Combination of lactic acid bacteria and its use for the prevention and/or treatment of infections and inflammatory conditions



The present invention relates to a combination of lactic acid bacteria and its use for making a food supplement, a hygiene product or a pharmaceutical preparation for the prevention and/or treatment of infections and inflammatory conditions caused by bacteria, viruses or fungi, especially in the mouth, vagina, urethra, nose, eyes and ears.

Lactic acid bacteria are Gram-positive bacteria that produce lactic acid by the fermentation of glucose. *Streptococcus thermophilus* is also included in this definition by convention.

It is well known that strains of lactic acid bacteria that produce H_2O_2 can act as regulators of the bacterial flora in body orifices and on mucous membranes. It has been demonstrated that H_2O_2-producing lactic acid bacteria can antagonize *E. coli*, *N. gonorrhoea*, *G. vaginalis*, *C. trachomatis*, *U. urealyticum* and *B. bivius*. However, these bacteria are only of limited benefit when used in medical practice. This can be seen from the fact that preparations based on lactic acid bacteria (e.g. vaginal pessaries) intended for the treatment of infections by the above microorganisms (e.g. vaginitis) are not held in high regard by doctors, who prefer to



treat their patients with antibiotics or chemotherapeutic agents.

To the best of the inventor's knowledge, no antibacterial or flora-regulating action in body orifices and on mucous membranes has been attributed to arginine-utilizing lactic acid bacteria.

It has now been found surprisingly that the activity of H_2O_2-producing lactic acid bacteria is considerably potentiated by the addition of one or more strains of lactic acid bacteria that are capable of utilizing arginine. Arginine forms part of various small peptides found in biological fluids and it also occurs as free arginine. Many bacterial species utilize it for their own nutrition and growth. Arginine-utilizing lactic acid bacteria can therefore deprive other, pathogenic or potentially pathogenic bacteria of a certain quantity of arginine, which - though not enough to terminate their growth - makes them more susceptible to the action of the H_2O_2 produced by the lactic acid bacteria.

The present invention therefore provides a combination of lactic acid bacteria comprising:

(a) a first component consisting of at least one strain of H_2O_2-producing lactic acid bacteria, and

(b) a second component consisting of at least one strain of arginine-utilizing lactic acid bacteria.

The strain of lactic acid bacteria in component (a) is preferably chosen from a group made up of the strains of the species *Lactobacillus crispatus*,

Lactobacillus salivarius and *Lactobacillus casei*, while the strain of lactic acid bacteria in component (b) is chosen from a group made up of the strains of the species *Lactobacillus brevis*, *Lactobacillus gasseri* and *Lactobacillus fermentum*. More especially, the strain of lactic acid bacteria in component (b) is the *Lactobacillus brevis* CD2 strain deposited in the DSM - Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany, on February 6, 1998 with access number DSM 11988 under the Budapest Treaty, or mutants or derivatives thereof.

The ratio of the number of bacteria in component (a) to the number of bacteria in component (b) is preferably from 1 : 100 to 100 : 1, and more especially from 1 : 5 to 5 : 1, the most preferred ratio being 1 : 1.

The combination can be administered in the unit dosage form comprising from 1×10^2 to 5×10^{11} bacteria of component (a) and from 1×10^2 to 5×10^{11} bacteria of component (b), preferably 1×10^9 bacteria of component (a) and 3×10^9 bacteria of component (b).

The combination can also be administered in the form of tablets, sucking tablets, sweets, chewing gum, gelatin capsules, pessaries, suppositories and micro-enemas, as well as pellets, dental creams and gels, denture powders, mouthwashes, dentifrices, sprays, suspensions and ointments.

According to another embodiment of the invention, the combination additionally comprises at least one other strain of lactic acid bacteria chosen from a group made up of:

Lactobacillus acidophilus, Lactobacillus buchneri, Lactobacillus casei, Lactobacillus catenaforme, Lactobacillus cellobiosus, Lactobacillus crispatus, Lactobacillus curvatus, Lactobacillus delbrueckii, Lactobacillus jensenii, Lactobacillus leichmannii, Lactobacillus minutus, Lactobacillus plantarum, Lactobacillus salivarius, Lactobacillus brevis, Lactobacillus gasseri, Lactobacillus fermentum, Bifidobacterium adolescentis, Bifidobacterium angulatum, Bifidobacterium bifidum, Bifidobacterium breve, Bifidobacterium catenulatum, Bifidobacterium dentium, Bifidobacterium eriksonii, Bifidobacterium infantis, Bifidobacterium longum, Bifidobacterium plantarum and *Streptococcus thermophilus.*

The combination can also comprise vitamins, quaternary ammonium bases, mineral salts, antioxidants and anti-plaque agents.

The invention also relates to the use of a combination of lactic acid bacteria comprising:

(a) a first component consisting of at least one strain of H_2O_2-producing lactic acid bacteria and

(b) a second component consisting of at least one strain of arginine-utilizing lactic acid bacteria, for making a food supplement, a hygiene product or a pharmaceutical preparation for the prevention and/or

treatment of infections, and inflammatory conditions caused by bacteria, viruses or fungi, especially in the mouth, vagina, urethra, nose, eyes and ears. These infections and inflammatory conditions include gingivitis, periodontitis, mucositis and stomatitis caused by drugs and/or physical agents, Behçet's syndrome, diakeratosis of the oral cavity, glossitis, sore throat, sialadenitis, sialolithiasis, pemphigus, *Lichen planus*, Sjögren's syndrome, vaginosis, vaginitis, urethritis, prostatitis, proctitis, otitis, conjunctivitis, rhinitis, sinusitis, leucoplakia, aphthae, herpes, and infections with *Helicobacter pilori* in the oral cavity.

The combination can also be used to advantage for the treatment of the oral cavity as an oral deodorant, antiinflammatory, anti-caries and/or anti-plaque agent.

The following examples serve to illustrate the various aspects of the invention in more detail but should not be construed as in any way limiting the invention.

Example 1

The inhibitory effect of: H_2O_2-producing lactic acid bacteria (Component A); arginine-utilizing lactic acid bacteria (Component B); and the combination of the two strains, specifically in the ratio 1 : 1 (Combination AB); on the growth of potentially pathogenic bacteria was evaluated.

Briefly, the culture of lactic acid bacteria to be tested was first adjusted to a neutral pH, because an acidic pH itself inhibits bacterial growth. The suspension was subjected to sterile filtration, and the filtrate was used to impregnate a number of discs of absorbent paper (30 µl of filtrate per disc). The discs were placed on a plate of selective growth medium that had been inoculated with 0.1 ml of *Gardnerella vaginalis* (a strain which causes vaginosis and which was isolated in a laboratory) together with a control disc that was impregnated only with 30 µl of distilled water. After incubation for 24 h at 37°C, the inhibition of the growth of the pathogens was evaluated by measuring the diameter of the halo around the disc in millimetres.

A second series of tests was carried out with *Streptococcus mutans* as the target pathogen, this species being the causative agent of dental plaque and caries.

The characterization of the bacteria as H_2O_2 producers was done by a classical benzidine peroxidase reaction, which reveals H_2O_2-producing colonies of bacteria by a blue coloration. The activity of arginine dehydrolase was determined to evaluate the ability of the lactic acid bacteria to utilize arginine (M.C. Manca de Nadra, *Milchwissenschaft*, **37** (1982) pp. 669-670].

The lactic acid bacteria were obtained from the American Type Culture Collection (ATCC), Rockville, USA.

Bacterial strain	Halo of inhibition (anti-*G. vaginalis* activity, mm)
H_2O_2 producer (Component A)	
Lactobacillus crispatus (ATCC 39197)	75
Lactobacillus salivarius (ATCC 11741)	60
Lactobacillus crispatus + *Lactobacillus salivarius*	63
Arginine utilizer (Component B)	
Lactobacillus brevis (ATCC 14869)	0
Lactobacillus fermentum (ATCC 14931)	2
Lactobacillus brevis + *Lactobacillus fermentum*	0
Combination AB (ratio of A to B 1 : 1)	
Lactobacillus crispatus + *Lactobacillus brevis*	112
Lactobacillus crispatus + *Lactobacillus fermentum*	100

Lactobacillus salivarius + *Lactobacillus brevis*	117
Lactobacillus salivarius + *Lactobacillus fermentum*	104

Bacterial strain	Halo of inhibition (anti-*S. mutans* activity, mm)
H_2O_2 producer (Component A)	
Lactobacillus crispatus (ATCC 39197)	98
Lactobacillus salivarius (ATCC 11741)	102
Lactobacillus crispatus + *Lactobacillus salivarius*	99
Arginine utilizer (Component B)	
Lactobacillus brevis (ATCC 14869)	0
Lactobacillus fermentum (ATCC 14931)	1
Lactobacillus brevis + *Lactobacillus fermentum*	1

Combination AB (ratio of A to B 1 : 1)

Lactobacillus crispatus + *Lactobacillus brevis*	118
Lactobacillus crispatus + *Lactobacillus fermentum*	126
Lactobacillus salivarius + *Lactobacillus brevis*	121
Lactobacillus salivarius + *Lactobacillus fermentum*	120

Example 2

Sucking tablets with the following unit composition were prepared:

Combination AB (*Lactobacillus salivarius* + *Lactobacillus brevis, ratio 1 : 1*)	4000 million
Mannitol	400 mg
Saccharin	5 mg
Polyoxyethylene	50 mg
Mg stearate	15 mg
Talc	25 mg
Silica	5 mg

These tablets were administered to four volunteers who were told not to clean their teeth or use chewing gum during the previous week. The subjects took three tablets a day for one week, after meals,

allowing the tablets to dissolve in their mouth. Clinical evaluations were performed for both the dental plaque index and the gingival plaque index.

For the dental plaque the following scoring system was used on six teeth (first upper molar on the right; upper central incisor on the left; first upper premolar on the left; first lower molar on the left; lower central incisor on the right; first lower premolar on the right):

0 - no plaque at all

1 - no visible plaque

2 - visible plaque

3 - very obvious plaque.

The following scoring system was adopted for evaluating the gingival plaque, using the margin of the six teeth mentioned above:

0 - no inflammation

1 - slight inflammation

2 - moderate inflammation, with bleeding on contact

3 - marked inflammation, with a tendency to spontaneous bleeding.

The combined data obtained for the six teeth of the four volunteers were as follows:

Subject	Dental plaque index		Gingival plaque index	
	0	7 days	0	7 days
No. 1	8	2	7	1
No. 2	9	3	10	3
No. 3	15	5	6	2
No. 4	15	7	8	3

Example 3

Four subjects with a clinical and histological diagnosis of recurrent aphthous ulcers were treated for ten days with six sucking tablets a day, whose composition is given in Example 2.

All the patients treated showed a complete cure of the ulcers at the end of the ten-day course, and none of them had new ulcers during the following month.

To improve the flavour and appearance of the bacterial combination AB, suitable colouring agents and sweeteners such as saccharin, mint oil and xylitol can be added, as is customary and well known to those skilled in the art.

The combination AB can be administered in the form of pellets, sweets, chewing gum, gelatin capsules, dental creams and gels, denture powders, mouthwashes, dentifrices, tablets, pessaries, suppositories, sprays, suspensions and micro-enemas.

Example 4

Preparation of a toothpaste

Toothpaste base

Percentage composition:

Calcium phosphate dihydrate	37.5%
Glycerol (85% in water)	30.0%
Flavour (peppermint oil)	1.0%
Sodium carboxymethylcellulose	1.0%
Purified water	20.8%
Sodium saccharin (1% aq. solution)	2.5%
Sodium lauryl sulphate	2.0%
Purified water	5.2%
	100.0%

Composition by weight:

Calcium phosphate dihydrate	337.5 g
Glycerol (85% in water)	270.0 g
Flavour (peppermint oil)	9.0 g
Sodium carboxymethylcellulose	9.0 g
Purified water	187.2 g
Sodium saccharin (1% aq. solution)	22.5 g
Sodium lauryl sulphate	18.0 g
Purified water	48,8 g
	900.0 g

The glycerol was added to the calcium phosphate dihydrate, which had been ground and passed through a 50-mesh screen. The mixture was allowed to undergo hydration, giving a dense homogeneous paste.

The flavour (1% of peppermint oil) was added at this point.

The sodium carboxymethylcellulose was hydrated in water overnight (concentration of 4.6% in purified water).

The solution of sodium saccharin was added to the resulting thick gel.

The polymer gel was poured into a mortar already containing the calcium phosphate that had been hydrated with glycerol, and the components were vigorously mixed.

An approximately 28% solution of sodium lauryl sulphate in purified water was prepared separately.

The sodium lauryl sulphate solution was then added to the thick dicalcium phosphate paste.

The resulting thick homogeneous paste, which had good rheological properties, was mixed for a few minutes and then refined by passing it through a refiner with rollers. This gave a snow-white homogeneous paste with a pleasant mint aroma.

38.5 g of lyophilized lactic acid bacteria (L. *salivarius* + *L. brevis*, 1 : 1; 10^{10} CFU/g) were passed through a 50-mesh screen and added in small portions to the rest (770 g) of the above toothpaste base. The resulting homogeneous paste had a slightly pale brown colour and a mint odour.

Example 5

Preparation of fast-release vaginal tablets

Vaginal tablets coated with an effervescent layer were prepared by wet granulation. The tablets weighed 2100 mg each and contained 100 mg of the combination of lactic acid bacteria as the active substance.

Each vaginal tablet had the following composition:

Lyophilized and screened lactic acid bacteria (30 x 10^9 of *L. brevis*, 30 x 10^9 of *L. salivarius* and 90 x 10^9 of *L. plantarum* per gram)	100.0 mg
Lactose	1368.0 mg
Cornstarch	246.0 mg
Adipic acid	192.0 mg
Sodium bicarbonate	150.0 mg
Magnesium stearate	30.0 mg
Stearic acid	9.0 mg
Colloidal silica	5.0 mg
	2100.0 mg

CLAIMS

1. Combination of lactic acid bacteria comprising

(a) a first component consisting of at least one strain of H_2O_2-producing lactic acid bacteria, and

(b) a second component consisting of at least one strain of arginine-utilizing lactic acid bacteria,

wherein component (a) is selected from the group consisting of the strains of the species *Lactobacillus crispatus*, *Lactobacillus salivarius* and *Lactobacillus casei*, and component (b) is selected from the group consisting of the strains of the species *Lactobacillus brevis*, *Lactobacillus gasseri* and *Lactobacillus fermentum*, provided that when component (a) is *Lactobacillus casei*, component (b) is not *Lactobacillus gasseri* or *Lactobacillus fermentum* and when component (a) is *Lactobacillus crispatus*, component (b) is not *Lactobacillus fermentum*.

2. Combination according to Claim 1, in which the strain of lactic acid bacteria in component (b) is the *Lactobacillus brevis* CD2 strain deposited in the DSM - Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany, on February 6, 1998 with access number DSM 11988 under the Budapest Treaty, or mutants or derivatives thereof.

3. Combination according to any one of Claims 1-2, in which the ratio of the number of

bacteria in component (a) to the number of bacteria in component (b) is from 1 : 100 to 100 : 1.

4. Combination according to Claim 3, in which the said ratio is from 1 : 5 to 5 : 1.

5. Combination according to Claim 4, in which the said ratio is 1 : 1.

6. Combination according to any one of the previous claims in unit dosage units comprising from 1×10^2 to 5×10^{11} bacteria of component (a) and from 1×10^2 to 5×10^{11} bacteria of component (b).

7. Combination according to Claim 6, comprising 1×10^9 bacteria of component (a) and 3×10^9 bacteria of component (b).

8. Combination according to Claim 6 or 7, in the form of tablets, sucking tablets, sweets, chewing gum, gelatin capsules, pessaries, suppositories and micro-enemas.

9. Combination according to any one of Claims 1-5, in the form of pellets, dental creams and gels, denture powders, mouthwashes, dentifrices, sprays, suspensions and ointments.

10. Combination according to any one of the previous claims, additionally comprising at least one other strain of lactic acid bacteria, chosen from a group made up of *Lactobacillus acidophilus, Lactobacillus buchneri, Lactobacillus casei, Lactobacillus catenaforme, Lactobacillus cellobiosus, Lactobacillus crispatus, Lactobacillus curvatus, Lactobacillus delbrueckii, Lactobacillus jensenii,*

Lactobacillus leichmannii, *Lactobacillus minutus*, *Lactobacillus plantarum*, *Lactobacillus salivarius*, *Lactobacillus brevis*, *Lactobacillus gasseri*, *Lactobacillus fermentum*, *Bifidobacterium adolescentis*, *Bifidobacterium angulatum*, *Bifidobacterium bifidum*, *Bifidobacterium breve*, *Bifidobacterium catenulatum*, *Bifidobacterium dentium*, *Bifidobacterium eriksonii*, *Bifidobacterium infantis*, *Bifidobacterium longum*, *Bifidobacterium plantarum* and *Streptococcus thermophilus*, provided that when component (a) is *Lactobacillus casei*, said other strain is not *Lactobacillus gasseri* or *Lactobacillus fermentum* and when component (a) is *Lactobacillus crispatus*, said other strain is not *Lactobacillus fermentum*.

11. Combination according to any one of the previous claims, additionally comprising vitamins, quaternary ammonium bases, mineral salts, antioxidants and anti-plaque agents.

12. Use of a combination of lactic acid bacteria comprising:

(a) a first component consisting of at least one strain of H_2O_2-producing lactic acid bacteria; and

(b) a second component consisting of at least one strain of arginine-utilizing lactic acid bacteria,

wherein component (a) is selected from the group consisting of the strains of the species *Lactobacillus crispatus*, *Lactobacillus salivarius* and *Lactobacillus casei*, and component (b) is selected from the group consisting of the strains of the species *Lactobacillus*

brevis, *Lactobacillus gasseri* and *Lactobacillus fermentum*, provided that when component (a) is *Lactobacillus casei*, component (b) is not *Lactobacillus gasseri* or *Lactobacillus fermentum* and when component (a) is *Lactobacillus crispatus*, component (b) is not *Lactobacillus fermentum*, for making a food supplement, a hygiene product or a pharmaceutical preparation for the prevention and/or treatment of infections and inflammatory conditions caused by bacteria, viruses or fungi, especially in the mouth, vagina, urethra, nose, eyes and ears.

13. Use according to Claim 12, in which the infections and inflammatory conditions include gingivitis, periodontitis, mucositis and stomatitis caused by drugs and/or physical agents, Behçet's syndrome, diakeratosis of the oral cavity, glossitis, sore throat, sialadenitis, sialolithiasis, pemphigus, *Lichen planus*, Sjögren's syndrome, vaginosis, vaginitis, urethritis, prostatitis, proctitis, otitis, conjunctivitis, rhinitis, sinusitis, leucoplakia, aphthae, herpes, and infections with *Helicobacter pilori* in the oral cavity.

14. Use according to Claim 12 or 13 for the treatment of the oral cavity as a deodorant, antiinflammatory, anti-caries and/or anti-plaque agent.

15. The composition according to claim 1 or 2, wherein component (a) is *Lactobacillus crispatus* and component (b) is *Lactobacillus brevis*.

16. The composition according to claim 1 or 2, wherein component (a) is *Lactobacillus salivarius* and component (b) is *Lactobacillus brevis*.

17. The composition according to claim 1, wherein component (a) is *Lactobacillus salivarius* and component (b) is *Lactobacillus fermentum*.

18. The composition according to claims 15-17, wherein the ratio of the number of bacteria in component (a) to the number of bacteria in component (b) is 1:1.