6. ABSTRACT OF THE INVENTION

The invention relates to the field of food, feed and food supplements comprising high vitamin B12 levels, whereby the vitamin B12 is produced by fermentation of *Lactobacillus* strains on finger millet. Methods for increasing vitamin B12 production of *Lactobacillus* strains are also provided.

5. CLAIMS

We claim:

1. A method for producing a medium fermented by *Lactobacillus* with increased total cobalamin levels, comprising the following steps:
   (a) preparing a fermentation medium from all or a part of an extract of *Eleusine coracana* flour;
   (b) inoculating said fermentation medium with bacteria of one or more cobalamin producing *Lactobacillus* strains; and
   (c) allowing fermentation to take place, thereby producing said fermentation medium with increased total cobalamin levels.
2. The method according to claim 1, wherein said cobalamin producing *Lactobacillus* strain is a wild type strain.
3. The method according to claim 1, wherein said cobalamin producing *Lactobacillus* strain is a probiotic strain.
4. The method according to claim 1, wherein said cobalamin producing *Lactobacillus* strain is a member of the species *L.reuteri*
5. The method according to claim 1 further comprising the step of adding glutamine and cobalt to the fermentation medium during any of steps (a), (b), and (c).
6. The method according to claim 1 wherein said finger millet extract is finger millet flour.
7. A method for preparing food, feed or food supplement product, comprising producing a fermented medium with increased cobalamin levels in accordance with claim 1, and using all or part of the medium to prepare said product.

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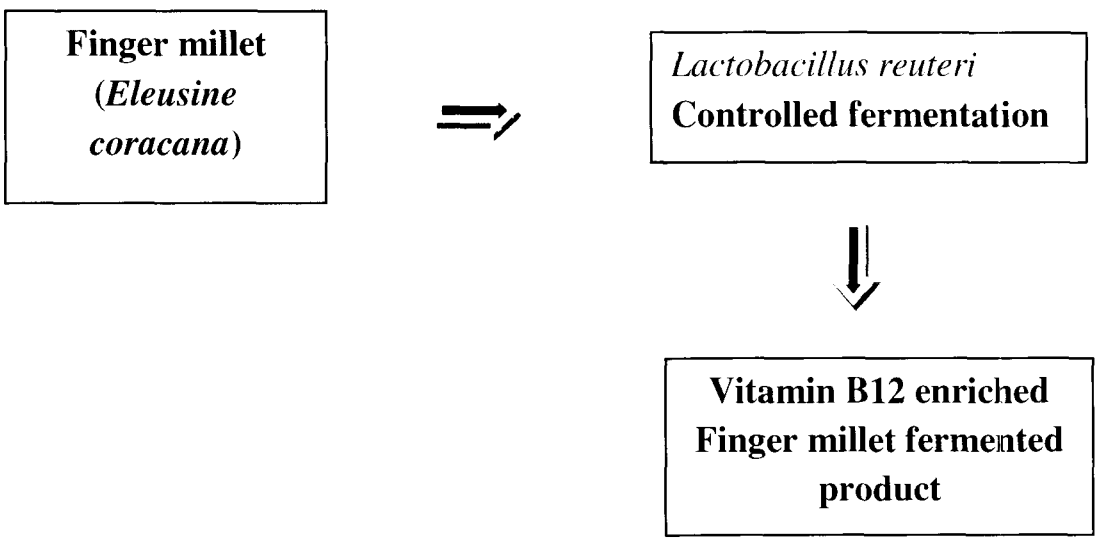

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Fortified product with

a) Vitamin B_{12}

b) Vitamin B_6

↓

Bioavailability of Vitamin B12 synthesized estimated in fermented food

↓ ↓

Identification and Quantification

a) Vitamin B_{12} and other vitamers which may be present in pictogram and microgram level as secondary metabolites.

Invitro

a) Caco-2 cell models

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4. DESCRIPTION

FIELD OF THE INVENTION

The invention relates to the field of microbiology and food and food-supplement production using microbial fermentation of finger millet. Provided are methods of producing high vitamin B12 levels by fermenting finger millet flour, as well as food and food supplements comprising or consisting of fermented finger millet flour and/or cobalamin (vitamin B12) obtained from such fermentation methods. Also, the use of finger millet flour (or parts thereof and/or dilutions and/or concentrations thereof) as a fermentation medium or fermentation supplement of cobalamin producing bacteria is provided herein. A further embodiment is the use of glutamine or cobalt (in combination with finger millet flour) for increasing cobalamin production during microbial fermentation.

General Definitions

"Lactic acid bacteria" (LAB) refers to bacteria, which produce lactic acid or another organic acid (such as propionic acid) as an end product of fermentation, such as, but not limited to, bacteria of the genus *Lactobacillus, Streptococcus, Lactococcus, Oenococcus, Leuconostoc, Pediococcus, Carnobacterium, Propionibacterium, Enterococcus* and *Bifidobacterium.*

"Food grade" are components which can safely be ingested (e.g. orally) by humans or animals.

"Probiotics" or "probiotic strain(s)" refers to strains of bacteria, which have a beneficial effect on the host when ingested by a subject and which are generally regarded as safe (GRAS) to humans.

BACKGROUND OF THE INVENTION

Vitamin B12 is an important vitamin for humans and can be obtained only from animal source. It is used to treat pernicious anaemia, peripheral neuritis and is used as a dietary supplement. Vitamin B12 is also an important animal feed supplement as growth enhancer.

The term vitamin B12 is used to describe compounds of the cobalt corrinoid family, in particular those of the cobalamin group. The most used compound of this group is cyanocobalamin and as such the term vitamin B12 is sometimes used to refer to cyanocobalamin. In this specification the term vitamin B12 should be attributed its broad meaning so as to include all the cobalt corrinoids of the cobalamin group, which include in particular cyanocobalamin, hydroxocobalamin, methylcobalamin and 5'-deoxyadenosylcobalamin, characterised by a cyano, hydroxyl, methyl or 5'-deoxyadenosyl radical respectively. The methylcobalamin and 5'deoxyadenosylcobalamin compounds are known to be unstable to light in isolated form and are easily transformed to hydroxocobalamin in aqueous solution.

Vitamin B12 is produced industrially by microbial fermentation using Pseudomonas denitrificans and Propionibacterium species (Martens et al., 2002). Contrary to Pseudomonas, Propionibacterium is food grade as described in EP0824152A2. Conversion of the produced natural vitamin B12 to cyanocobalamin form involves chemical processes like cyanidation followed by purification with organic solvents. This process is expensive apart from being unsafe for operators and environment.

DETAILED DESCRIPTION OF THE INVENTION

Methods of producing cobalamin according to the invention

The present invention provides methods for increasing cobalamin levels produced by cobalamin producing *Lactobacillus* species. The inventors found that total cobalamin levels, produced during fermentation of *Lactobacillus* species are significantly increased when finger millet extracts and/or finger millet flour or parts thereof or are used or added to the fermentation medium. In addition, foods such as cereal-containing or cereal derived foods are commonly preserved by fermentation, so that in accordance with the invention a method for food preservation (i.e., for reducing perishability food) is provided, whereby the resulting product has as further benefit an increased cobalamin level.

Thus in one embodiment of the invention a method for producing cobalamin and/or for increasing total cobalamin levels produced by *Lactobacillus* species is provided comprising the following steps:

(a) providing an extract from a finger millet flour of a *Eleusine coracana* species;

(b) using all or part of the finger millet flour extract to prepare a fermentation medium;

(c) inoculating said fermentation medium with one or more *Lactobacillus* strains;

(d) allowing fermentation to take place; and optionally

(e) using all or part of the fermented medium for the preparation of a food-, feed- or food supplement product with high cobalamin levels.

EXAMPLES

The invention will be further described by way of the following examples in order to further elucidate the process.

Example 1

1. Fermentation on finger millet flour

In this example it was determined whether finger millet flour fermentation yielded cobalamin production

Material and Methods

1.1.1 Strain and Inoculum Preparation

The following strain was used:

Lactobacillus reuteri (1%) inoculum was used for inoculation of the developed media. *L.reuteri* was isolated from human breast milk.

1.1.2 Preparation of fermentation medium and CDM

Finger millet media was made from *Eleusine coracana* (CO9, CO13, CO14 and OUAT2 varieties) separately. The finger millet grains were powdered using a kitchen blender. The powdered samples were stored at -20°C until further use. Before inoculation, the media components were autoclaved separately and then mixed to assure sterility.

Fermentation

The finger millet flour was inoculated with *L.reuteri.*

1.2 Results

The cobalamin production levels were determined for *L.reuteri* in finger millet flour. Results were shown in Table 1 for *L.reuteri.*

TABLE 1

Fermentation of *L.reuteri* strain in finger millet flour

Samples	Total cobalamin production (µg/g)
Finger millet (CO9)	20.6
Finger millet (CO13)	35.95
Finger millet (CO14)	38.3
Finger millet (OUAT2)	21.95

Example 2

In this example the possibility of extending the findings described above for other natural substrates were investigated. The same experiment was tested using oats (*Avena sativa*), soybean (*Glycine max*), spinach (*Spinacia oleracea*), moringa (*Moringa oleifera*) and mulai keerai (*Amaranthus* sp.).

Samples	Total cobalamin production (µg/g)
Oats	26.7
Soybean	33.05
Spinach	27.95

Moringa	29.8
Mulai keerai	24.05

Example 3

Vitamin B12 production was determined after fermentation of *L. reuteri* on chemically defined medium (CDM). The experiments were performed as described above. Chemically defined media was made from cobalt nitrate and glutamic acid.

B12 measurements

Vitamin B12 content was determined according to the Chemiluminescence assay, using luminol.

Samples	Total cobalamin production (μg/g)
Cobalt	61.4
Glutamic acid	44.85

3.2 Conclusion

The bacterium was able to produce B12 on finger millet, natural substrates and CDM.

Example 4

Based on the three examples above a food product with beneficial properties is made. The food product is fermented cereal flour that produces cobalamin. Moreover, *Lactobacilli* with probiotic properties to ferment finger millet flour and increase the cobalamin content.

PATENT CITATIONS

Cited Patent	Filing date	Publication date	Applicant	Title
EP0824152A2	12 Aug 1997	18 Feb 1998	Gist-Brocades B.V.	Microbial production of vitamin B12 and production and use of compositions comprising high concentrations of vitamin B12 activity
EP2227552B1	5 Dec 2008	2 Nov 2011	Purac Biochem B.V.	Increased folate production levels by fermenting melon juice

with Lactobacillus

NON-PATENT CITATIONS

Reference

1. Martens, J. H., Barg, H., Warren, M., & Jahn, D., 2002 "Microbial production of vitamin B12". Applied Microbiology and Biotechnology, 58(3), pp. 275-285.