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(54) Title: MICROCAPSULES LOADED WITH PROBIOTICS AND PRODUCTION THEREOF

(57) Abstract: The invention is related to the production method of the microencapsulated form of probiotics with the chitosan-alginate polymers, the microcapsules loaded with probiotics obtained by this method and their use in food, agriculture and cosmetics. These microcapsules do not make alterations in the colour and appearance of the product on which they are applied and have antifungal and anti-mycotoxin features.



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MI CROCAPSULES LOADED WITH PROBIOTICS AND PRODUCTION THEREOF

Technical Field

- 5 The invention is related to the production method of the microencapsulated form of probiotics with the chitosan-alginate polymers, the microcapsules loaded with probiotics obtained by this method and their use in food, agriculture and cosmetics.

Known State of the Art (Prior Art)

- 10 Mycotoxins are toxic metabolic products produced by some moulds such as *Aspergillus*, *Penicillium* and *Fusarium*, which can be found in foods and agricultural products generally as a result of contamination. Mycotoxins being formed during the cultivation, storage, processing or transportation of foods cause varying degrees of toxicosis (mycotoxicosis) in humans and/or
- 15 animals. Preventing the growth of mould in foods is difficult, however, the amount of these can be minimized during the processing and storage of foods by providing hygienic conditions. Forming of moulds which produce toxins is an exceptional problem in the developing countries, that is because in these countries, there are no controlled storage conditions such as in the developed countries. Besides, in tropical zones having warm and humid climate, the risk of
- 20 mould growth in food increases.

- After the mycotoxin is formed, mycotoxins can be removed from the food products by binders. However, since this method is used after mycotoxin formation, it deteriorates the quality of the product, causes changes in taste and leads to chemical pollution. Also, since most of the binders
- 25 used today are mycotoxin-specific, they do not present sufficient efficiency with products contaminated with more than one mycotoxin.

- Due to reasons as such, the use of microorganisms which support consumer health and which have immune system-stimulating effects in the production of food and agricultural products
- 30 increases. It is known that the probiotics which constitute a significant group of these microorganisms play an important role in the prevention and treatment of gastrointestinal

system disorders and formation of normal microflora. Use of probiotics in food stuff is longstanding. However, addition of probiotics directly into the product causes change in taste and quality.

- 5 Also, it is known that the probiotic technology boosts the immune system, repairs the natural defence mechanism, prevents collagen structure damage and slows the aging process by providing the water balance of the skin.

10 When the inventions similar to the subject invention are analysed, the following documents are found:

- US20060008511A1: In this patent document, a preparation, which comprises a probiotic encapsulated with a mix of xanthan gum and chitosan gum as ingredients, developed for pets, is described.
- US20120128821A1: In this patent document, a method related to isolating probiotic from donkey's milk is described.

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Brief Description and Purposes of the Invention

20 The invention is related to microcapsules obtained by microencapsulating endospore form of probiotics with chitosan-alginate polymers. The microcapsules loaded with said probiotics prevent mycotoxigenic moulds and the mycotoxins which the moulds produce through biological struggle. In this study, encapsulation of probiotics is carried out especially in order to prevent probiotics from being damaged by gastric acid or in order to increase the quality in fermented products.

The microcapsules loaded with probiotics related to the subject invention;

- 25
- Prevent the contamination of mycotoxin to food products at the rate of 95% with different combinations of probiotics,
 - Do not cause any alteration in the colour and appearance of the product they are applied to,
 - Affect many mycotoxin species of different types since they suppress the mould growth
- 30 and stress mechanism in food and agricultural products,

- Have antifungal effect,
 - Do not necessitate extra disinfection since at the same time, they will also have an antifungal effect on the product they are being applied to,
 - Are natural and harmless,
- 5** - Provide protection in storage up to 360 days by extending the shelf life of the product they are applied to.

Detailed Description of the Invention

10 The present invention is related to chitosan-alginate microcapsules loaded with probiotics which present antifungal and anti-mycotoxin activity in fields such as food, agriculture, cosmetics and healthcare.

The production method of the microcapsules loaded with the probiotic related to the invention contains the procedure steps of;

- Dissolving the alginate in water which is between 0,002- 0,1% ratio by weight,
 - Autoclaving the alginate solution at 110-130°C temperature, preferably at 121°C and then sterilizing the same,
- 15**
- Mixing the probiotics with alginate,
 - Adding calcium chloride (CaCl_2) between 0,01-1 M, preferably 0,1 M to the probiotic-alginate mix and stirring,
- 20**
- Dissolving chitosan in 0,25-3% concentration by weight in 0.5-5% organic acid by volume (lactic acid, acetic acid, ascorbic acid, citric acid, tartaric acid, malic acid etc.), preferably in 1% organic acid (In this stage, the pH is adjusted between 3.5-7).
 - Autoclaving the chitosan solution at 110-130°C temperature, preferably at 121°C and then sterilizing the same,
- 25**
- Thereafter, combining the 3:1 chitosan solution and the alginate-calcium chloride (CaCl_2) solution containing the probiotics in a container dropwise,
 - Adding tween 80 (Polysorbate 80) between 0.01-2% by volume and stirring the mixture until homogenization is obtained.

The probiotics used in the invention are chosen from the microorganisms of; *Bacillus laterosporus*, *Bacillus sphaericus*, *Bacillus subtilis*, *Bacillus coagulans*, *Streptococcus thermophilus*, *Azotobacter*, *Azospirillum*, *Agrobacterium*, *Gluconobacter*, *Flavobacterium*, *Herbaspirillum*, *Bacillus megaterium*, *Thiobacillus*, , *B. polymyxa*, *B. brevis*, *B. licheniformis*, *B.*

5 *circulans*, *B. cereus*, *B. thuringiensis*, *B. longum*, *B. breve*, *B. infantis*, *L. helveticus*, *L. rhamnosus*, *L. plantarum*, *L. casei* , *L. acidophilus* , *Lactobacillus delbreckii*, *Lactobacillus ellobiosus*, *Lactobacillus lactis*, *Lactobacillus acidophilus*, *Lactobacillus reuteri*, *Lactobacillus brevis*, *Lactobacillus casei*, *Lactobacillus curvatus*, *Lactobacillus fermentum*, *Lactobacillus plantarum*, *Lactobacillus helveticus*, *Streptococcus cremoris*, *Streptococcus thermophilus*, *Streptococcus*

10 *intemedius*, *Streptococcus lactis*, *Streptococcus diacetylactis*, *Enterococcus faecalis*, *Lactococcus spp*, *Lactococcus lactis subsp.*, *Pediococcus spp.*, *Pediococcus cerevisiae*, *Pediococcus acidilactici*, *Pediococcus pentosaceus*, *Bifidobacterium spp.* *Bifidobacterium animalis*, *Bifidobacterium adolescentis*, *Bifidobacterium bifidum*, *Bifidobacterium breve*, *Bifidobacterium infantis*, *Bifidobacterium longum*, *Leuconostoc spp.* *L. salivarius*, *L. paracasei*, *L. gasseri*, *L. reuteri*, *B.*

15 *Bifidum*, *B. longum*, *B. infantis*, *Lb. delbrueckii*, *Lb. plantarum*, *Lb. pentosus*, *Lb. brevis*, *P. damnosus*, *Lb. collinoides*, *Lb. pentosus*, *Pediococcus spp.*, *Lb. buchneri*, *Leuconostoc mesenteroides*, *Pedococcus pentosaceus*, *Lb. casei*, *Lb. kefir*, *Lb. acidophilus*, *Lb. helveticus*, *Lb. casei*, *Lb. bulgaricus*, *Lb. lactis*, *Lb. plantarum*, *Lb. brevis* , *Acetobacter spp.*, *Streptococcus genera*, *Streptococcus spp.*, *Streptococcus lactis*, *S. thermophilus*, *S. durans*, *S. cremoris*,

20 *Lactobacillus alimentarius*, *L. Alimentarius*, *L. multaromicus*, *L. sanfrancisco*, *Lactococcus lactis ssp*, *S. cerevisia*, *Lactobacillus sakei*, *Lactobacillus alimentarius*, *Lactobacillus paralimentarius*, *Lactobacillus paracasei*, *Lactobacillus buchneri*, *Enterococcus faecium*, *Enterococcus mundtii*, *Enterococcus faecalis*, *Enterococcus casseliflavus*, *Lactobacillus pentosus*, *Enterococcus faecium*, *Pediococcus pentosaceus*, *Lactobacillus farciminis*, *Pichia kudriavzevii*, *Lactobacillus farciminis*,

25 *Lactobacillus casei*, *Lactobacillus alimentarius*, *Pichia kudriavzevii* , *Candida humilis*, *L.lactis subsp. cremoris*, *Lb.delbrueckii subsp. lactis*, *Lb.helveticus*, *Lb.casei*, *Lb.delbrueckii subsp.* , *Leuc.mesenteroides subsp. cremoris*, *Lb. johsonii*, *Lb.kefirenofacies*, *Lb.curvatus*, *P.acidilactici*, *P.pentosaceus*, *Lb.alimentarius*, *C.piscicola*, *Leuc.mesenteroides*, *P.acidilactici*, *P.cerevisiae*, *Lb.pentosus*, *P.acidilactici*, *T.halophilus*, *Lb.sanfransiscensis*, *Lb.farciminis*, *Lb.fermentum*,

30 *Lb.amylovorus*, *Lb.reuteri*, *Lb.pontis*, *Lb.panis*, *Lb.alimentarius*, *W.cibaria*, *O.oeni*, *L.coryniformis*, *L.curvatus*, *L.jugurti*, *L.jensenii*, *L.bucheneri*, *L.cellobiosus*, *L.coprophilus*, *L.hilgardii*, *L.leichmannii*, *L.dextranicum*, *P.acidilactici*, *P.pentosaceus*, *S.thermophilus*, *L.lactis*

subsp. diacety lactis, *L.lactis subsp. hordniae*, *L.garvieae*, *L.rafinolactis*, *V.fluvialis*,
V.salmoninarum, *Leuconostoc* sp., *L.cremoris*, *L.dextranicum*, *L.mesenteroides*,
L.paramesenteroides, *L.gelidum*, *L.carnosum*, *Carnobacterium* sp., *C.divergens*, *C.mobile*,
C.gallinarum, *C.piscicola*, *Vagococcus* sp., *V.fluvialis*, *V.salmoninarum*, *L.garvieae*, *Lactococcus*
5 *diacetylactis*, *Propionibacterium freudenreichii*, *Pediococcus* sp, *S.uvarum*, *Lb.coryniformis*,
Candida crusei, *Weissella confusa*, *Hansenula silvicola*, *Debaryomyces hansenii*, *Trichosporon*
beigelli, *Bacillus amyloliquefaciens*, *Torulopsis* sp., *Candida mycoderma*, *Lb.buchneri*,
P.acidilactici, *Pediococcus pentasaceus*, *L.cellobiosus*, *E.mundtii/E.gallinarum*, *E.casseliflavus*,
P.urinae-equi, *Lb.murinus*, *Candida milleri*, *E.burtonii*, *E.fibulinger*, *Issatchenkia orientalis*,
10 *Candida pelliculosa*, *C.tropicalis*, *Pediococcus acidilactici*, *Pseudopiantarum*, *Pediococcus*
acidilactici, *Pediococcus pentosaceus*, *Leuconostoc pseudomesenteroides*, *Weissella cibaria*, *Lb.*
paraplantarum, *Issatchenkia orientalis*, *Candida glabrata*, *Pediococcus acidilactici*,
Kluyveromyces marxianus, *Pichia kudriavzevii* , *Saccharomyces servazzi*, *Torulaspora delbrueckii*,
Kazachstania unispora, *Saccharomyces barnettii*.

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The application of the product related to the invention to food and agricultural products is as follows:

- 10 ml of the solution containing 10^9 cfu bacteria is mixed with 1 L water in order to provide protection against mycotoxin in the garden or during the storage period,
- The same is applied on the products by means of a spraying method.

20

CLAIMS

1. The invention is a production method of polymeric microcapsules containing a probiotic which is protective against mould and mycotoxin formation, characterized in that it comprises the procedure steps of;
- Dissolving the alginate in water which is between 0,002- 0,1% ratio by weight,
 - Autoclaving the alginate solution at 110-130°C temperature, preferably at 121°C and then sterilizing the same,
 - Mixing the probiotics with alginate,
 - Adding calcium chloride (CaCl_2) between 0,01-1 M, preferably 0,1 M to the probiotic-alginate mix and stirring,
 - Dissolving chitosan in 0,25-3% concentration by weight in 0.5-5% organic acid by volume,
 - Autoclaving chitosan solution at 110-130°C temperature, and then sterilizing the same,
 - Thereafter, combining the 3:1 chitosan solution and the alginate-calcium chloride (CaCl_2) solution containing the probiotics in a container dropwise,
 - Adding tween 80 (Polysorbate 80) between 0.01-2% by volume and stirring the mix until homogenization is obtained.
2. A method according to Claim 1, characterized in that the organic acid is lactic acid, acetic acid, ascorbic acid, citric acid, tartaric acid or malic acid.
3. A method according to Claim 1, characterized in that the pH is adjusted to be between 3.5-7 in the procedure step in which the chitosan is dissolved in organic acid.
4. A method according to Claim 1, characterized in that the alginate solution is autoclaved at 121°C and then sterilized.
5. A method according to Claim 1, characterized in that the chitosan solution is autoclaved at 121°C and then sterilized.
6. A method according to Claim 1, characterized in that 0,1 M calcium chloride (CaCl_2) is added to the probiotic-alginate mix and stirred.
7. The method in which 0,25-3% chitosan by weight is dissolved in 1% organic acid.
8. A method according to Claim 1, characterized in that said probiotics are bacteria and/or yeast.

9. A method according to Claim 8, characterized in that the probiotics are chosen from the microorganisms of; *Bacillus laterosporus*, *Bacillus sphaericus*, *Bacillus subtilis*, *Bacillus coagulans*, *Streptococcus thermophilis*, *Azotobacter*, *Azospirillum*, *Agrobacterium*, *Gluconobacter*, *Flavobacterium*, *Herbaspirillum*, *Bacillus megaterium*, *Thiobacillus*, , *B.*
- 5 *polymyxa*, *B. brevis*, *B. licheniformis*, *B. circulans*, *B. cereus*, *B. thuringiensis*, *B. longum*, *B. breve*, *B. infantis*, *L. helveticus*, *L. rhamnosus*, *L. plantarum*, *L. casei* , *L. acidophilus* , *Lactobacillus delbreckii*, *Lactobacillus ellobiosus*, *Lactobacillus lactis*, *Lactobacillus acidophilus*, *Lactobacillus reuteri*, *Lactobacillus brevis*, *Lactobacillus casei*, *Lactobacillus curvatus*, *Lactobacillus fermentum*, *Lactobacillus plantarum*, *Lactobacillus helveticus*,
- 10 *Streptococcus cremoris*, *Streptococcus thermophiles*, *Streptococcus intermedius*, *Streptococcus lactis*, *Streptococcus diacetylactis*, *Enterococcus faecalis*, *Lactococcus spp.*, *Lactococcus lactis subsp.*, *Pediococcus spp.*, *Pediococcus cerevisiae*, *Pediococcus acidilactici*, *Pediococcus pentosaceus*, *Bifidobacterium spp.* *Bifidobacterium animalis*, *Bifidobacterium adolescentis*, *Bifidobacterium bifidum*, *Bifidobacterium breve*,
- 15 *Bifidobacterium infantis*, *Bifidobacterium longum*, *Leuconostoc spp.* *L. salivarius*, *L. paracasei*, *L. gasseri*, *L. reuteri*, *B. Bifidum*, *B. longum*, *B. infantis*, *Lb. delbrueckii*, *Lb. plantarum*, *Lb. pentosus*, *Lb. brevis*, *P. damnosus*, *Lb. collinoides*, *Lb. pentosus*, *Pediococcus spp.*, *Lb. buchneri*, *Leuconostoc mesenteroides*, *Pedococcus pentosaceus*, *Lb. casei*, *Lb. kefir*, *Lb. acidophilus*, *Lb. helveticus*, *Lb. casei*, *Lb. bulgaricus*, *Lb. lactis*,
- 20 *Lb. plantarum*, *Lb. brevis* , *Acetobacter spp.*, *Streptococcus genera*, *Streptococcus spp.*, *Streptococcus lactis*, *S. thermophilus*, *S. durans*, *S. cremoris*, *Lactobacillus alimentarius*, *L. Alimentarius*, *L. multaromicus*, *L. sanfrancisco*, *Lactococcus lactis ssp*, *S. cerevisia*, *Lactobacillus sakei*, *Lactobacillus alimentarius*, *Lactobacillus paralimentarius*, *Lactobacillus paracasei*, *Lactobacillus buchneri*, *Enterococcus faecium*, *Enterococcus mundtii*, *Enterococcus faecalis*, *Enterococcus casseliflavus*, *Lactobacillus pentosus*,
- 25 *Enterococcus faecium*, *Pediococcus pentosaceus*, *Lactobacillus farciminis*, *Pichia kudriavzevii*, *Lactobacillus farciminis*, *Lactobacillus casei*, *Lactobacillus alimentarius*, *Pichia kudriavzevii* , *Candida humilis*, *L.lactis subsp. cremoris*, *Lb.delbrueckii subsp. lactis*, *Lb.helveticus*, *Lb.casei*, *Lb.delbrueckii subsp.* , *Leuc.mesenteroides subsp. cremoris*, *Lb.*
- 30 *johsonii*, *Lb.kefirenofacies*, *Lb.curvatus*, *P.acidilactici*, *P.pentosaceus*, *Lb.alimentarius*, *C.piscicola*, *Leuc.mesenteroides*, *P.acidilactici*, *P.cerevisiae*, *Lb.pentosus*, *P.acidilactici*, *T.halophilus*, *Lb.sanfransiscensis*, *Lb.farciminis*, *Lb.fermentum*, *Lb.amylovorus*,

- Lb.reuteri*, *Lb.pontis*, *Lb.panis*, *Lb.alimentarius*, *W.cibaria*, *O.oeni*, *L.coryniformis*, *L.curvatus*, *L.jugurti*, *L.jensenii*, *L.bucheneri*, *L.cellobiosus*, *L.coprophilus*, *L.hilgardii*, *L.leichmannii*, *L.dextranicum*, *P.acidilactici*, *P.pentosaceus*, *S.thermophilus*, *L.lactis* subsp. *diacety lactis*, *L.lactis* subsp. *hordniae*, *L.garvieae*, *L.rafinolactis*, *V.fluvialis*, *V.salmoninarum*, *Leuconostoc* sp., *L.cremoris*, *L.dextranicum*, *L.mesenteroides*, *L.paramesenteroides*, *L.gelidum*, *L.carnosum*, *Carnobacterium* sp., *C.divergens*, *C.mobile*, *C.gallinarum*, *C.piscicola*, *Vagococcus* sp., *V.fluvialis*, *V.salmoninarum*, *L.garvieae*, *Lactococcus diacetylactis*, *Propionibacterium freudenreichii*, *Pediococcus* sp., *S.uvarum*, *Lb.coryniformis*, *Candida crusei*, *Weissella confusa*, *Hansenula silvicola*, *Debaryomyces hansenii*, *Trichosporon beigelli*, *Bacillus amyloliquefaciens*, *Torulopsis* sp., *Candida mycoderma*, *Lb.buchneri*, *P.acidilactici*, *Pediococcus pentasaceus*, *L.cellobiosus*, *E.mundtii/E.gallinarum*, *E.casseliflavus*, *P.urinae-equi*, *Lb.murinus*, *Candida milleri*, *E.burtonii*, *E.fibulinger*, *Issatchenkia orientalis*, *Candida pelliculosa*, *C.tropicalis*, *Pediococcus acidilactici*, *Pseudopiantarum*, *Pediococcus acidilactici*, *Pediococcus pentosaceus*, *Leuconostoc pseudomesenteroides*, *Weissella cibaria*, *Lb. paraplantarum*, *Issatchenkia orientalis*, *Candida glabrata*, *Pediococcus acidilactici*, *Kluyveromyces marxianu*, *Pichia kudriavzevi*, *Saccharomyces servazzi*, *Torulaspora delbrueckii*, *Kazachstania unispora*, *Saccharomyces barnettii*.
- 5**
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- 15**
- 20** 10. Microcapsule loaded with the probiotic which is protective against mould and mycotoxin formation, produced by the method according to Claim 1.
- 25** 11. Use of the microcapsule loaded with probiotics according to Claim 10 in agriculture.
- 30** 12. Use of the microcapsule loaded with probiotics according to Claim 11 with grape, corn, almond, hazelnut, peanut derivatives, walnut, fresh/dry fruits/vegetables or grain products.
- 25** 13. Use of the microcapsule loaded with probiotics according to Claim 10 with stored agricultural products.
- 30** 14. Use of the microcapsule loaded with probiotics according to Claim 10 with food products.
15. Use of the microcapsule loaded with probiotics according to Claim 14 with food products such as cheese, olives, yoghurt, meat products, fruits and vegetables.
- 30** 16. Use of the microcapsule loaded with probiotics according to Claim 10 with cosmetic products.

17. Use of the microcapsule loaded with probiotics according to Claim 17 with cosmetic products such as toiletry, creams, gels and emulsions.