A. viscosus* OMZ105.

The tested LAB strains were associated daily (since contained in the yogurt base meal), starting on day 23. 2 frozen vials, each containing $2x10^{11}$ viable cells of the test strain, were mixed in 200 ml of yogurt in order to have at least 10^9 CFU/ml. *S. thermophilus* NCC1536, a non S-HA adhering strain, was used as a negative control.

The yogurt and diet 2000a meals, of 1 ml and 400 mg respectively, were offered alternatively 18 times per day at 20-min intervals (Table 10). Therefore, each animal received in total 18 ml of yogurt and 7.2 g of powdered diet.

The meals were dispensed in the food cups of a programmed feeder machine that automatically offered to the animals the right meal at the exact time.

Table 10. Eating times.

N° of the meal	High cariogenic meals	Yogurt meals	N° of the meal
1	10:00	10:20	2
3	10:40	11:00	4
5	11:20	11:40	6
7	12:00	12:20	8
9	12:40	13:00	10
11	13:20	13:40	12
13	14:00	14:20	14
15	14:40	15:00	16
17	15:20	15:40	18
19	16:00	16:20	20
21	16:40	17:00	22
23	17:20	17:40	24
25	18:00	18:20	26
27	18:40	19:00	28
29	19:20	19:40	30
31	20:00	20:20	32
33	20:40	21:00	34

Bacteriological evaluation

Five rats per treatment were swabbed on day 58. The swab suspensions were either plated on Petri dishes for CFU (colony forming units) counts or immobilized on glass slides for immunofluorescence for TCN (total cells number) counts.

Procedure for CFU determination

- Swab rats' teeth with a sterile cotton-tipped stick and place it immediately in 5 ml of sterile NaCl 0.9%
- Vortex for 1 min and sonicate for 5 s at 50 W
- Spirally plate the properly diluted suspensions on CBA, MS and HJL agar
- Incubate CBA and MS plates at 37 °C and HJL plates at 45 °C.

Procedure for TCN determination

- Put 10 µl of the undiluted swab suspension prepared for CFU determination per well on a 24 wells glass slide (Dynatech Produkte AG, Embrach Embraport, Switzerland), and air dry
- Fix by soaking in methanol for 2 min and air dry
- Incubate with 10 µl of the proper antibody or serum diluted in ELISA buffer (section 4.2.2.5) and incubate at 37 °C for 30 min
- Aspirate each drop from the side of the well and wash by soaking the slide first in ELISA buffer and then in distilled water. Air dry
- Apply 10 µl of goat-anti-rabbit IgG (H + L) – FITC (Sigma) diluted 1:400 and incubate at 37 °C for 30 min
- Wash as before and air dry
- Apply 49 µl of mounting fluid (section 4.2.2.5), cover with a glass slip and count the fluorescent cells with a fluorescence microscope

Statistics

Data were treated with two-way ANOVA ((Snedecor and Cochran, 1980)).

RESULTS from the continuous association of the dairy strains by the way of a chilled dairy product feeding.

Bacteriological evaluation (Table 11)

- ***Colonization of the strain.*** 1.7 (+/- 1.1) x10^7 CFU were counted for the plaque forming *A. viscosus* OMZ105, when the dairy product was supplemented with the non-adherent *S. thermophilus* control (NCC1536). The dairy strains could not be counted by microbiological methods. By immunofluorescence, however, a qualitative evaluation was tempted. The three adherent dairy strains could be recognized in the plaque samples from treatments 2 and 3. Since they were co-aggregated with other oral bacteria and mouth debris, thus generating big clusters, a precise quantification was impossible.

- ***Variations in the total flora (TF).*** The three treatments containing the adherent tested strains *S. thermophilus* NCC1561 and *L. lactis* NCC2211 displayed significant reduced numbers of colony forming units on CBA compared with the control group containing the non-adherent strain *S.*

thermophilus NCC1536 (Table 11). Treatment 2 reduced the CFU counts with a significant factor of $P_F<0.01$ and treatment 3 even more significantly ($P_F<0.001$).

- ***Modulation of A. viscosus OMZ105 tooth colonization by the tested strains.*** In the case of the plaque forming bacterium *A. viscosus* OMZ105, an apparently less pronounced but more significant decrease in the number of colony forming units was observed for the three treatments containing the tested adherent strains with respect to treatment 1 ($P_F<0.01$) (Table 11). By contrast the percentages of *A. viscosus* on the total CFU counts were not significantly different. Approximately 50% of the total CFU for all four treatments were identified as *A. viscosus* colonies.

Table 11. Mean values per rat of colony forming units for the total flora (TF) and *A. viscosus* OMZ105 and their respective percentages (N = 5).

Treatment	TF on CBA x 10^6	OMZ105 on CBA x 10^6	% OMZ105 on CBA	TF on MS x 10^4
1 (NCC1536)	32.5 +/- 13.87	17.3 +/- 11.01	52.8 +/- 23.09	41.9 +/- 41 n.s.
2 (NCC1561)	17.6 +/- 6.57 **	8.0 +/- 1.96 **	47.2 +/- 9.37 n.s.	5.6 +/- 3.8 n.s.
3 (NCC2211)	13.9 +/- 4.85 ***	5.8 +/- 2.07 **	43.4 +/- 18.16 n.s.	1.7 +/- 1.11 n.s.
SEM	2.99	2.17	5.65	10.33
P_F	0.001	0.01	n.s.	n.s.
LSD 0.05*	9.21	6.69	-	-
LSD 0.01**	12.92	9.37	-	-
LSD 0.001***	18.25	-	-	-

Treatments 2-3 were compared with treatment 1. SEM = standard error of the mean; n.s. = not significant. CBA = Columbia Blood Agar; MS = Mitis-salivarius agar. OMZ105: *A. naeslundii* genospecies 2.

In this *in vivo* assay, the strains that were supplied daily, displayed clear inhibitory effect on the total microflora, whose CFU significantly diminished. This diminution can be explained by the growth antagonism of the dairy strains versus oral species. For instance *in vitro*, all of them, including the non S-HA adhering *S. thermophilus* NCC1536, can inhibit the growth of *A. viscosus* OMZ105. However, *in vivo* such an effect can only be displayed by the S-HA adhering strains, since it was detected in the treatments 2-4 compared to the first.

In particular, since the animals had been infected with *A. viscosus* OMZ105, the quantification of this plaque-forming organism was possible at the end of the experimental period, and the decrease of its CFU could be closely monitored.

The percentages of *A. viscosus* OMZ105 on the total CFU did not decrease in parallel, therefore one can deduce that the growth antagonism effect was also displayed versus other species, i.e. Veillonellae, and consequently it is a global effect which is observed.

Thus the strains CNCM I-1985 and CNCM-1986 are able to modulate the oral microbial ecology, significatively decreasing the colonization extent of *A. naeslundii* genospecies 2, with which the rats had been infected.

Example 3: Production and initial analysis of surfactant substances from *S. thermophilus* NCC1561 *and S. thermophilus* NCC1536.

S. thermophilus NCC1561 and *S. thermophilus* NCC1536 were grown overnight in 1 l of Belliker at 42 °C. For biosurfactant production, the procedure described in Busscher et al. (1997) *Appl. Environ. Microbiol.* **63**, 3810-3817, (Busscher *et al.*, 1997)was used.

Preparation of the surfactant substances

Procedure

- Wash cells three times in PBS
- Resuspend in 200 ml of distilled water or PBS
- Produce the biosurfactant by gently stirring the suspension for 2 or 4 h at room temperature
- Separate bacteria by centrifugation at 10000 rpm for 10 min
- Centrifuge supernatant twice at 10000 rpm for 10 min
- Freeze-dry and weigh both the pellet and the surfactant substances solutions.

The crude biosurfactant suspension was first analyzed by SDS-PAGE and then submitted to surface tension measurements.

Procedure for SDS-PAGE

SDS-PAGE was carried out with a precast 12.5% ExcelGel (Amersham Pharmacia Biotech). Silver staining was performed with the Plusone Silver Staining Kit (Amersham Pharmacia Biotech).

Procedure for surface tension measurement

The surface tension of the biosurfactant suspensions was measured with a TVT1 Drop Volume Tensiometer (Lauda, Lauda-Königshofen, Germany), which is based on the drop volume principle. Briefly, the method consists in the exact determination of the volume of a suspension drop that detaches from a capillary. This volume (critical volume) is proportional to the surface tension (σ), whose value is calculated with the relation:

$$\sigma = V\, g\, \Delta\rho\, F / 2\pi r_{cap}$$

where:

- σ is the interfacial tension
- V is the drop volume
- G is the acceleration constant
- $\Delta\rho$ is the difference of the densities of both adjacent phases
- F is the correction factor
- r_{cap} is the radius of the capillary

The measurements were done in duplicate at 37 °C against air. Each measurement consisted of 10 cycles. A solution of 6 mg/ml of crude product released, made the water surface tension decrease from 70 to 51 mN/m (Table 13). SDS-PAGE profile of the bacteria released products, showed that there were many different substances of proteinaceous nature in the solution.

Table 13. Surface tension values of the biosurfactant suspensions compared to water and PBS. The values are the mean of two experiments, each one consisting of ten measurements.

	Crude extract concentration	Surface tension (mN/m)
Water	-	69.07 +/- 0.01
PBS	-	68.13 +/- 0.30
S. thermophilus NCC1561	6 mg/ml	51.47 +/- 0.17
S. thermophilus NCC1536	6 mg/ml	51.67 +/- 1.32

RESULT

S. thermophilus NCC1561 and *S. thermophilus* NCC1536 cells are able to release substances with a surfactant activity. It is therefore possible that the biosurfactant produced by *S. thermophilus* NCC1561 makes the bacterium itself and the other oral strains established close to it detach from the tooth surface. By contrast, this action would not be displayed by *S. thermophilus* NCC1536, since this strain does not adhere to the teeth.

Example 4: TOOTHPASTE

Toothpaste is prepared by adding 10^5 cfu/ml of at least one of the lactic bacteria strain CNCM I-1984, CNCM I-1985, CNCM I-1986, CNCM I-1987 in a lyophilised form, to the following mixture containing: 1.65% Cetyl pyridinium chloride, 33.0% Sorbitol (70% soln), 25.0% Glycerin, 2.0% Sodium carboxymethyl cellulose, 0.25% Sodium fluoride, 26.3% Silica (RP 93), 8.1% Thickening Silica (Sident 22), 0.5% Sodium saccharine, 3.2% Poloxamer (Pluronic F108).

This toothpaste is intended for the prophylaxis or the treatment of root caries, dental plaque and other infections induced by *A.naeslundii* species.

Example 5: YOGHURT

5 1 MRS culture medium are sterilised for 15 min at 121°C and then inoculated with 5% by volume of an active culture of at least one of the

S.thermophilus strain CNCM I-1984, CNCM I-1985 containing approximately 10^9 cfu/ml. After incubation for 8 h at 41°C, a starter containing $4.5.10^8$ cfu/ml is obtained.

5 l reconstituted skimmed milk having a dry matter content of 10%, to which 0.1% yeast extract has been added, are sterilised for 15 min at 121°C and inoculated with 2% of an active culture of commercial thickening *Streptococcus thermophilus* containing approximately 10^9 cells/ml. After incubation for 4 h at 41°C, a starter containing $4.5.10^8$ cells/ml is obtained.

One batch of whole milk containing 3.7% fats strengthened with 2.5% skimmed milk powder and then pasteurised for 30 min at 90°C is then inoculated with 2% by volume of the starter of at least one of the strains CNCM I-1984, CNCM I-1985 and 3% by volume of the starter of thickening *Streptococcus thermophilus*. The inoculated milk is stirred, poured into pots and incubated for 4 h at 41°C.

The yoghurt obtained has a good firm and smooth texture and is intended for the health of the mouth.

Example 6: CHEWING GUM

A chewing gum for preventing or treating root caries, dental plaque or other *A.naeslundii*-related deseases can be prepared adding an active culture of at least one of the *S.thermophilus* strain CNCM I-1984, CNCM I-1985 so that it contains approximately 10^4 to 10^9 cfu/g, to the following typical ingredients: 67.5 % Xylitol, 20 % Gum base, 5 % Calcium carbonate, 3 % Glycerin, 2 % Pluronic F127, 1 % Cellulose gum, 0.5 % Balast compounds and 1 % Flavor.

Example 7: PET FOOD COMPOSITION

A pet food for mouth health is obtained by preparing a feed mixture made up of corn, corn gluten chicken and fish meal, salts, vitamins and minerals. The feed mixture is fed into a preconditioner and moistened. The moistened feed leaving the preconditioner is then fed into an extruder-cooker and gelatinised. The gelatinised matrix leaving the extruder is forced through a die and extruded. The extrudate is cut into pieces suitable for feeding to dogs, dried at about 110°C for about 20 minutes and cooled to form pellets which have a water activity of about 0.6.

The pellets are sprayed with 3 coating mixtures. Each coating mixture contains active culture of at least one of the *S.thermophilus* strains CNCM I-1984, CNCM I-1985 but one coating mixture uses hydrogenated soy fat as a coating substrate, one coating mixture uses water as a coating substrate and one coating mixture uses protein digest as a coating substrate. The pellets contain approximately 10^4 to 10^9 cfu/g of said strains.

Claims

1. The use of lactic bacteria strain that is exogenous to the oral microflora, which has been selected for its ability to adhere to the pellicle of the teeth and to produce a growth inhibition factor, for the preparation of a composition intended for reducing dental plaque and for treating or preventing root caries and other diseases related to *Actinomyces naeslundii* in mammals.

2. The use according to claim 1, wherein the lactic bacteria strain is from dairy origin.

3. The use according to claims 1 or 2, wherein the lactic bacteria strain is selected from the group consisting of *Streptococcus thermophilus*, *Lactococcus lactis subsp. lactis*, and *Lactococcus lactis subsp. lactis biovar diacetylactis*.

4. The use according to one of claims 1 to 3, wherein the lactic bacteria strain is selected from the group consisting of the strains CNCM I-1984, CNCM I-1985, CNCM I-1986, CNCM I-1987.

5. The use according to one of claims 1 to 4, wherein the composition is an edible composition comprising an effective quantity of lactic bacteria for reducing dental plaque and/or for treating or preventing root caries and other diseases related to *Actinomyces naeslundii* in mammals.

6. The use according one of claims 1 to 5, wherein the composition contains at least 10^4-10^9 cfu /g of the lactic bacteria strain.

7. The use according to one of claims 1 to 6, wherein the lactic bacteria strain is combined with a bacteriocin.

8. A composition for maintaining the health of the mouth by reducing the colonization of *Actinomyces naeslundii* in mammals, said composition containing at least one lactic bacteria strain that is exogenous to the oral microflora, which has been selected for its ability to adhere the pellicle of the teeth and to produce a growth inhibition factor.

9. A composition for maintaining the health of the mouth by reducing the colonization of *Actinomyces naeslundii* in mammals, *said composition* comprises :
   - a lactic bacteria strain which has been selected for its ability to adhere to the pellicle of the teeth and to produce a growth inhibition factor, and
   - a bacteriocin.

10. A composition according to claims 8 or 9, comprising at least one lactic bacteria strain selected from the group consisting of *Streptococcus thermophilus*, *Lactococcus lactis subsp. lactis*, and *Lactococcus lactis subsp. lactis biovar diacetylactis*.

11. A composition according to one of claims 8 to 10, comprising at least one lactic bacteria strain selected from the group consisting of the strains CNCM I-1984, CNCM I-1985, CNCM I-1986, CNCM I-1987.

12. A composition according to one of claims 8 to 11, comprising at least 10^4-10^9 cfu /g of the lactic bacteria strain.

13. A method for the prevention or the treatment of *Actinomyces naeslundii*-related diseases in mammals, comprising the step of feeding a mammal a composition containing at least one lactic bacteria strain selected for its ability to adhere to the tooh surface and to produce a growth inhibition factor.

14. A method according to claim 13, for reducing dental plaque and treating or preventing root caries in mammals.