

DOCUMENT MADE AVAILABLE UNDER THE PATENT COOPERATION TREATY (PCT)

International application number:	PCT/EP2020/083770
International filing date:	27 November 2020 (27.11.2020)
Document type:	Certified copy of priority document
Document details:	Country/Office: EP
	Number: 19219287.0
	Filing date: 23 December 2019 (23.12.2019)
Date of receipt at the International Bureau:	03 December 2020 (03.12.2020)

Remark: Priority document submitted or transmitted to the International Bureau in compliance with Rule 17.1(a),(b) or (b-bis)

CERTIFICATE OF AVAILABILITY OF A CERTIFIED PATENT DOCUMENT IN A DIGITAL LIBRARY

The International Bureau certifies that a copy of the patent application indicated below has been available to the WIPO Digital Access Service since the date of availability indicated, and that the patent application has been available to the indicated Office(s) as of the date specified following the relevant Office code:

Document details: Country/Office: **EP**

Filing date: **23 Dec 2019 (23.12.2019)**

Application number: **19219287**

Date of availability of document: **24 Dec 2019 (24.12.2019)**

The following Offices can retrieve this document by using the access code:

**AR, AT, AU, BE, BR, CA, CL, CN, CO, DK, EA, EE, EP, ES, FI, GB,
GE, IB, IL, IN, JP, KR, MA, MX, NL, NO, NZ, SE, US**

Date of issue of this certificate: **03 Dec 2020 (03.12.2020)**

Bescheinigung

Die angehefteten
Unterlagen stimmen mit der
als ursprünglich eingereicht
geltenden Fassung der auf
dem nächsten Blatt
bezeichneten europäischen
Patentanmeldung überein.

Certificate

The attached documents are
exact copies of the text in
which the European patent
application described on the
following page is deemed to
have been filed.

Attestation

Les documents joints à la
présente attestation sont
conformes au texte,
considéré comme
initialement déposé, de la
demande de brevet
européen qui est spécifiée à
la page suivante.

Patentanmeldung Nr.

Patent application No.

Demande de brevet n°

19219287.0 / EP19219287

The organisation code and number of your priority application, to be used for filing abroad under the Paris Convention, is EP19219287.

Der Präsident des Europäischen Patentamts;
Im Auftrag

For the President of the European Patent Office

Le Président de l'Office européen des brevets
p.o.



V. Joseph

Anmeldung Nr:
Application no.: 19219287.0
Demande no :

Anmeldetag:
Date of filing: 23.12.19
Date de dépôt :

Anmelder / Applicant(s) / Demandeur(s):

Evonik Operations GmbH
Rellinghauser Straße 1-11
45128 Essen/DE

Bezeichnung der Erfindung / Title of the invention / Titre de l'invention:

(Falls die Bezeichnung der Erfindung nicht angegeben ist, oder falls die Anmeldung in einer Nicht-Amtssprache des EPA eingereicht wurde, siehe Beschreibung bezüglich ursprünglicher Bezeichnung.

If no title is shown, or if the application has been filed in a non-EPO language, please refer to the description for the original title.

Si aucun titre n'est indiqué, ou si la demande a été déposée dans une langue autre qu'une langue officielle de l'OEB, se référer à la description pour le titre original.)

PREPARATION COMPRISING PROBIOTIC STRAINS FOR GLUTEN DEGRADATION

In Anspruch genommene Priorität(en) / Priority(Priorities) claimed / Priorité(s) revendiquée(s)

Staat/Tag/Aktenzeichen / State/Date/File no. / Pays/Date/Numéro de dépôt:

Am Anmeldetag benannte Vertragsstaaten / Contracting States designated at date of filing / Etats contractants désignés lors du dépôt:

AL AT BE BG CH CY CZ DE DK EE ES FI FR GB GR HR HU IE IS IT LI LT LU LV MC MK MT NL NO PL
PT RO RS SE SI SK SM TR

Preparation comprising probiotic strains for gluten degradation

The current invention concerns preparations comprising probiotic strains belonging to the genera *Bacillus sp.*, *Lactobacillus sp.*, and *Pediococcus sp.* as viable cells or cytoplasmic extract thereof, and proteases and their use for safe gluten degradation in humans and during food production.

- 5 Gluten is the main protein network of cereals such as wheat, rye, and barley. Gluten includes monomeric α -gliadins, γ -gliadins, Ω -gliadins, which carry peptide sequences with immunogenic and/or toxic potential (the most prominent examples are listed in table 1).

Gliadin	Position	Sequence
α 9-gliadin	57-68	QLQFPFPQQLPY
A-gliadin	62-75	PQPQLPYPQPQSFP
Y-gliadin	134-153	QQLPQPQPQSQSFPQQQRPF
α 2-gliadin	57-89	LQLQFPFPQQLPYPQPQLPYPQPQLPYPQPQPF

Table 1: Immunogenic gliadin peptides

- 10 Incomplete digestion of gliadins can release these peptides, leading to adverse reactions in susceptible individuals. The literature also shows the release of immunogenic peptides from glutenins, the other protein fraction constituting gluten. The spectrum of gluten-related disorders includes celiac disease (CD), wheat allergy (WA), non-celiac gluten sensitivity (NCGS), and gluten-sensitive irritable bowel syndrome [1]. Though these disorders have pathogenic differences, they show related symptoms and, as they are not curable, are treated by avoidance of gluten / gluten-
- 15 containing foods. Moreover, various other health conditions (e.g. schizophrenia, atopy, fibromyalgia, endometriosis, obesity, non-specific gastrointestinal symptoms) have been suggested to benefit from gluten avoidance [2]. These facts explain the rise of gluten-free diets (GFD); and such practice also extends to a large and increasing number of healthy, symptom-free people. For example, reportedly 33 % of the US population wants to avoid gluten, and 41 % of an athlete
- 20 population reported being on a GFD for more than 50 % of the time [3].

- Practicing a GFD is however associated with challenges and adverse effects, which need to be considered in a risk and benefit evaluation, especially when there is no clear indication to maintain a GFD, i.e. where gluten avoidance is rather a lifestyle choice than a medical necessity. A GFD is often imbalanced, e.g. due to the avoidance of cereal products, with micronutrient and fiber
- 25 deficiencies, alongside an excess of calories and an increased content of sugar and saturated fats found in many gluten-replacement foods [4-6]. Potential harms of a GFD therefore include growth/development retardation for children and adolescents, various malnutrition-associated disorders, hyperlipidemia, hyperglycemia, and coronary artery disease [6]. Moreover, long-term adherence to a GFD can cause intestinal microbiome dysbiosis with subsequent adverse health
- 30 effects [7].

A GFD is at present the only effective therapy for CD, WA, and NCGS patients. Particularly for CD, ingestion of gluten or similar proteins are the trigger for the development and exacerbation of the disease, whereby strict avoidance of gluten ingestion is of critical importance. However, even food products considered or claimed as being gluten-free often contain (trace) amounts of gluten that

are above a safe limit of gluten intake (typically < 20 ppm for CD patients). To ensure food safety for CD patients, strategies have been conceived to support gluten avoidance or detoxification. A key determinant of the intestinal fate of gluten and the physiological response to it is the intestinal microbiota, as has been revealed from experiments with differentially colonized mice [8] and from
 5 comparisons of microbiota from CD patients versus healthy individuals [9, 10]. In line with this, several microbiota-targeted technologies have been developed with the aim to ameliorate gluten-related disorders. These technologies can be categorized into: 1. Oral application of *Lactobacillus* spp. or *Bifidobacterium* spp. to correct dysbiosis associated with GFD or gluten-related disorders, 2. Oral application of *Lactobacillus* spp. or *Bifidobacterium* spp. as non-specific support for gluten-
 10 related disorders via undefined mechanisms., 3. Oral application of *Lactobacillus* spp. or *Bifidobacterium* spp. to support the degradation of gluten, 4. Oral application of peptide hydrolases to support the degradation of gluten ("glutenases"). Degradation of toxic/immunogenic gluten epitopes requires combined activities of peptidases PepN, PepO, Pepl, PepX, PepP [11].

AU2015261774 AA claims the use of *Lactobacillus casei* IPLA12038 to prevent or treat CD. This
 15 strain has been described to degrade a certain amount of the 33-mer, but not any other important immunogenic peptide, within 12 hours and to possess the following enzymatic activities: PepN 8.03 mEU/mg; PepQ 9.5 mEU/mg; Pepl 0.58 mEU/mg; PepX 3.19 mEU/mg. The strain does not survive acidic conditions (pH < 3.0) and therefore does not offer a technical solution for gluten-related disorders.

20 WO2017139659 A1 claims cleavage of XPQ motifs and only two immunogenic peptides (33-mer and 26-mer) by subtilisins from *Rothia* species.

AU2008341708 AA claims the use of *Bifidobacterium longum* CECT 7347 in the treatment of gluten-related disorders; no reference is made to any peptidase or protease activities of this strain against gluten or critical epitopes therein.

25 WO17134240 A1 claims compositions containing the species *Faecalibacterium prausnitzii*, *Butyricicoccus pullicaecorum*, *Roseburia inulinivorans*, *Roseburia hominis*, *Akkermansia muciniphila*, *Lactobacillus plantarum* and *Anaerostipes caccae* for the treatment of CD.

US2017000830 AA claims compositions containing *Lactococcus* species and enzymes for amelioration of gluten sensitivity. Francavilla et al. reported improvement of irritable bowel
 30 syndrome (IBS)-like symptoms of CD patients on a GFD after application of a combination of five strains from the genera *Lactobacillus* and *Bifidobacterium* [12]. This treatment was associated with a shift in gut microbiota composition; effects of the strains on gluten digestion were however not reported. Meanwhile, the same group assessed *in vitro* peptidase activities of *Lactobacillus* strains, showing activities of up to 10 mU/mg for PepN, 10 mU/mg for Pepl, 5 mU/mg for PEP, 25 mU/mg
 35 for PepQ for strains of the species *Lactobacillus plantarum*, *Lactobacillus bulgaricus*, *Lactobacillus rhamnosus*, *Lactobacillus paracasei*, and *Lactobacillus casei* [13]. Combined application of ten of these strains led to hydrolysis of gliadin epitopes listed in Table 1 after 24 hours of incubation. Survival of the strains under gastric and small intestinal conditions was not determined, the

effectiveness of these strains on gluten digestion in the gastrointestinal tract of humans can therefore not be predicted.

Herran et al. isolated 27 bacterial strains belonging to the species *L. salivarius*, *L. rhamnosus*, *L. reuteri*, *L. casei*, *L. oris*, *L. gasseri*, *L. fermentum*, *L. crispatus*, *L. brevis*, *B. subtilis*, *B.*

- 5 *amyloliquefaciens*, *B. pumilus*, and *B. licheniformis* from the small intestine of humans that showed proteolytic activity against the 33-mer only after a very long incubation time of 24 hours and not against other peptides [14]. Similarly, weak activity against this epitope was found for other strains of human small intestinal origin, again including the species *B. subtilis*, *B. pumilus*, and *B. licheniformis* [15].

- 10 The commercial use of peptide hydrolases with the intention to detoxify gluten during food processing [16] and in humans [17] has been described. However, related products containing these enzymes have minimal evidence of efficacy [17].

- Conclusively, the problems of gluten-related disorders remain unsolved, as all the above-mentioned applications have several problems and have not been successfully translated into the
15 clinic. Applications 1 and 2 may merely provide an indirect benefit, as the underlying problem of gluten toxicity is not addressed directly. Application 3 has so far been limited to *ex vivo* digestion of gluten, assessment of solely protease activity against gluten/gliadin proteins, leading to only partial digestion, or assessment of digestion of only very few immunogenic gluten peptides after very long incubation times of 12 hours or longer.

- 20 The lack of successful clinical translation can be explained by poor survival of the combined microbial strains under realistic conditions in the gastrointestinal tract, and due to limited activity against gluten peptides in food matrices.

Microbial enzyme treatments for CD patients (Application 4) have the major limitation of poor proteolytic resistance, extent and duration of enzymatic activity during gastrointestinal transit [18].

- 25 Moreover, they are even considered as hazardous because they can only partially degrade gluten and thereby potentially release toxic epitopes [17].

It was aimed to overcome these limitations by developing an inventive and effective technology based on the following considerations:

- 30
 - Gluten/gliadin/glutenin degradation during human digestion is not beneficial *per se*, because incomplete degradation can lead to the formation of toxic and/or immunogenic peptides
 - Concerns have been expressed on the safety of currently available means to trigger *gluten degradation in vivo*, as these may do so only partially and can thereby induce or worsen gluten toxicity
- 35
 - Any attempt to trigger gluten degradation *in vivo* needs to make sure that such degradation is complete and leads to safe degradation products
 - Given the diversity of gluten-inherent peptide sequences with immunogenic potential, a combination of peptide hydrolases from different microbes is required to ensure complete degradation of all peptides

- Such combination is preferably provided by a consortium of probiotic microorganisms that are metabolically active and synergize with each other in relevant parts of the gastrointestinal tract (i.e. the stomach and duodenum) to promote a safe, rapid, and complete digestion of gluten proteins from relevant food matrices to non-toxic, non-immunogenic small peptides or amino acids
- We conceived that such a synergism can be achieved by combining acid- and bile-resistant strains with suitable protein/peptide substrate specificities and found that combinations of certain *Lactobacillus* sp., *Bacillus* sp., and in some cases also *Pediococcus* sp., including their cytoplasmic extracts, from specific ecological niches are particularly useful for this

In this invention we disclose the application of consortia comprised by *Lactobacillus* and *Bacillus* species, with optional addition of *Pediococcus* strains as a technology for use in the degradation of gluten in the gastrointestinal tract of humans and animals as well as during food production.

The means how such technology can bring a benefit to people in need thereof as well as to people willing to minimize their exposure to gluten for precautionary or other reasons is as follows:

- (i) Safe clearance of intentionally or accidentally ingested gluten as a cure or complementing therapy for CD, WA, and NCGS patients. The possibility to return to a conventional, gluten-containing diet.
- (ii) Safe clearance of intentionally or accidentally ingested gluten as a cure or complementing therapy for people with non-specific intestinal or extra-intestinal symptoms that may result from ingested gluten. The possibility to return to a conventional, gluten-containing diet.
- (iii) Offering a solution for symptom-free people wanting to minimize their gluten exposure as an alternative to adhering to a GFD.

25

The present invention is directed to preparations comprising *Lactobacillus* and *Bacillus* species, with optional addition of *Pediococcus* strains. These new preparations promote the digestion of gluten to non-toxic and non-immunogenic peptides/amino acids in the human gastrointestinal tract and during the production of gluten-containing food stuffs.

30 Subject of the present invention is therefore a preparation comprising consortia of at least one probiotic strain selected from the genus *Bacillus* and at least one probiotic strain selected from the genus *Lactobacillus*, for use in safe and complete degradation of gluten,

- a) wherein said consortium of strains can degrade the 12-mer peptide QLQPFQPQLPY (Seq-ID No 1), the 14-mer peptide PQPQLPYPQPQSFP (Seq-ID No 2), the 20-mer peptide QQLPQPQQPQQSFPQQQRPF (Seq-ID No 3), the 33-mer peptide LQLQPFQPQLPYQPQLPYQPQLPYQPQP (Seq-ID No 4).

35

In a preferred configuration, the consortium of strains can degrade the 12-mer peptide QLQPFQPQLPY (Seq-ID No 1), the 14-mer peptide PQPQLPYPQPQSFP (Seq-ID No 2), the 20-

mer peptide QQLPQPQQPQQSFPQQQRPF (Seq-ID No 3), the 33-mer peptide LQLQFPQPQLPYPQPQLPYPQPQLPYPQPQPF (Seq-ID No 4) by at least 1% or at least 10 %, or at least 20 % or at least 30 % or at least 40 %, or at least 50 % or at least 60 % or at least 70 %, or at least 80 %, or at least 90 %, preferably at least 95 %, more preferably at least 98 %.

5 A further preferred configuration is a preparation for use,

b) wherein said consortium of strains is capable of degrading gluten to a digest that does not cause an immunogenic or toxic response in the small intestine or small intestinal explant of a subject or animal affected by a gluten-related disorder, and/or

10 c) wherein all strains of the consortium survive (less than 2 log CFU loss) simulated gastric (pH 1.0-4.0) and intestinal (pH 5.4-6.8; 0.05-0.6 % bile acids, or the concentration of bile acids present in humans under in vivo conditions) conditions, and/or

d) wherein members of the consortia have complementary PepP, PepO, PepX, Pepl, and PepN activities with at least one peptidase activity equal to or more than 3 U/g (PepP), 5 U/g (PepO), 20 U/g (PepX), 17 U/g (Pepl), 20 U/g (PepN).

15 Bacteria of the species *Bacillus subtilis*, *Bacillus pumilus*, *Bacillus licheniformis* and *Bacillus megaterium*, *Bacillus amyloliquefaciens* and *Lactobacillus plantarum*, *Lactobacillus paracasei*, *Lactobacillus sanfranciscensis*, *Lactobacillus brevis*, *Lactobacillus reuteri*, and in some cases also *Pediococcus sp.* were found to be especially suitable for this effect.

Therefore, in a preferred embodiment, the *Bacillus* strains are selected from *Bacillus pumilus*,
20 *Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus megaterium*, preferably selected from *Bacillus pumilus* DSM 33297, DSM 33335, DSM 33301, *Bacillus subtilis* DSM 33353, DSM 33298, *Bacillus licheniformis* DSM 33354, *Bacillus megaterium* DSM 33300, DSM 33336.

The *Lactobacillus* strains are selected from *Lactobacillus plantarum*, *Lactobacillus casei*,
25 *Lactobacillus paracasei*, *Lactobacillus brevis*, *Lactobacillus sanfranciscensis*, *Lactobacillus reuteri*, preferably selected from *Lactobacillus plantarum* DSM 33368, DSM 33369, DSM 33370, DSM 33367, DSM 33366, DSM 33364, DSM 33363, DSM 33362, DSM 33365, *Lactobacillus paracasei* DSM 33376, DSM 33375, DSM 33373, *Lactobacillus reuteri* DSM 33374, *Lactobacillus brevis* DSM 33377, *Lactobacillus sanfranciscensis* DSM 33378, DSM 33379.

In a preferred embodiment, the preparation for use according to the present invention comprises
30 one or more of the following strains:

L. plantarum DSM 33370, DSM 33363, DSM 33364, DSM 33365; *L. paracasei* DSM 33373; *L. brevis* DSM 33377; *Bacillus pumilus* DSM 33297, DSM 33335, *Bacillus licheniformis* DSM 33354, *Bacillus megaterium* DSM 33300, and *Bacillus subtilis* DSM 33353, or

L. plantarum DSM 33362, DSM 33367, DSM 33368; *L. paracasei* DSM 33375; *L. sanfranciscensis*
35 DSM 33379; *Bacillus pumilus* DSM 33301, *Bacillus megaterium* DSM 33300, DSM 33336, *Bacillus subtilis* DSM 33298, DSM 33353, or

L. plantarum DSM 33366, DSM 33369; *Lactobacillus reuteri* DSM 33374; *L. paracasei* DSM 33376; *Pediococcus pentosaceus* DSM 33371; *L. sanfranciscensis* DSM 33378; *Bacillus licheniformis* DSM 33354, *Bacillus pumilus* DSM 33301, *Bacillus megaterium* DSM 33300, DSM 33336, and *Bacillus subtilis* DSM 33298, or

- 5 *L. plantarum* DSM 33363 and DSM 33364, DSM 33365, *L. paracasei* DSM 33373, *Bacillus pumilus* DSM 33297 and *Bacillus megaterium* DSM 33300, or

L. plantarum DSM 33363 and DSM 33364, DSM 33365, *L. paracasei* DSM 33373, *Bacillus subtilis* DSM 33298, and *Bacillus pumilus* DSM 33301.

- 10 The cells of the strains of the current invention may be present in the compositions of the current invention, as spores (which are dormant), as vegetative cells (which are growing), as transition state cells (which are transitioning from growth phase to sporulation phase), as cellular extracts or as a combination of at least two of these types of cells. In a preferred embodiment, the probiotic strain is present in a dormant form or as vegetative cells. In alternative embodiment, cytoplasmic
15 extracts or cell-free supernatants or heat-killed biomass of the probiotic strains are used.

In a further preferred embodiment, the preparation further comprises one or more probiotic strains, preferably selected from *Pediococcus sp.*, *Weissella sp.*, more preferably *Pediococcus pentosaceus* DSM 33371.

- In a further preferred embodiment, the preparation further comprises one or more of the following:
20 microbial proteases purified from *Aspergillus niger*, *Aspergillus oryzae*, *Bacillus sp.*, *Lactobacillus sp.*, *Pediococcus sp.*, *Weissella sp.*, *Rothia mucilaginosa*, *Rothia aeria*, subtilisins, nattokinase, arabinoxylans, barley grain fibre, oat grain fibre, rye fibre, wheat bran fibre, inulins, fructooligosaccharides (FOS), galactooligosaccharides (GOS), resistant starch, beta-glucans, glucomannans, galactoglucomannans, guar gum, xylooligosaccharides, alginate.

- 25 The invention is also directed to preparations for use for treating or preventing gluten-related disorders, preferably selected from celiac disease, non-celiac gluten sensitivity, wheat allergy, and gluten-sensitive irritable bowel syndrome in a subject or animal in need thereof.

- Moreover, the invention is directed to preparations for use for producing gluten-free foods, from gluten-containing cereals wheat, barley, rye, and oat, preferably containing less than 20 ppm
30 gluten.

In a preferred embodiment, the preparation for use further comprises a substance, which acts as permeabilizer of the microbial cell membrane of members of *Bacillus sp.*, *Lactobacillus sp.*, *Pediococcus sp.*, *Weissella sp.*, preferably alginate.

- In an alternative embodiment, one or more of the probiotic strains selected from *Bacillus sp.*,
35 *Lactobacillus sp.*, *Pediococcus sp.* and *Weissella sp.* are immobilized individually or as consortia. Immobilization can be realized on solid surfaces such as cellulose and chitosan, as entrapment within a porous matrix such as polysaccharide gels like alginates, k-carrageenan, agar, chitosan and polygalacturonic acid or other polymeric matrixes like gelatin, collagen and polyvinyl alcohol or

by flocculation and microencapsulation or electrospraying technologies. Strains of the genera *Lactobacillus*, *Bacillus*, and *Pediococcus* were screened for survival under simulated gastrointestinal conditions and for PepN, PepO, PEP, PepX, PepQ activities. The following strains survived under simulated gastrointestinal conditions (less than 2 log reduction of CFU) and showed exceptionally high peptidase activities such as PepN, PepO, PEP, PepX, PepQ:

Lactobacillus plantarum DSM 33370, DSM 33362, DSM 33365, DSM 33363, DSM 33364, DSM 33367, DSM 33366, DSM 33369, DSM 33368

Lactobacillus reuteri DSM 33374

Lactobacillus brevis DSM 33377

Lactobacillus paracasei DSM 33373, DSM 33375, DSM 33376

Lactobacillus sanfranciscensis DSM 33378, DSM 33379

Bacillus subtilis DSM 33298, DSM 33353

Bacillus pumilus DSM 33301, DSM 33297, DSM 33335

Bacillus megaterium DSM 33300, DSM 33336

Bacillus licheniformis DSM 33354

Pediococcus pentosaceus DSM 33371

Thus *Bacillus/Lactobacillus/Pediococcus* strains that are preferably used for preparations according to the present invention are selected from the following groups:

The *Bacillus subtilis* strains as deposited under DSM 33298, DSM 33353 at the DSMZ; the *Bacillus pumilus* strains as deposited under DSM 33301, DSM 33297, DSM 33335 at the DSMZ; the *Bacillus megaterium* strains as deposited under DSM 33300, DSM 33336 at the DSMZ; the *Bacillus licheniformis* strain as deposited under DSM 33354 at the DSMZ; the *Pediococcus pentosaceus* strain as deposited under DSM 33371 at the DSMZ; the *Lactobacillus sanfranciscensis* strains as deposited under DSM 33378, DSM 33379 at the DSMZ; the *Lactobacillus plantarum* strains as deposited under DSM 33370, DSM 33362, DSM 33365, DSM 33363, DSM 33364, DSM 33367, DSM 33366, DSM 33369, DSM 33368 at the DSMZ; the *Lactobacillus reuteri* strain as deposited under DSM 33374 at the DSMZ; the *Lactobacillus brevis* strain as deposited under DSM 33377 at the DSMZ; the *Lactobacillus paracasei* strains as deposited under DSM 33373, DSM 33375, DSM 33376 at the DSMZ.

When strains *L. plantarum* DSM 33370, DSM 33363, DSM 33364, DSM 33365; *L. paracasei* DSM 33373; *L. brevis* DSM 33377; *Bacillus pumilus* DSM 33297, DSM 33335, *Bacillus licheniformis* DSM 33354, *Bacillus megaterium* DSM 33300, *Bacillus subtilis* DSM 33353 were combined (combination 1), all the four tested gluten epitopes listed in Table 1 were completely degraded within 12 hours.

Likewise, combination 2, which comprised *L. plantarum* DSM 33362, DSM 33367, DSM 33368; *L. paracasei* DSM 33375; *L. sanfranciscensis* DSM 33379; *Bacillus pumilus* DSM 33301, *Bacillus megaterium* DSM 33300, DSM 33336, *Bacillus subtilis* DSM 33298, DSM 33353,

and combination 3, which comprised *L. plantarum* DSM 33366 and DSM 33369, *Lactobacillus reuteri* DSM 33374; *L. paracasei* DSM 33376; *Pediococcus pentosaceus* DSM 33371; *L. sanfranciscensis* DSM 33378; *Bacillus licheniformis* DSM 33354, *Bacillus pumilus* DSM 33301, *Bacillus megaterium* DSM 33300, DSM 33336, *Bacillus subtilis* DSM 33298 led to complete
 5 degradation of gluten epitopes listed in Table 1 within 12 hours.

Furthermore, a combination of *Lactobacillus plantarum* DSM 33363 and DSM 33364, *Lactobacillus plantarum* DSM 33365, *Lactobacillus paracasei* DSM 33373, and *Bacillus pumilus* DSM 33297 and *Bacillus megaterium* DSM 33300 were similarly effective as strain combinations 1-3.

Therefore, a preferred configuration of the present invention is directed to a preparation comprising
 10 strain combinations selected from the following:

L. plantarum DSM 33370, DSM 33363, DSM 33364, DSM 33365; *L. paracasei* DSM 33373; *L. brevis* DSM 33377; *Bacillus pumilus* DSM 33297, DSM 33335, *Bacillus licheniformis* DSM 33354, *Bacillus megaterium* DSM 33300 and *Bacillus subtilis* DSM 33353, or

L. plantarum DSM 33362, DSM 33367, DSM 33368; *L. paracasei* DSM 33375; *L. sanfranciscensis* DSM 33379; *Bacillus pumilus* DSM 33301, *Bacillus megaterium* DSM 33300, DSM 33336, *Bacillus subtilis* DSM 33298 and DSM 33353, or
 15

L. plantarum DSM 33366 and DSM 33369, *Lactobacillus reuteri* DSM 33374; *L. paracasei* DSM 33376; *Pediococcus pentosaceus* DSM 33371; *L. sanfranciscensis* DSM 33378; *Bacillus licheniformis* DSM 33354, *Bacillus pumilus* DSM 33301, *Bacillus megaterium* DSM 33300, DSM 33336 and *Bacillus subtilis* DSM 33298, or
 20

Lactobacillus plantarum DSM 33363 and DSM 33364, *Lactobacillus plantarum* DSM 33365, *Lactobacillus paracasei* DSM 33373, and *Bacillus pumilus* DSM 33297 and *Bacillus megaterium* DSM 33300.

Numerous other combinations among the above listed strains of *Bacillus* sp., *Lactobacillus* sp., and
 25 *Pediococcus* sp. showed comparable performance to those already indicated.

More specifically, preferred configurations of this invention comprise strain combinations that in sum provide high enzymatic PepN, PepI, PepO, PepX, and PepP activity; consequently, such combinations contain at least one strain of each of the following groups 1-5, wherein group members have particularly high enzymatic activity for PepN (group 1), PepI (group 2), PepO (group
 30 3), PepX (group 4), PepP (group 5).

A subject of the present invention is therefore a preparation comprising at least one strain of each of the following groups 1-5:

Group 1: *Bacillus subtilis*, *Bacillus pumilus*, *Bacillus licheniformis*, *Bacillus megaterium* *Pediococcus pentosaceus*, *Lactobacillus plantarum*, *Lactobacillus brevis*, *Lactobacillus paracasei*, *Lactobacillus paracasei*, *Lactobacillus reuteri*, *Lactobacillus plantarum*,
 35 *Lactobacillus plantarum*,

Group 2: *Lactobacillus paracasei* *Lactobacillus plantarum*,

Group 3: *Bacillus subtilis*, *Bacillus pumilus*,

Group 4: *Lactobacillus sanfranciscensis*, *Pediococcus pentosaceus*, *Lactobacillus plantarum*, *Lactobacillus reuteri*, *Lactobacillus paracasei*,

5 Group 5: *Bacillus megaterium*, *Lactobacillus sanfranciscensis*, *Pediococcus pentosaceus*, *Lactobacillus brevis*, *Lactobacillus plantarum*, *Lactobacillus reuteri*, *Lactobacillus paracasei*.

Therefore, a preferred embodiment is a preparation wherein the strains are selected from the following groups 1-5:

10 Group 1: *Bacillus subtilis* DSM 33298, *Bacillus pumilus* DSM 33297, *Bacillus licheniformis* DSM 33354, *Bacillus megaterium* DSM 33336, *Pediococcus pentosaceus* DSM 33371, *Lactobacillus plantarum* DSM 33370, *Lactobacillus brevis* DSM 33377, *Lactobacillus paracasei* DSM 33376, *Lactobacillus paracasei* DSM 33375, *Lactobacillus reuteri* DSM 33374, *Lactobacillus plantarum* DSM 33367, *Lactobacillus plantarum* DSM 33363, *Lactobacillus paracasei* DSM 33373, *Lactobacillus plantarum* DSM 33365, *Lactobacillus plantarum* DSM 33362,

15 Group 2: *Lactobacillus paracasei* DSM 33375, *Lactobacillus paracasei* DSM 33373, *Lactobacillus plantarum* DSM 33365,

Group 3: *Bacillus subtilis* DSM 33353, *Bacillus pumilus* DSM 33335, *Bacillus pumilus* DSM 33301,

20 Group 4: *Lactobacillus sanfranciscensis* DSM 33378, *Lactobacillus sanfranciscensis* DSM 33379, *Pediococcus pentosaceus* DSM 33371, *Lactobacillus plantarum* DSM 33370, *Lactobacillus plantarum* DSM 33369, *Lactobacillus reuteri* DSM 33374, *Lactobacillus plantarum* DSM 33363, *Lactobacillus paracasei* DSM 33373, *Lactobacillus plantarum* DSM 33365,

25 Group 5: *Bacillus megaterium* DSM 33300, *Lactobacillus sanfranciscensis* DSM 33378, *Pediococcus pentosaceus* DSM 33371, *Lactobacillus brevis* DSM 33377, *Lactobacillus plantarum* DSM 33368, *Lactobacillus reuteri* DSM 33374, *Lactobacillus plantarum* DSM 33367, *Lactobacillus plantarum* DSM 33366, *Lactobacillus plantarum* DSM 33364, *Lactobacillus paracasei* DSM 33373, *Lactobacillus plantarum* DSM 33365.

30

Another preferred configuration of the present invention are formulations to be used in the preparation of food stuffs from cereals by e.g. fermentation and baking processes.

Therefore, the invention is also related to a food supplement or food product or pharmaceutical product, comprising a preparation according to the present invention.

35 One subject of the present invention is the use of a preparation according to the present invention as a food supplement or its use in foodstuffs. Preferred foodstuffs according to the invention are chocolate products, gummies, mueslis, muesli bars, and dairy products.

A further subject of the current invention is also the use of a preparation of the current invention as a synbiotic ingredient in food products.

- 5 A further subject of the present invention is a foodstuff composition containing a preparation according to the present invention and at least one further food ingredient, preferably selected from proteins, carbohydrates, fats, further probiotics, prebiotics, enzymes, vitamins, immune modulators, milk replacers, minerals, amino acids, coccidiostats, acid-based products, medicines, and combinations thereof.

The foodstuff composition according to the present invention does also include dietary supplements, e. g. in the form of a pill, capsule, tablet, powder or liquid.

- 10 A further subject of the current invention is a pharmaceutical composition containing a preparation according to the present invention and a pharmaceutically acceptable carrier.

The preparations according to the present invention, when administered to human beings, preferably improve the health status, in particular gut health, cardiovascular health, mental health, or immune health of a human being.

- 15 An advantageous configuration according to the present invention is a composition for improving the health status of a human being by one or more of the following:

- decreasing the amount of toxic gluten epitopes, preferably those listed in Table 1.
- decreasing the amount of initial gluten to less than 20 ppm.

Working Examples

Example 1. Probiotic microorganisms resistant to gastrointestinal conditions

Simulated gastric and intestinal fluids were used as described by Fernández et al. [19]. Stationary-phase-grown cells were harvested at 8000 g for 10 min, washed with physiologic solution, and suspended in 50 ml of simulated gastric juice (cell density of 10 log CFU/ml), which contains NaCl (125 mM/l), KCl (7 mM/l), NaHCO₃ (45 mM/l), and pepsin (3 g/l) (Sigma–Aldrich CO., St. Louis, MO, USA) [20]. The final pH was adjusted to 2.0, 3.0, and 8.0. The value of pH 8.0 was used to investigate the influence of the components of the simulated gastric juice, apart from the effect of low pH. The suspension was incubated at 37°C under anaerobic conditions and agitation to simulate peristalsis. Aliquots of this suspension were taken at 0, 90, and 180 min, and viable count was determined. The effect of gastric digestion was also determined by suspending cells in reconstituted skimmed milk (RSM) (11% solids, w/v) before inoculation of simulated gastric juice at pH 2.0. The final pH after the addition of RSM was ca. 3.0. This condition was assayed to simulate the effect of the food matrix during gastric transit [20]. After 180 min of gastric digestion, cells were harvested and suspended in simulated intestinal fluid, which contains 0.1% (w/v) pancreatin and 0.15% (w/v) Oxgall bile salt (Sigma-Aldrich Co.) at pH 8.0. The suspension was incubated at 37°C under agitation and aliquots were taken at 0, 90, and 180 min [21]. 119 out of < 400 tested strains showed a decrease of less than 2 log of initial 1x10¹⁰ CFU/ml and defined as resistant to simulated gastrointestinal conditions.

Example 2. Protease and peptidase activities of single strains resistant to gastrointestinal conditions

All 119 strains (*Lactobacillus* sp., 63 strains; *Weissella* sp., 3 strains; *Pediococcus* sp., 1 strain; and *Bacillus* sp., 51 strains) showing resistance to simulated gastrointestinal conditions were tested for their peptidase and proteinase activities towards synthetic substrates. To assay the peptidase activities, cultures of each strain from the late exponential phase of growth (ca. 9.0 log CFU/ml) were used. Aliquots (0.3 g [dry weight]) of washed cell pellets were re-suspended in 50 mM Tris-HCl (pH 7.0), incubated at 30°C for 30 min, and centrifuged at 13,000 × g for 10 min to remove enzymes loosely associated to the cell wall. The cytoplasmic extract was prepared by incubating bacterial suspensions with lysozyme in 50 mM Tris-HCl (pH 7.5) buffer containing 24% sucrose at 37°C for 60 min, under stirring conditions (ca. 160 rpm). Spheroblasts were resuspended in isotonic buffer and sonicated for 40 s at 16 A/s (Sony Prep model 150; Sanyo, United Kingdom). The extracts were concentrated 10-fold by freeze-drying, re-suspended in 5 mM Tris-HCl (pH 7.0), and dialyzed for 24 h at 4°C. General aminopeptidase type N (PepN), proline iminopeptidase (PepI), X-prolyl dipeptidyl aminopeptidase (PepX) endopeptidase (PepO) and prolyl endopeptidase (PepP) activities of the cytoplasmic extracts of lactobacilli were measured by using Leu-p-nitroanilides (p-NA), Pro-p-NA, Gly-Pro-p-NA, Z-Gly-Gly-Leu-p-NA and Z-Gly-Pro-4-nitroanilide substrates (Sigma Chemical Co), respectively. The assay mixture contained 900 µl of 2.0 mM substrate in 0.05 M potassium phosphate buffer, pH 7.0, and 100 µl of cytoplasmic extract. The mixture was incubated at 37°C for 180 min, and the absorbance was measured at 410 nm. The data were compared to standard curves set up

by using p-nitroaniline. One unit of activity was defined as the amount of enzyme required to liberate 1 μ mol of p-nitroaniline for min under the assay conditions. Based on Principal Component Analysis (PCA) data from the above peptidase activities, some strains clearly separated from the other ones (Fig. 1). Fig. 2 reports the strains showing very high peptidase activities (at least for one peptidase activity). PepN activity ranged from 0.0 (U002-C04; U541-C05; U776-C02; DSM 33301; U021-C01; DSM32540; U567-C04) to 31.400 ± 0.09 U (DSM 33362) (median value 3.08). The strains with low peptidase activity (with the internal numbers / or deposited at the DSMZ: U002-C04; U541-C05; U776-C02; DSM 33301; U021-C01; DSM32540; U567-C04) were not further evaluated. The other most active strains were DSM 33367, DSM 33374, DSM 33370, DSM 33371, DSM 33377, DSM 33365, DSM 33373, *Bacillus pumilus* DSM 33297, *Bacillus subtilis* DSM 33298, DSM 33376, DSM 33375, DSM 33363, *Bacillus licheniformis* DSM 33354, and *Bacillus megaterium* DSM 33336 (Fig. 1, Fig. 2). The median value of Pepl was of 1.66. The most active strains (Pepl activity > 18 U) were DSM 33375, DSM 33373, and DSM 33365. PepX activity ranged from 0.0 to ca. 24 U. The most active strains were DSM 33379, DSM 33371, DSM 33370, DSM 33369, DSM 33374, DSM 33365, DSM 33373, and DSM 33363 (Fig.1 and Fig. 2) (median value of 1.81). The median value of PepO was of 0.54. The most active strains (PepO activity > 5 U) were DSM 33353, DSM 33335, and DSM 33301. PepP activity ranged from 0.0 to 6.23 U (DSM 33368) (median value 0.22). The other most active strains (PepP activity > 3 U) were *Bacillus megaterium* DSM 33300, DSM 33378, DSM 33371, DSM 33377, DSM 33367, DSM 33374, DSM 33366, DSM 33365, DSM 33373, and DSM 33364.

Figure 1 shows the score (A) and loading (B) plots of the first and second principal components after principal component analysis (PCA) based on the general aminopeptidase type N (PepN), proline iminopeptidase (Pepl), X-prolyl dipeptidyl aminopeptidase (PepX), endopeptidase (PepO) and prolyl endopeptidase (PepP) activities of the cytoplasmic extracts of the 119 *Bacillus*, *Lactobacillus*, *Pediococcus*, and *Weissella* strains. PepN, Pepl, PepX, PepP were measured by using Leu-p-nitroanilides (p-NA), Pro-p-NA, Gly-Pro-p-NA, Z-Gly-Gly-Leu-p-NA and Z-Gly-Pro-4-nitroanilide substrates, respectively. Strains showing very high peptidase activities (at least for one peptidase) were reported in red.

Figure 2 shows peptidase activities (PepN, Pepl, PepX, PepO and PepP) of selected single *Bacillus* (B.), *Lactobacillus* (L.) and *Pediococcus* (P.) strains. One unit (U) of activity was defined as the amount of enzyme required to liberate 1 μ mol of p-nitroanilide per min under the assay conditions.

Example 3. Peptidase activities of mixture of strains against immunogenic epitopes

Bacillus, *Lactobacillus*, and *Pediococcus* strains showing very high peptidase activities (at least for one peptidase) were assessed as mixed strains to combine intense and complementary enzyme activities. Various mixtures were used to assay their capacity to in vitro degrade immunogenic epitopes responsible for gluten intolerance.

The hydrolysis of peptides was carried out using combinations of cytoplasmic extracts of previously selected bacteria strains. Immunogenic epitopes corresponding to fragments 57-68 (Q-L-Q-P-F-P-

Q-P-Q-L-P-Y) of α 9-gliadin, 62-75 (P-Q-P-Q-L-P-Y-P-Q-P-Q-S-F-P) of A-gliadin, 134-153 (Q-Q-L-P-Q-P-Q-Q-P-Q-Q-S-F-P-Q-Q-Q-R-P-F) of γ -gliadin, and 57-89 (L-Q-L-Q-P-F-P-Q-P-Q-L-P-Y-P-Q-P-Q-L-P-Y-P-Q-P-Q-L-P-Y-P-Q-P-Q-P-F) (33-mer) of α 2-gliadin were chemically synthesized and used at an initial concentration of 1 mM. Hydrolysis was monitored by RP-HPLC. Single peaks from RP-HPLC were analysed by nano-ESI tandem mass spectrometry (nano-ESI-MS/MS). The mixtures of strains that showed the best hydrolysis of synthetic immunogenic epitopes were numbers 3, 4 and 5 (Figure 3), which fully hydrolysed all toxic peptides. Figure 3 shows peptidase activities of mixtures of strains against immunogenic epitopes.

Strain mixtures were as follows:

1. *L. plantarum* DSM 33362, DSM 33363, DSM 33364, DSM 33366; *L. sanfranciscensis* DSM 33379; *Bacillus pumilus* DSM 33297, DSM 33335, *Bacillus licheniformis* DSM 33354, *Bacillus megaterium* DSM 33300, *Bacillus subtilis* DSM 33353.
2. *L. paracasei* DSM 33375, DSM 33376; *L. plantarum* DSM 33369, DSM 33368; *L. sanfranciscensis* DSM 33378; *Bacillus licheniformis* DSM 33354, *Bacillus megaterium* DSM 33300, DSM 33336, *Bacillus pumilus* DSM 33297, DSM 33301.
3. *L. plantarum* DSM 33370, DSM 33363, DSM 33364, DSM 33365; *Lactobacillus paracasei* DSM 33373, *L. brevis* DSM 33377; *Bacillus pumilus* DSM 33297, DSM 33335, *Bacillus licheniformis* DSM 33354, *Bacillus megaterium* DSM 33300, *Bacillus subtilis* DSM 33353.
4. *L. plantarum* DSM 33362, DSM 33367, DSM 33368; *L. paracasei* DSM 33375; *L. sanfranciscensis* DSM 33379; *Bacillus pumilus* DSM 33301, *Bacillus megaterium* DSM 33300, DSM 33336, and *Bacillus subtilis* DSM 33298, DSM 33353.
5. *L. plantarum* DSM 33366, DSM 33369, *Lactobacillus reuteri* DSM 33374; *L. paracasei* DSM 33376; *Pediococcus pentosaceus* DSM 33371, *L. sanfranciscensis* DSM 33378; *Bacillus licheniformis* DSM 33354, *Bacillus pumilus* DSM 33301, *Bacillus megaterium* DSM 33300, DSM 33336, *Bacillus subtilis* DSM 33298.
6. *L. plantarum* DSM 33370, DSM 33367, *Lactobacillus reuteri* DSM 33374; *L. brevis* DSM 33377; *Bacillus pumilus* DSM 33301, *Bacillus megaterium* DSM 33300, DSM 33336, *Bacillus subtilis* DSM 33298.

Example 4. Degradation of gluten under simulated gastrointestinal conditions by different consortia

The gluten degradation under simulated gastrointestinal digestion was assessed. The following consortia, including a total of 22 strains (*Lactobacillus plantarum* DSM 33370, DSM 33362, DSM 33363, DSM 33364, DSM 33365, DSM 33366, DSM 33368, DSM 33369 and DSM 33367; *Lactobacillus reuteri* DSM 33374; *Lactobacillus paracasei* DSM 33376, *Lactobacillus paracasei* DSM 33373, DSM 33375; *Lactobacillus brevis* DSM 33377, *Pediococcus pentosaceus* DSM 33371; *Bacillus pumilus* DSM 33297, *Bacillus pumilus* DSM 33335, DSM 33301, DSM 33335, *Bacillus licheniformis* DSM 33354, *Bacillus megaterium* DSM 33300, DSM 33336, and *Bacillus subtilis* DSM 33298, DSM 33353) were prepared:

1. *L. plantarum* DSM 33370, DSM 33365, DSM 33363 and DSM 33364, *L. paracasei* DSM 33373 *L. brevis* DSM 33377, *Bacillus pumilus* DSM 33297, DSM 33335, DSM 33301;
2. *L. plantarum* DSM 33362 and DSM 33367, DSM 33368, *L. paracasei* DSM 33375, *Bacillus subtilis* DSM 33298, *Bacillus licheniformis* DSM 33354, and *Bacillus megaterium* DSM 33300;
3. *L. plantarum* DSM 33366, DSM 33369, *Lactobacillus reuteri* DSM 33374, *L. paracasei* DSM 33376, *Pediococcus pentosaceus* DSM 33371, *Bacillus megaterium* DSM 33336, and *Bacillus subtilis* DSM 33353;
4. *L. plantarum* DSM 33365, DSM 33363 and DSM 33364, *L. paracasei* DSM 33373, *Bacillus subtilis* DSM 33298 and *Bacillus pumilus* DSM 33301;
5. *L. brevis* DSM 33377, *Pediococcus pentosaceus* DSM 33371, *L. plantarum* DSM 33369, *Bacillus pumilus* DSM 33297 and *Bacillus megaterium* DSM 33300;
6. *L. paracasei* DSM 33375, *L. plantarum* DSM 33367, DSM 33368; *Bacillus pumilus* DSM 33335, and *Bacillus licheniformis* DSM 33354;
7. *L. plantarum* DSM 33370, DSM 33362, and DSM 33366, *Lactobacillus reuteri* DSM 33374, *Bacillus megaterium* DSM 33336, and *Bacillus subtilis* DSM 33353.

Five grams of wheat bread (chewed for 30 s and collected in a beaker with 10 mL of NaK-phosphate 0.05 M, pH 6.9) or related dough were suspended in simulated gastric juice containing NaCl (125 mM), KCl (7 mM), NaHCO₃ (45 mM), and pepsin (3 g/L) (Sigma–Aldrich CO., St. Louis, MO, USA).

The suspension was added of the pooled selected strains as live (with a final cell density of approximately 9.0 log CFU/mL) and lysed bacteria (corresponding to 9.0 log cells/mL). A control dough, without addition of bacterial mixture, was also subjected to simulated digestion. The suspension was incubated at 37°C, under stirring to simulate peristalsis. After 180 min of gastric digestion, the suspension was added with simulated intestinal fluid, which contained 0.1% (w/v) pancreatin and 0.15% (w/v) Oxgall bile salt (Sigma–Aldrich Co.) at pH 8.0. Besides pancreatin and bile salt, the fluid contained enzymatic preparation E1, E2 (each at 0.2 g/kg), Veron HPP (10 g/100 kg of protein) and Veron PS (25 g/100 kg of protein) enzymes. Proteases of *Aspergillus oryzae* (500,000 haemoglobin units on the tyrosine basis/g; enzyme 1 [E1]) and *Aspergillus niger* (3,000 spectrophotometric acid protease units/g; enzyme 2 [E2]), routinely used for bakery applications, were supplied by BIO-CAT Inc. (Troy, VA). Veron HPP and Veron PS are bacterial proteases from *Bacillus subtilis* (AB Enzymes). Enzymatic mixture (E1, E2, Veron PS, Veron HPP) was not added in the control dough. Intestinal digestion was carried out for 48 h at 37°C under stirring conditions (ca. 200 rpm). After digestion, samples were put on ice and the concentration of hydrolysed gluten was determined using R5 antibody-based sandwich and competitive ELISA (R5-ELISA) [22]. R5-ELISA analysis was carried out with the RIDASCREEN® Gliadin competitive detection kit according to the instructions of the manufacturer (R-Biopharm AG, Germany). Moreover, ELISA Systems Gluten Residue Detection Kit (Windsor, Australia) was used for quantification of residual gluten. The presence of epitopes in digested samples was monitored after 6, 16, 24, 36 and 48 h of incubation through HPLC analysis. Liquid chromatography coupled with nano electrospray ionization-ion trap tandem mass spectrometry (nano-ESI-MS/MS) was also used to confirm the hydrolysis of gluten and the absence of toxic epitopes.

As estimated by ELISA test, after 6 h of digestion the concentration of hydrolysed gluten was in the range of 810 ± 0.02 ppm for the control and 310 ± 0.06 ppm for mixture 3 (Figure 4). After 16 and 24 h of digestion, gluten content was $\geq 100 \pm 0.08$ ppm for all the theses. Compared to other mixtures, only mixture 4 showed the complete hydrolysis of gluten after 36 h of digestion. At the end of incubation (48 h), complete hydrolysis was also found for mixtures 5 and 6.

Regarding the residual gluten, all the mixtures showed complete detoxification after 24 h of digestion. However, the mixtures number 5, 6 and 7 were able to decrease residual gluten at a level ≤ 10 ppm after 16 h of fermentation; most importantly, the mixture 4 showed complete gluten degradation after 16 h of digestion (Figure 5). The mixture of strains that caused the most efficient hydrolysis of synthetic immunogenic epitopes was number 4, because it completely hydrolysed toxic peptides after 16 h of incubation (Figure 6).

Figure 5 shows concentration of residual gluten (ppm) after 6, 16, 24, 36 and 48 h of simulated gastrointestinal digestion, as estimated by a specific ELISA test. Control: dough without bacterial cells and enzymes; Mixture 1 (M1): *L. plantarum* DSM 33365, *L. paracasei* DSM 33373, *L. plantarum* DSM 33363, DSM 33364 and DSM 33370, *L. brevis* DSM 33377, *Bacillus pumilus* DSM 33297, DSM 33301 and DSM 33335; Mixture 2 (M2): *L. plantarum* DSM 33368, DSM 33362 and DSM 33367, *L. paracasei* DSM 33375, *Bacillus subtilis* DSM 33298, *Bacillus licheniformis* DSM 33354, and *Bacillus megaterium* DSM 33300; Mixture 3 (M3): *L. plantarum* DSM 33366 and DSM 33369, *L. reuteri* DSM 33374, *L. paracasei* DSM 33376, *P. pentosaceus* DSM 33371, *Bacillus megaterium* DSM 33336, and *Bacillus subtilis* DSM 33353; Mixture 4 (M4): *L. plantarum* DSM 33365, *L. paracasei* DSM 33373, *L. plantarum* DSM 33363 and DSM 33364, *Bacillus subtilis* DSM 33298 and *Bacillus pumilus* DSM 33301; Mixture 5 (M5): *L. brevis* DSM 33377, *P. pentosaceus* DSM 33371, *L. plantarum* DSM 33369, *Bacillus pumilus* DSM 33297 and *Bacillus megaterium* DSM 33300; Mixture 6 (M6): *L. plantarum* DSM 33368 and DSM 33367, *L. paracasei* DSM 33375, *Bacillus pumilus* DSM 33335 and *Bacillus licheniformis* DSM 33354; Mixture 7 (M7): *L. plantarum* DSM 33362, DSM 33366 and DSM 33370, *L. reuteri* DSM 33374, *Bacillus megaterium* DSM 33336, and *Bacillus subtilis* DSM 33353. M1-M7 were added of E1, E2, Veron PS, Veron HPP commercial enzymes.

Figure 6 shows RP-HPLC peptide profiles of control (panel A), Mixture 4 (panel B) and Mixture 7 (panel C) digested wheat bread samples. M4 and M5 were added of E1, E2, Veron PS, Veron HPP commercial enzymes.

Example 6. Assessment of immunogenicity of gluten digests by using duodenal explants from celiac disease patients

Immunogenicity of the digests was ex vivo estimated by testing the cytokine expression in duodenal biopsy specimens from patients with celiac disease (CD). All CD patients expressed the HLA-DQ2 phenotype. CD was diagnosed according to European Society for Pediatric Gastroenterology, Hepatology, and Nutrition criteria [23]. Immediately after excision, all biopsy specimens were placed in ice-chilled culture medium (RPMI 1640; Gibco-Invitrogen, UK) and transported to the laboratory

within 30 min. Duodenal biopsy specimens were cultured for 4 h using the organ tissue culture method originally described by Browning and Trier [24]. Briefly, the biopsy specimens were oriented villous side up on a stainless-steel mesh and positioned over the central well of an organ tissue culture dish (Falcon, USA). The well contained RPMI supplemented with 15% foetal calf serum (Gibco-Invitrogen) and 1% penicillin-streptomycin (Gibco-Invitrogen, UK). Dishes were placed into an anaerobic jar and incubated at 37 °C.

Digested samples of control dough (positive control) (wheat bread digested without the addition of bacterial cells and microbial enzymes), Mixture 4 (wheat bread digested with the addition of live and lysed cells of *L. plantarum* DSM 33365, DSM 33363 and DSM 33364, *L. paracasei* DSM 33373, *Bacillus subtilis* DSM 33298 and *Bacillus pumilus* DSM 33301 and E1, E2, Veron PS, Veron HPP commercial enzymes) and Mixture 7 (wheat bread digested with the addition of live and lysed cells of *L. plantarum* DSM 33362, and DSM 33366, *Lactobacillus reuteri* DSM 33374, *L. plantarum* DSM 33370, *Bacillus megaterium* DSM 33336, and *Bacillus subtilis* DSM 33353 and E1, E2, Veron PS, Veron HPP commercial enzymes) were subjected to gliadin and glutenin polypeptide extraction and used for assessing their ability to induce cytokine expression in duodenal biopsy specimens from CD patients. Four biopsy specimens from each CD patient were cultured with culture medium under four conditions: (i) with doughs containing the Mixture 4 (wheat bread digested with the addition of live and lysed cells of *L. plantarum* DSM 33365, DSM 33363 and DSM 33364, *L. paracasei* DSM 33373, *Bacillus subtilis* DSM 33298 and *Bacillus pumilus* DSM 33301 and E1, E2, Veron PS, Veron HPP commercial enzymes) digested for 48 h; (ii) with dough containing the Mixture 7 (wheat bread digested with the addition of live and lysed cells of *L. plantarum* DSM 33370, DSM 33362, and DSM 33366, *Lactobacillus reuteri* DSM 33374, *Bacillus megaterium* DSM 33336, and *Bacillus subtilis* DSM 33353 and E1, E2, Veron PS, Veron HPP commercial enzymes) digested for 48 h; (iii) with control dough digested for 48 h (Control); and (iv) with culture medium (RPMI 1640+gastric and intestinal juice, negative control). Biopsy specimens from each patient were rinsed and stored in RNeasy lysis buffer (Qiagen GmbH, Germany) at -80°C to preserve the RNA. Total RNA was extracted from the tissues using the RNeasy minikit (Qiagen GmbH) according to the manufacturer's instructions. The concentration of mRNA was estimated by determination of the UV absorbance at 260 nm. Aliquots of total RNA (500 ng) were reverse transcribed using random hexamers, TaqMan reverse transcription reagents (Applied Biosystems, Monza, Italy), and 3.125 U/μl of MultiScribe reverse transcriptase to a final volume of 50 μl. The cDNA samples were stored at -20°C. RT-PCR was performed in 96-well plates using an ABI Prism 7500HT fast sequence detection system (Applied Biosystems). Data collection and analyses were performed using the machine software. PCR primers and fluorogenic probes for the target genes (IFN-γ, IL-2, and IL-10) and the endogenous control (gene coding for glyceraldehyde-3-phosphate dehydrogenase [GAPDH]) were purchased as a TaqMan gene expression assay and a pre-developed TaqMan assay (Applied Biosystems), respectively. The assays were supplied as a 20x mix of PCR primers and TaqMan Minor Groove Binder 6-carboxyfluorescein dye-labelled probes with a non-fluorescent quencher at the 3' end of the probe. Two-step reverse transcription-PCR was performed using first-strand cDNA with a final concentration of 1x TaqMan gene expression assay mix and 1x TaqMan universal PCR master mix. The final reaction volume was 25 μl. Each sample was analysed in triplicate, and all experiments

were repeated twice. A non-template control (RNase-free water) was included with every plate. The following thermal cycler conditions were used: 2 min at 50 °C (uracil DNA glycosylase activation), 10 min at 95 °C, and 40 cycles of 15 s at 95 °C and 1 min at 60 °C. Initially, a standard curve and a validation experiment were performed for each primer/probe set. Six serial dilutions (20 to 0.1 ng/μl) of IFN-γ, IL-2, or IL-10 cDNA were used as a template for each primer/probe set. A standard curve was generated by plotting the threshold cycle (CT) values against the log of the amount of input cDNA. The CT value is the PCR cycle at which an increase in reporter fluorescence above the baseline level is first detected. The average value for the target gene was normalized using an endogenous reference gene (the GAPDH gene). A healthy duodenal biopsy specimen was used to calibrate all the experiments. The levels of IFN-γ, IL-2, and IL-10 proteins secreted into the supernatant were quantified by ELISA in 96-well round-bottom plates (Tema Ricerca, Milan, Italy) according to the manufacturer's recommendations.

As expected, the duodenal biopsy specimens incubated with positive control produced significantly ($P < 0.05$) higher expression of interleukin 2 (IL-2), interleukin 10 (IL-10) (B), and interferon gamma (IFN-γ) mRNA than the negative control (RPMI 1640 + gastric and intestinal juice) (Figure 7). Compared to negative control, the digested samples with the Mixture 4 showed the same ($P > 0.05$) level of IL-2, IL-10 and IFN-γ. The Mixture 7 was characterized by lower synthesis of IL-2 than the positive control, but, compared to mixture 4 and negative control, by higher synthesis of IL-2. Similar trends were also found for IL-10 and IFN-γ. The results of this project provide evidence that the selected strains have the potential to improve the digestion of gluten in gluten-sensitive patients and to hydrolyse immunogenic peptides during gastrointestinal digestion, which decreases gluten toxicity for CD patients.

Figure 7 shows concentration (ng/μl) of interleukin 2 (IL-2) (A), interleukin 10 (IL-10) (B), and interferon gamma (IFN-γ) (C) in duodenal biopsy specimens from patients with CD. Control, wheat bread digested without the addition of bacterial cells and microbial enzymes; RPMI+gastric and intestinal juice, negative control; Mixture 4, wheat bread digested with the addition of live and lysed cells of *L. plantarum* DSM 33365, *L. plantarum* DSM 33363 and DSM 33364, *L. paracasei* DSM 33373, *Bacillus subtilis* DSM 33298 and *Bacillus pumilus* DSM 33301 and E1, E2, Veron PS, Veron HPP commercial enzymes) and Mixture 7, wheat bread digested with the addition of live and lysed cells of *L. plantarum* DSM 33362, DSM 33366 and DSM 33370, *L. reuteri* DSM 33374, *Bacillus megaterium* DSM 33336, and *Bacillus subtilis* DSM 33353 and E1, E2, Veron PS, Veron HPP commercial enzymes. CD1 to CD10, duodenal biopsy specimens from celiac patients.

References

1. Sapone A, Bai JC, Ciacci C, Dolinsek J, Green PH, Hadjivassiliou M, Kaukinen K, Rostami K, Sanders DS, Schumann M *et al*: **Spectrum of gluten-related disorders: consensus on new nomenclature and classification.** *BMC Med* 2012, **10**:13.
2. Niland B, Cash BD: **Health Benefits and Adverse Effects of a Gluten-Free Diet in Non-Celiac Disease Patients.** *Gastroenterol Hepatol (N Y)* 2018, **14**(2):82-91.
3. Lis DM, Stellingwerff T, Shing CM, Ahuja KD, Fell JW: **Exploring the popularity, experiences, and beliefs surrounding gluten-free diets in nonceliac athletes.** *Int J Sport Nutr Exerc Metab* 2015, **25**(1):37-45.
4. Wild D, Robins GG, Burley VJ, Howdle PD: **Evidence of high sugar intake, and low fibre and mineral intake, in the gluten-free diet.** *Aliment Pharmacol Ther* 2010, **32**(4):573-581.
5. Thompson T, Dennis M, Higgins LA, Lee AR, Sharrett MK: **Gluten-free diet survey: are Americans with coeliac disease consuming recommended amounts of fibre, iron, calcium and grain foods?** *J Hum Nutr Diet* 2005, **18**(3):163-169.
6. Larretxi I, Simon E, Benjumea L, Miranda J, Bustamante MA, Lasa A, Eizaguirre FJ, Churrua I: **Gluten-free-rendered products contribute to imbalanced diets in children and adolescents with celiac disease.** *Eur J Nutr* 2018.
7. De Palma G, Nadal I, Collado MC, Sanz Y: **Effects of a gluten-free diet on gut microbiota and immune function in healthy adult human subjects.** *Br J Nutr* 2009, **102**(8):1154-1160.
8. Galipeau HJ, McCarville JL, Huebener S, Litwin O, Meisel M, Jabri B, Sanz Y, Murray JA, Jordana M, Alaedini A *et al*: **Intestinal microbiota modulates gluten-induced immunopathology in humanized mice.** *Am J Pathol* 2015, **185**(11):2969-2982.
9. Caminero A, Galipeau HJ, McCarville JL, Johnston CW, Bernier SP, Russell AK, Jury J, Herran AR, Casqueiro J, Tye-Din JA *et al*: **Duodenal Bacteria From Patients With Celiac Disease and Healthy Subjects Distinctly Affect Gluten Breakdown and Immunogenicity.** *Gastroenterology* 2016, **151**(4):670-683.
10. Francavilla R, Ercolini D, Piccolo M, Vannini L, Siragusa S, De Filippis F, De Pasquale I, Di Cagno R, Di Toma M, Gozzi G *et al*: **Salivary microbiota and metabolome associated with celiac disease.** *Appl Environ Microbiol* 2014, **80**(11):3416-3425.
11. De Angelis M, Cassone A, Rizzello CG, Gagliardi F, Minervini F, Calasso M, Di Cagno R, Francavilla R, Gobbetti M: **Mechanism of degradation of immunogenic gluten epitopes from Triticum turgidum L. var. durum by sourdough lactobacilli and fungal proteases.** *Appl Environ Microbiol* 2010, **76**(2):508-518.
12. Francavilla R, Cristofori F, Verzillo L, Gentile A, Castellaneta S, Polloni C, Giorgio V, Verduci E, D'Angelo E, Dellatte S *et al*: **Randomized Double-Blind Placebo-Controlled Crossover Trial for the Diagnosis of Non-Celiac Gluten Sensitivity in Children.** *Am J Gastroenterol* 2018, **113**(3):421-430.
13. Francavilla R, De Angelis M, Rizzello CG, Cavallo N, Dal Bello F, Gobbetti M: **Selected Probiotic Lactobacilli Have the Capacity To Hydrolyze Gluten Peptides during Simulated Gastrointestinal Digestion.** *Appl Environ Microbiol* 2017, **83**(14).
14. Herran AR, Perez-Andres J, Caminero A, Nistal E, Vivas S, Ruiz de Morales JM, Casqueiro J: **Gluten-degrading bacteria are present in the human small intestine of healthy volunteers and celiac patients.** *Res Microbiol* 2017, **168**(7):673-684.
15. Caminero A, Herran AR, Nistal E, Perez-Andres J, Vaquero L, Vivas S, Ruiz de Morales JM, Albillos SM, Casqueiro J: **Diversity of the cultivable human gut microbiome involved in gluten metabolism: isolation of microorganisms with potential interest for coeliac disease.** *FEMS Microbiol Ecol* 2014, **88**(2):309-319.
16. Pyle GG, Paaso B, Anderson BE, Allen DD, Marti T, Li Q, Siegel M, Khosla C, Gray GM: **Effect of pretreatment of food gluten with prolyl endopeptidase on gluten-induced malabsorption in celiac sprue.** *Clin Gastroenterol Hepatol* 2005, **3**(7):687-694.
17. Krishnareddy S, Stier K, Recanati M, Lebwohl B, Green PH: **Commercially available glutenases: a potential hazard in coeliac disease.** *Therap Adv Gastroenterol* 2017, **10**(6):473-481.
18. Shan L, Marti T, Sollid LM, Gray GM, Khosla C: **Comparative biochemical analysis of three bacterial prolyl endopeptidases: implications for coeliac sprue.** *Biochem J* 2004, **383**(Pt 2):311-318.

19. Fernandez MF, Boris S, Barbes C: **Probiotic properties of human lactobacilli strains to be used in the gastrointestinal tract.** *J Appl Microbiol* 2003, **94**(3):449-455.
20. Zarate G, Chaia AP, Gonzalez S, Oliver G: **Viability and beta-galactosidase activity of dairy propionibacteria subjected to digestion by artificial gastric and intestinal fluids.** *J Food Prot* 2000, **63**(9):1214-1221.
- 5 21. De Angelis M, Siragusa S, Berloco M, Caputo L, Settanni L, Alfonsi G, Amerio M, Grandi A, Ragni A, Gobbetti M: **Selection of potential probiotic lactobacilli from pig feces to be used as additives in pelleted feeding.** *Res Microbiol* 2006, **157**(8):792-801.
- 10 22. Valdes I, Garcia E, Llorente M, Mendez E: **Innovative approach to low-level gluten determination in foods using a novel sandwich enzyme-linked immunosorbent assay protocol.** *Eur J Gastroenterol Hepatol* 2003, **15**(5):465-474.
23. of ES: **Revised criteria for diagnosis of coeliac disease. Report of Working Group of European Society of Paediatric Gastroenterology and Nutrition.** *Arch Dis Child* 1990, **65**(8):909-911.
- 15 24. Browning TH, Trier JS: **Organ culture of mucosal biopsies of human small intestine.** *J Clin Invest* 1969, **48**(8):1423-1432.

Claims

1. Preparation comprising consortia of at least one probiotic strain selected from the genus *Bacillus* and at least one probiotic strain selected from the genus *Lactobacillus*, for use in safe and complete degradation of gluten,
 a) wherein said consortium of strains can degrade the 12-mer peptide QLQPFQPQLPY (Seq-ID No 1), the 14-mer peptide PQPQLPYQPQSFP (Seq-ID No 2), the 20-mer peptide QQLPQPQQPQQSFPQQRP (Seq-ID No 3), the 33-mer peptide LQLQPFQPQLPYQPQLPYQPQLPYQPQLPYQPQPF (Seq-ID No 4).
2. Preparation for use according to claim 1, wherein the consortium of strains can degrade the 12-mer peptide QLQPFQPQLPY (Seq-ID No 1), the 14-mer peptide PQPQLPYQPQSFP (Seq-ID No 2), the 20-mer peptide QQLPQPQQPQQSFPQQRP (Seq-ID No 3), the 33-mer peptide LQLQPFQPQLPYQPQLPYQPQLPYQPQLPYQPQPF (Seq-ID No 4) by at least 1% or at least 10 %, or at least 20 % or at least 30 % or at least 40 %, or at least 50 % or at least 60 % or at least 70 %, or at least 80 %, or at least 90 %, preferably at least 95 %, more preferably at least 98 %.
3. Preparation for use according to any preceding claim,
 b) wherein said consortium of strains is capable of degrading gluten to a digest that does not cause an immunogenic or toxic response in the small intestine or small intestinal explant of a subject or animal affected by a gluten-related disorder, and/or
 c) wherein all strains of the consortium survive (less than 2 log CFU loss) simulated gastric (pH 1.0-4.0) and intestinal (pH 5.4-6.8; 0.05-0.6 % bile acids, or the concentration of bile acids present in humans under in vivo conditions) conditions, and/or
 d) wherein members of the consortia have complementary PepP, PepO, PepX, Pepl, and PepN activities with at least one peptidase activity equal to or more than 3 U/g (PepP), 5 U/g (PepO), 20 U/g (PepX), 17 U/g (Pepl), 20 U/g (PepN).
4. Preparation for use according to any preceding claim, wherein the *Bacillus* strains are selected from *Bacillus pumilus*, *Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus megaterium*, preferably selected from *Bacillus pumilus* DSM 33297, DSM 33335, DSM 33301, *Bacillus subtilis* DSM 33353, DSM 33298, *Bacillus licheniformis* DSM 33354, *Bacillus megaterium* DSM 33300, DSM 33336.
5. Preparation for use according to any preceding claim, wherein the *Lactobacillus* strains are selected from *Lactobacillus plantarum*, *Lactobacillus casei*, *Lactobacillus paracasei*, *Lactobacillus brevis*, *Lactobacillus sanfranciscensis*, *Lactobacillus reuteri*, preferably

selected from *Lactobacillus plantarum* DSM 33368, DSM 33369, DSM 33370, DSM 33367, DSM 33366, DSM 33364, DSM 33363, DSM 33362, DSM 33365, *Lactobacillus paracasei* DSM 33376, DSM 33375, DSM 33373, *Lactobacillus reuteri* DSM 33374, *Lactobacillus brevis* DSM 33377, *Lactobacillus sanfranciscensis* DSM 33378, DSM 33379.

5

6. Preparation for use according to any preceding claim, wherein the probiotic strains are present in a dormant form or as vegetative cells.
7. Preparation for use according to any preceding claim, wherein cytoplasmic extracts or cell-free supernatants or heat-killed biomass of the probiotic strains are used.
8. Preparation for use according to any preceding claim, wherein the preparation further comprises one or more probiotic strains, preferably selected from *Pediococcus sp.*, *Weissella sp.*, more preferably *Pediococcus pentosaceus* DSM 33371.
9. Preparation for use according to any preceding claim, wherein the preparation further comprises one or more of the following: microbial proteases purified from *Aspergillus niger*, *Aspergillus oryzae*, *Bacillus sp.*, *Lactobacillus sp.*, *Pediococcus sp.*, *Weissella sp.*, *Rothia mucilaginosa*, *Rothia aeria*, subtilisins, nattokinase, arabinoxylans, barley grain fibre, oat grain fibre, rye fibre, wheat bran fibre, inulins, fructooligosaccharides (FOS), galactooligosaccharides (GOS), resistant starch, beta-glucans, glucomannans, galactoglucomannans, guar gum, xylooligosaccharides, alginate.
10. Preparation for use according to any preceding claim for treating or preventing gluten-related disorders, preferably selected from celiac disease, non-celiac gluten sensitivity, wheat allergy, and gluten-sensitive irritable bowel syndrome in a subject or animal in need thereof.
11. Preparation for use according to any preceding claim for producing gluten-free foods, from gluten-containing cereals wheat, barley, rye, and oat, preferably containing less than 20 ppm gluten.
12. Preparation for use according to any preceding claim, further comprising a substance, which acts as permeabilizer of the microbial cell membrane of members of *Bacillus sp.*, *Lactobacillus sp.*, *Pediococcus sp.*, *Weissella sp.*, preferably alginate.
13. Preparation for use according to any preceding claim where one or more of the probiotic strains selected from *Bacillus sp.*, *Lactobacillus sp.*, *Pediococcus sp.* and *Weissella sp.* are immobilized individually or as consortia.
14. Preparation comprising at least one strain of each of the following groups 1-5:

40

- Group 1: *Bacillus subtilis*, *Bacillus pumilus*, *Bacillus licheniformis*, *Bacillus megaterium*, *Pediococcus pentosaceus*, *Lactobacillus plantarum*, *Lactobacillus brevis*, *Lactobacillus paracasei*, *Lactobacillus reuteri*, *Lactobacillus plantarum*, *Lactobacillus plantarum*,
- 5 Group 2: *Lactobacillus paracasei*, *Lactobacillus plantarum*,
- Group 3: *Bacillus subtilis*, *Bacillus pumilus*,
- Group 4: *Lactobacillus sanfranciscensis*, *Pediococcus pentosaceus*, *Lactobacillus plantarum*, *Lactobacillus reuteri*, *Lactobacillus paracasei*,
- Group 5: *Bacillus megaterium*, *Lactobacillus sanfranciscensis*, *Pediococcus pentosaceus*,
- 10 *Lactobacillus brevis*, *Lactobacillus plantarum*, *Lactobacillus reuteri*, *Lactobacillus paracasei*.
15. Preparation according to claim 14, wherein the strains are selected from the following groups 1-5:
- Group 1: *Bacillus subtilis* DSM 33298, *Bacillus pumilus* DSM 33297, *Bacillus licheniformis* DSM 33354, *Bacillus megaterium* DSM 33336, *Pediococcus pentosaceus* DSM 33371, *Lactobacillus plantarum* DSM 33370, *Lactobacillus brevis* DSM 33377, *Lactobacillus paracasei* DSM 33376, *Lactobacillus paracasei* DSM 33375, *Lactobacillus reuteri* DSM 33374, *Lactobacillus plantarum* DSM 33367, *Lactobacillus plantarum* DSM 33363, *Lactobacillus paracasei* DSM 33373, *Lactobacillus plantarum* DSM 33365, *Lactobacillus plantarum* DSM 33362,
- 15 *Lactobacillus plantarum* DSM 33362,
- Group 2: *Lactobacillus paracasei* DSM 33375, *Lactobacillus paracasei* DSM 33373, *Lactobacillus plantarum* DSM 33365,
- Group 3: *Bacillus subtilis* DSM 33353, *Bacillus pumilus* DSM 33335, *Bacillus pumilus* DSM 33301,
- 25 Group 4: *Lactobacillus sanfranciscensis* DSM 33378, *Lactobacillus sanfranciscensis* DSM 33379, *Pediococcus pentosaceus* DSM 33371, *Lactobacillus plantarum* DSM 33370, *Lactobacillus plantarum* DSM 33369, *Lactobacillus reuteri* DSM 33374, *Lactobacillus plantarum* DSM 33363, *Lactobacillus paracasei* DSM 33373, *Lactobacillus plantarum* DSM 33365,
- 30 Group 5: *Bacillus megaterium* DSM 33300, *Lactobacillus sanfranciscensis* DSM 33378, *Pediococcus pentosaceus* DSM 33371, *Lactobacillus brevis* DSM 33377, *Lactobacillus plantarum* DSM 33368, *Lactobacillus reuteri* DSM 33374, *Lactobacillus plantarum* DSM 33367, *Lactobacillus plantarum* DSM 33366, *Lactobacillus plantarum* DSM 33364, *Lactobacillus paracasei* DSM 33373, *Lactobacillus plantarum* DSM 33365.
- 35 16. Preparation according to claim 14 or 15, comprising the following strains:
- *L. plantarum* DSM 33370, DSM 33363, DSM 33364, DSM 33365; *L. paracasei* DSM 33373; *L. brevis* DSM 33377; *Bacillus pumilus* DSM 33297, DSM 33335, *Bacillus*

licheniformis DSM 33354, *Bacillus megaterium* DSM 33300 and *Bacillus subtilis* DSM 33353, or

- 5 – *L. plantarum* DSM 33362, DSM 33367, DSM 33368; *L. paracasei* DSM 33375; *L. sanfranciscensis* DSM 33379; *Bacillus pumilus* DSM 33301, *Bacillus megaterium* DSM 33300, DSM 33336, *Bacillus subtilis* DSM 33298 and DSM 33353, or
- *L. plantarum* DSM 33366 and DSM 33369, *Lactobacillus reuteri* DSM 33374; *L. paracasei* DSM 33376; *Pediococcus pentosaceus* DSM 33371; *L. sanfranciscensis* DSM 33378; *Bacillus licheniformis* DSM 33354, *Bacillus pumilus* DSM 33301, *Bacillus megaterium* DSM 33300, DSM 33336 and *Bacillus subtilis* DSM 33298, or
- 10 – *Lactobacillus plantarum* DSM 33363 and DSM 33364, *Lactobacillus plantarum* DSM 33365, *Lactobacillus paracasei* DSM 33373, and *Bacillus pumilus* DSM 33297 and *Bacillus megaterium* DSM 33300.
- 15 17. A food supplement or food product or pharmaceutical product, comprising a preparation according to any one of claims 14-16.

Summary

The current invention concerns preparations comprising probiotic strains belonging to the genera *Bacillus sp.*, *Lactobacillus sp.*, and optionally also *Pediococcus sp.* as viable cells or cytoplasmic extract thereof, and proteases and their use for safe gluten degradation in humans and during food production.

5

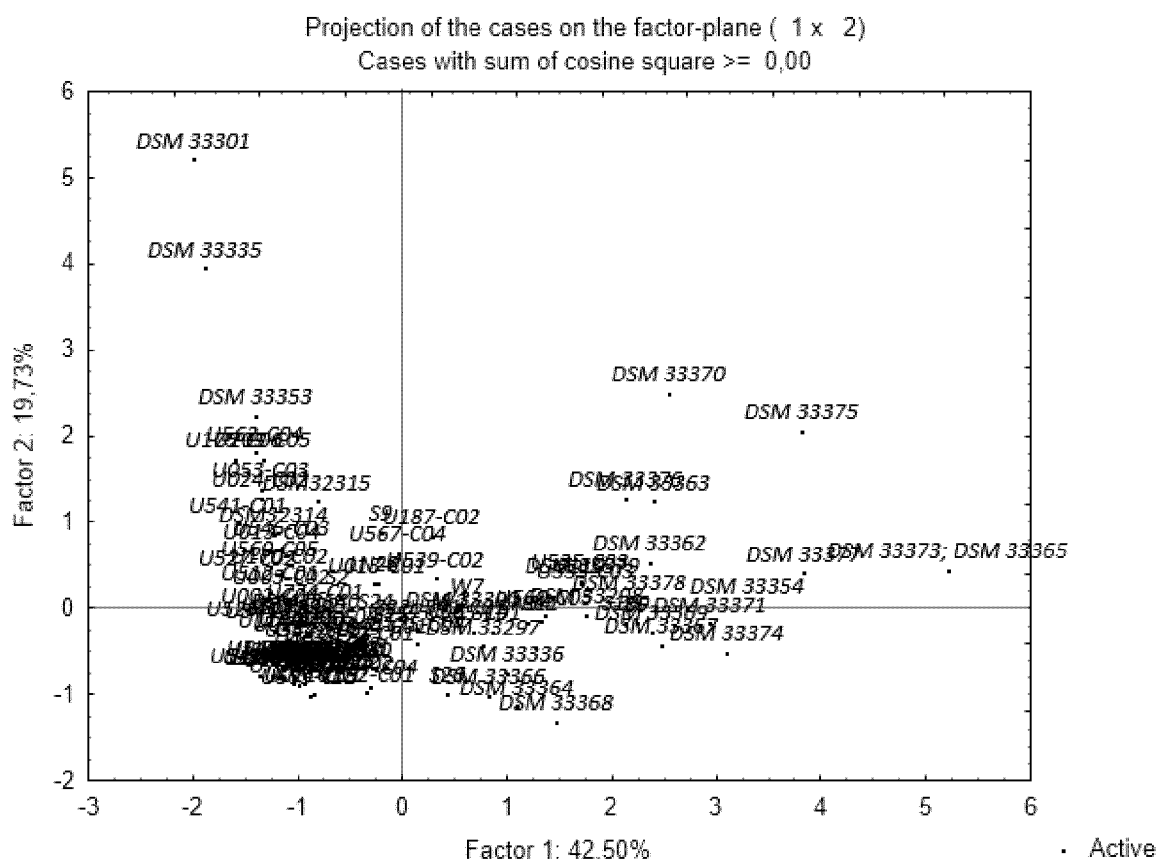


Figure 1 A: Score plots of the first and second principal components after principal component analysis (PCA) based on the general aminopeptidase type N (PepN), proline iminopeptidase (PepI), X-prolyl dipeptidyl aminopeptidase (PepX), endopeptidase (PepO) and prolyl endopeptidase (PepP) activities of the cytoplasmic extracts of the 119 *Bacillus*, *Lactobacillus*, *Pediococcus*, and *Weissella* strains. PepN, PepI, PepX, PepP were measured by using Leu-p-nitroanilides (p-NA), Pro-p-NA, Gly-Pro-p-NA, Z-Gly-Gly-Leu-p-NA and Z-Gly-Pro-4-nitroanilide substrates, respectively. Strains showing very high peptidase activities (at least for one peptidase) show a factor 2 of more than 2 and a factor 1 of at least 1.

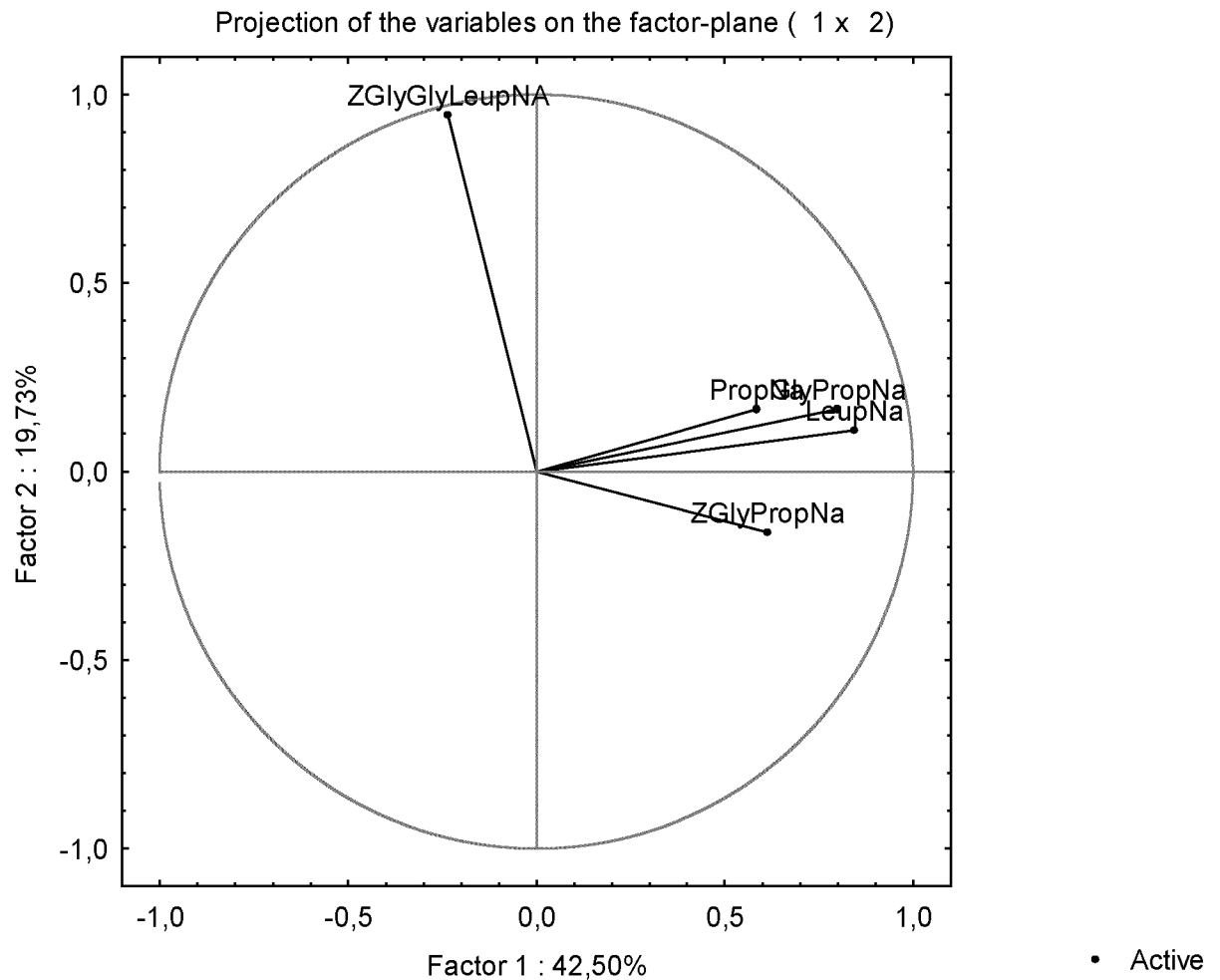
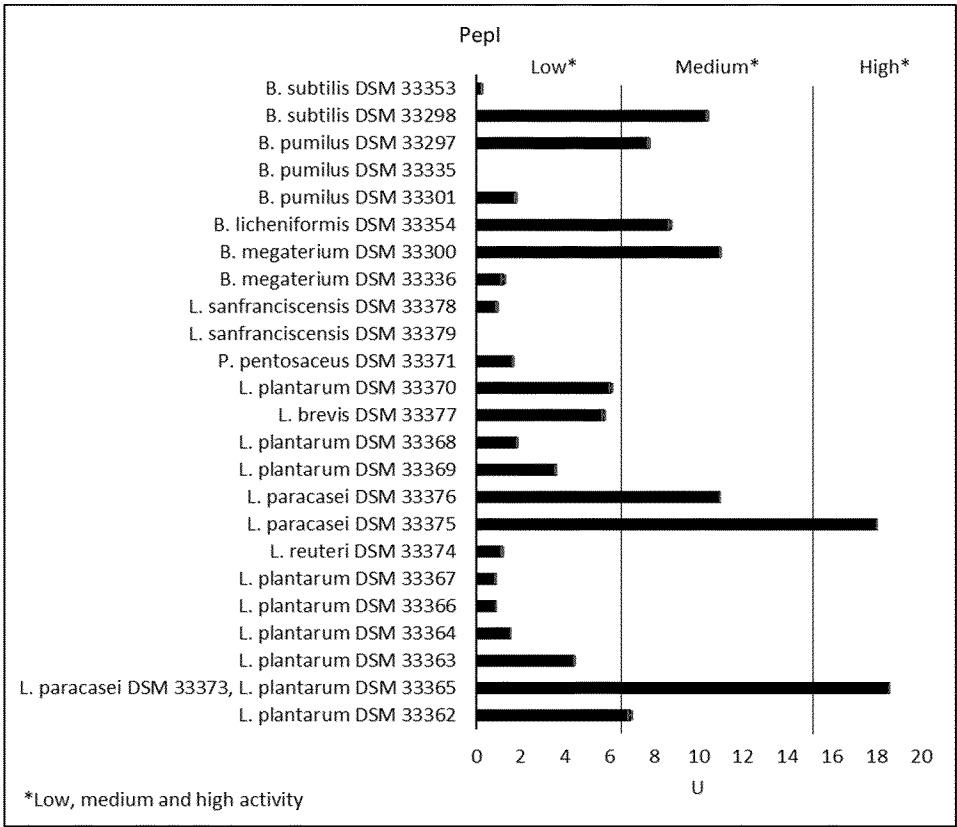
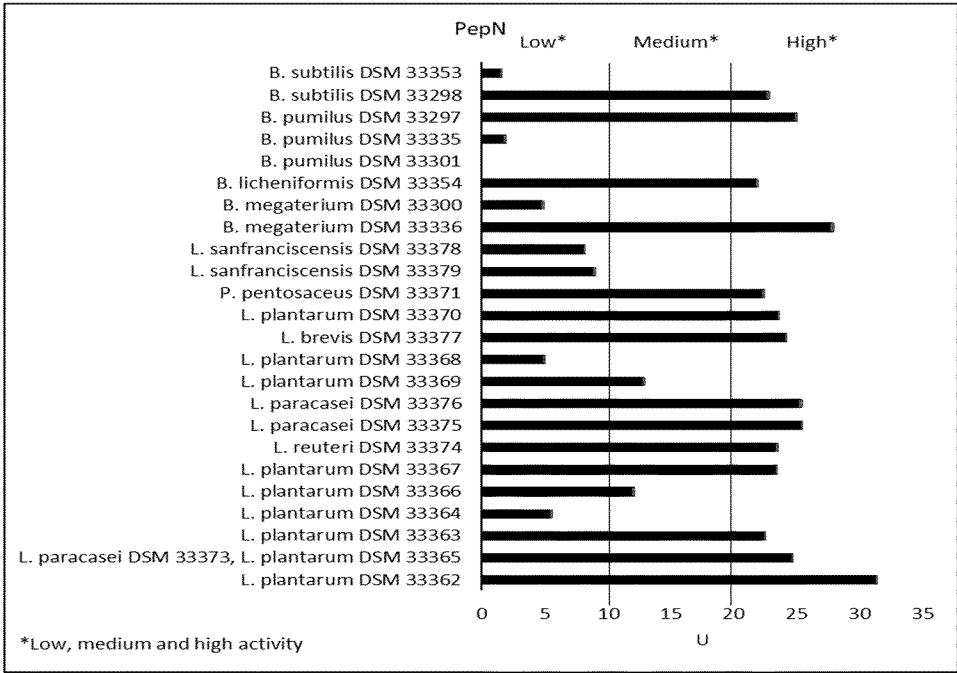
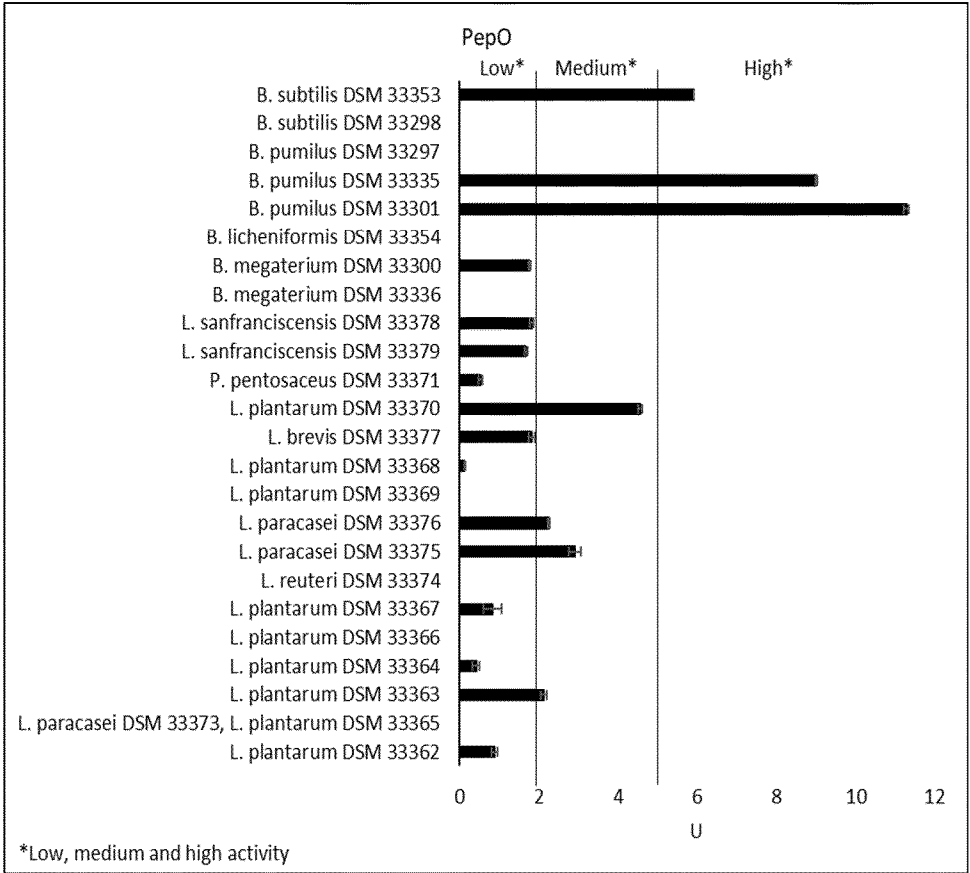
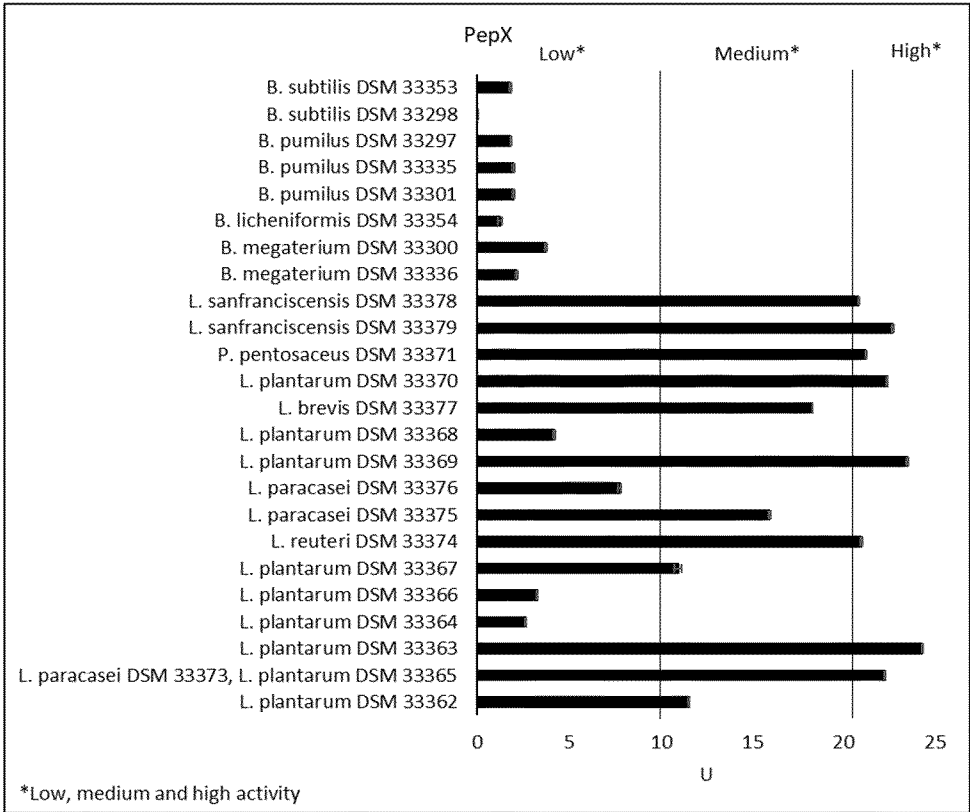


Figure 1 B: Loading plots of the first and second principal components after principal component analysis (PCA) based on the general aminopeptidase type N (PepN), proline iminopeptidase (PepI), X-prolyl dipeptidyl aminopeptidase (PepX), endopeptidase (PepO) and prolyl endopeptidase (PepP) activities of the cytoplasmic extracts of the 119 *Bacillus*, *Lactobacillus*, *Pediococcus*, and *Weissella* strains. PepN, PepI, PepX, PepP were measured by using Leu-p-nitroanilides (p-NA), Pro-p-NA, Gly-Pro-p-NA, Z-Gly-Gly-Leu-p-NA and Z-Gly-Pro-4-nitroanilide substrates, respectively.





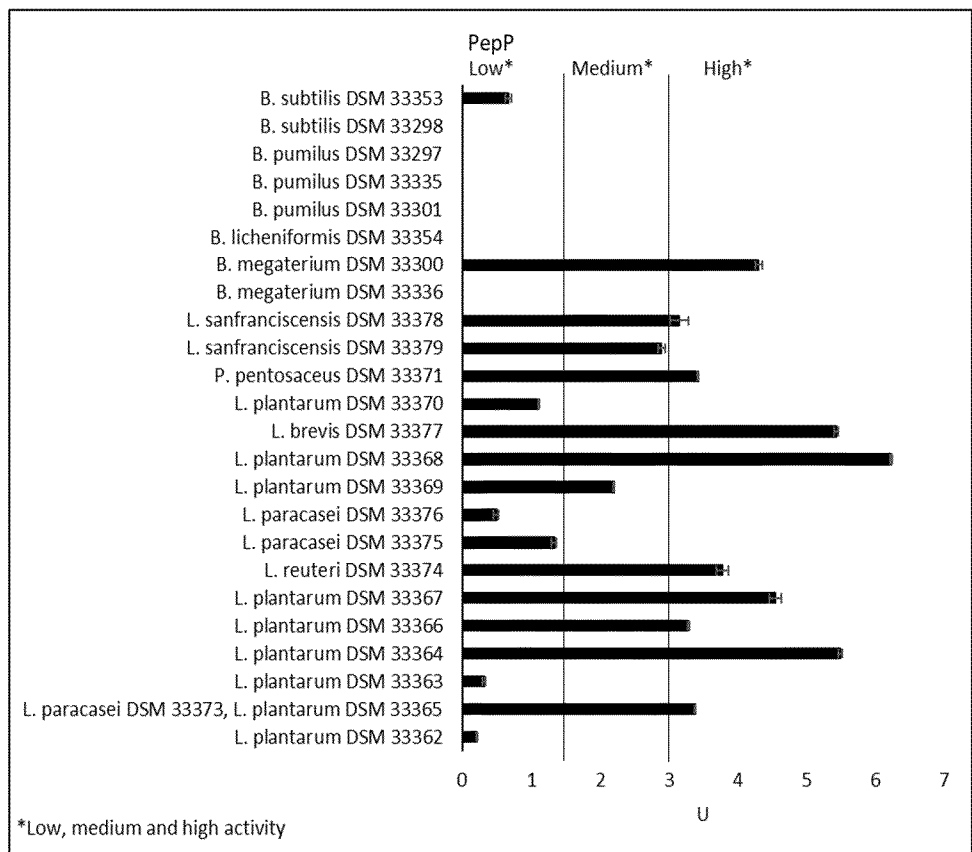


Figure 2: Peptidase activities (PepN, PepI, PepX, PepO and PepP) of selected single *Bacillus* (B.), *Lactobacillus* (L.) and *Pediococcus* (P.) strains. One unit (U) of activity was defined as the amount of enzyme required to liberate 1 μ mol of p-nitroanilide per min under the assay conditions.

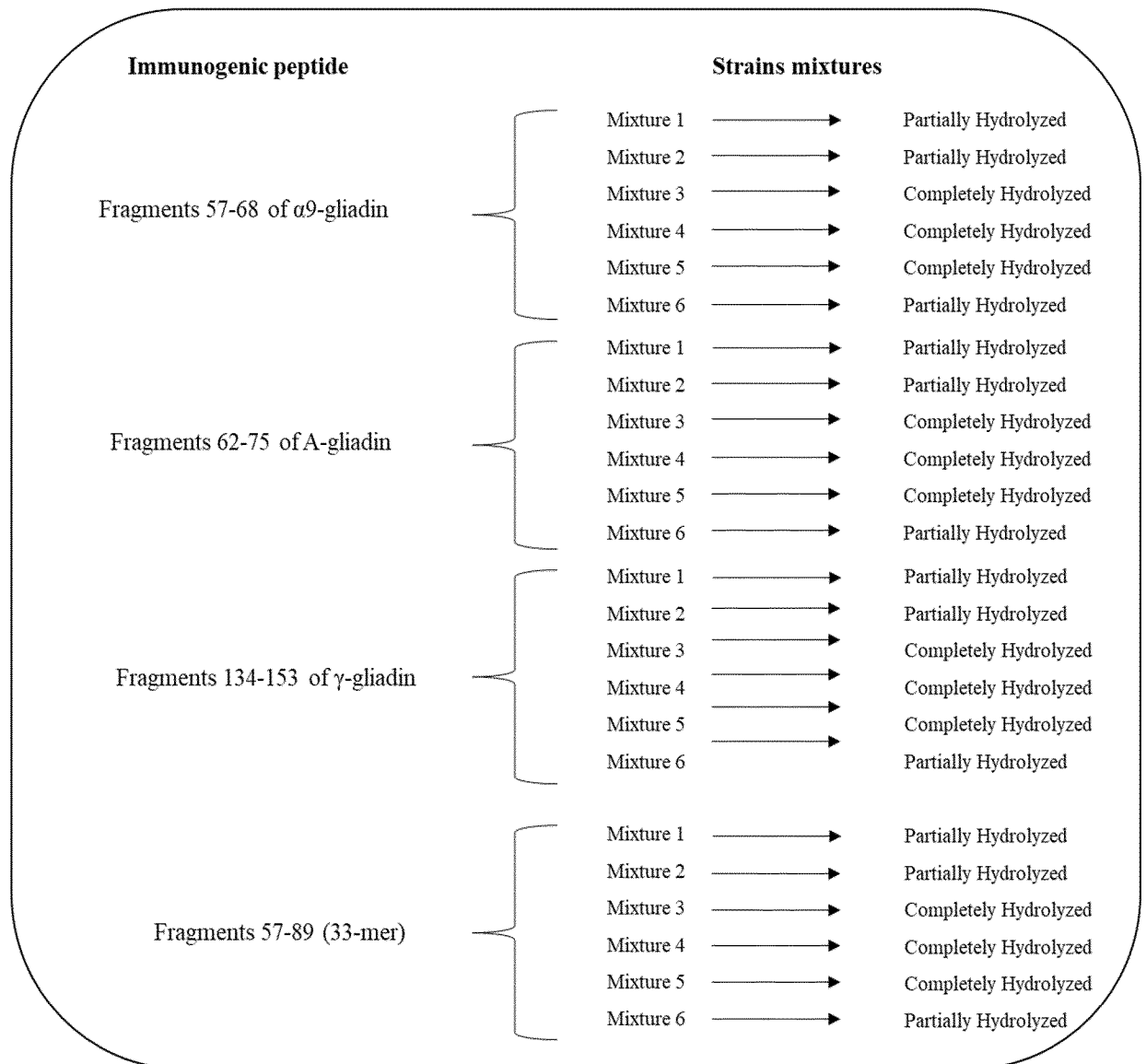


Figure 3: Peptidases activities of mixtures of strains against immunogenic epitopes.

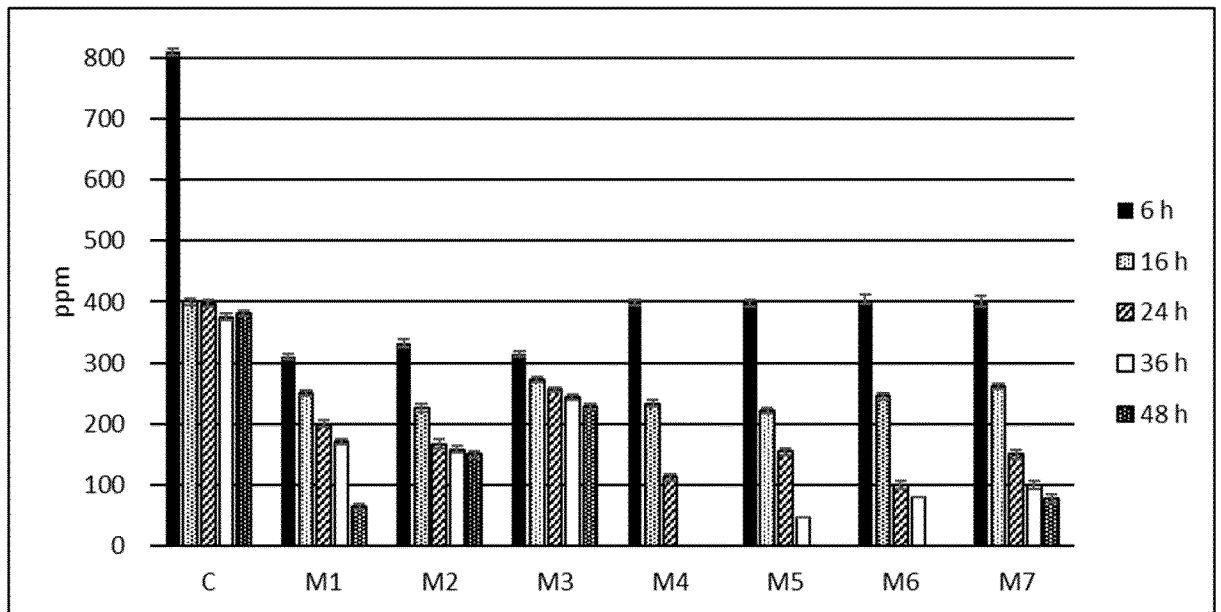


Figure 4: Concentration of hydrolysed gluten (peptides, ppm) after 6, 16, 24, 36 and 48 h of simulated gastrointestinal digestion, as estimated by a specific ELISA test. Control: dough digested without bacterial cells and commercial enzymes; Mixture 1 (M1): *L. plantarum* DSM 33365, *L. plantarum* DSM 33363, DSM 33364 and DSM 33370, *L. paracasei* DSM 33373, *L. brevis* DSM 33377, *Bacillus pumilus* DSM 33297, DSM 33335, DSM 33301; Mixture 2 (M2): *L. plantarum* DSM 33368, DSM 33362 and DSM 33367, *L. paracasei* DSM 33375, *Bacillus subtilis* DSM 33298, *Bacillus licheniformis* DSM 33354, and *Bacillus megaterium* DSM 33300; Mixture 3 (M3): *L. plantarum* DSM 33366 and DSM 33369, *L. reuteri* DSM 33374, *L. paracasei* DSM 33376, *P. pentosaceus* DSM 33371, *Bacillus megaterium* DSM 33336, and *Bacillus subtilis* DSM 33353; Mixture 4 (M4): *L. plantarum* DSM 33365, DSM 33363 and DSM 33364, *L. paracasei* DSM 33373, *Bacillus subtilis* DSM 33298 and *Bacillus pumilus* DSM 33301; Mixture 5 (M5): *L. brevis* DSM 33377, *P. pentosaceus* DSM 33371, *L. plantarum* DSM 33369, *Bacillus pumilus* DSM 33297 and *Bacillus megaterium* DSM 33300; Mixture 6 (M6): *L. plantarum* DSM 33368 and DSM 33367, *L. paracasei* DSM 33375, *Bacillus pumilus* DSM 33335, and *Bacillus licheniformis* DSM 33354; Mixture 7 (M7): *L. plantarum* DSM 33362, DSM 33366 and DSM 33370, *L. reuteri* DSM 33374, *Bacillus megaterium* DSM 33336, and *Bacillus subtilis* DSM 33353. M1-M7 were added of E1, E2, Veron PS, Veron HPP commercial enzymes.

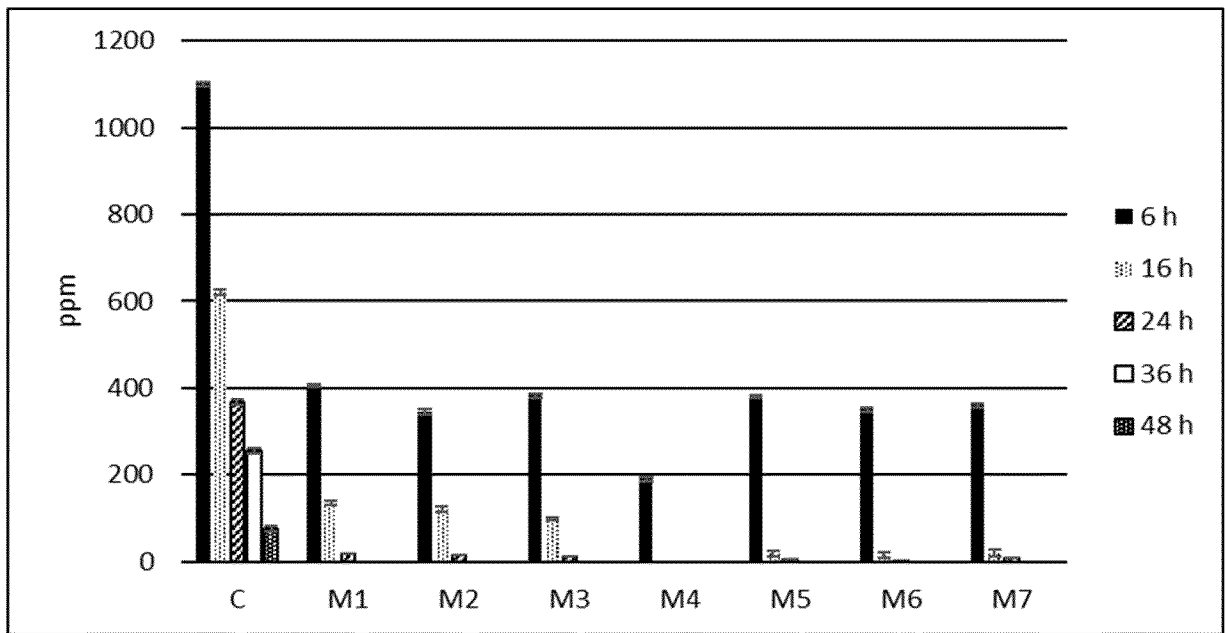


Figure 5: Concentration of residual gluten (ppm) after 6, 16, 24, 36 and 48 h of simulated

gastrointestinal digestion, as estimated by a specific ELISA test. Control: dough without bacterial

- 5 cells and enzymes; Mixture 1 (M1): *L. plantarum* DSM 33363, DSM 33364, DSM 33365 and DSM 33370, *L. paracasei* DSM 33373, *L. brevis* DSM 33377, *Bacillus pumilus* DSM 33297, DSM 33335, DSM 33301; Mixture 2 (M2): *L. plantarum* DSM 33368, DSM 33362 and DSM 33367, *L. paracasei* DSM 33375, *Bacillus subtilis* DSM 33298, *Bacillus licheniformis* DSM 33354, and *Bacillus megaterium* DSM 33300; Mixture 3 (M3): *L. plantarum* DSM 33366 and DSM 33369, *L. reuteri*
- 10 DSM 33374, *L. paracasei* DSM 33376, *P. pentosaceus* DSM 33371, *Bacillus megaterium* DSM 33336, and *Bacillus subtilis* DSM 33353; Mixture 4 (M4): *L. plantarum* DSM 33363 and DSM 33364, DSM 33365, *L. paracasei* DSM 33373, *Bacillus subtilis* DSM 33298 and *Bacillus pumilus* DSM 33301; Mixture 5 (M5): *L. brevis* DSM 33377, *P. pentosaceus* DSM 33371, *L. plantarum* DSM 33369, *Bacillus pumilus* DSM 33297 and *Bacillus megaterium* DSM 33300; Mixture 6 (M6): *L.*
- 15 *plantarum* DSM 33368 and DSM 33367, *L. paracasei* DSM 33375, *Bacillus pumilus* DSM 33335, and *Bacillus licheniformis* DSM 33354; Mixture 7 (M7): *L. plantarum* DSM 33362, DSM 33366 and DSM 33370, *L. reuteri* DSM 33374, *Bacillus megaterium* DSM 33336, and *Bacillus subtilis* DSM 33353. M1-M7 were added of E1, E2, Veron PS, Veron HPP commercial enzymes.

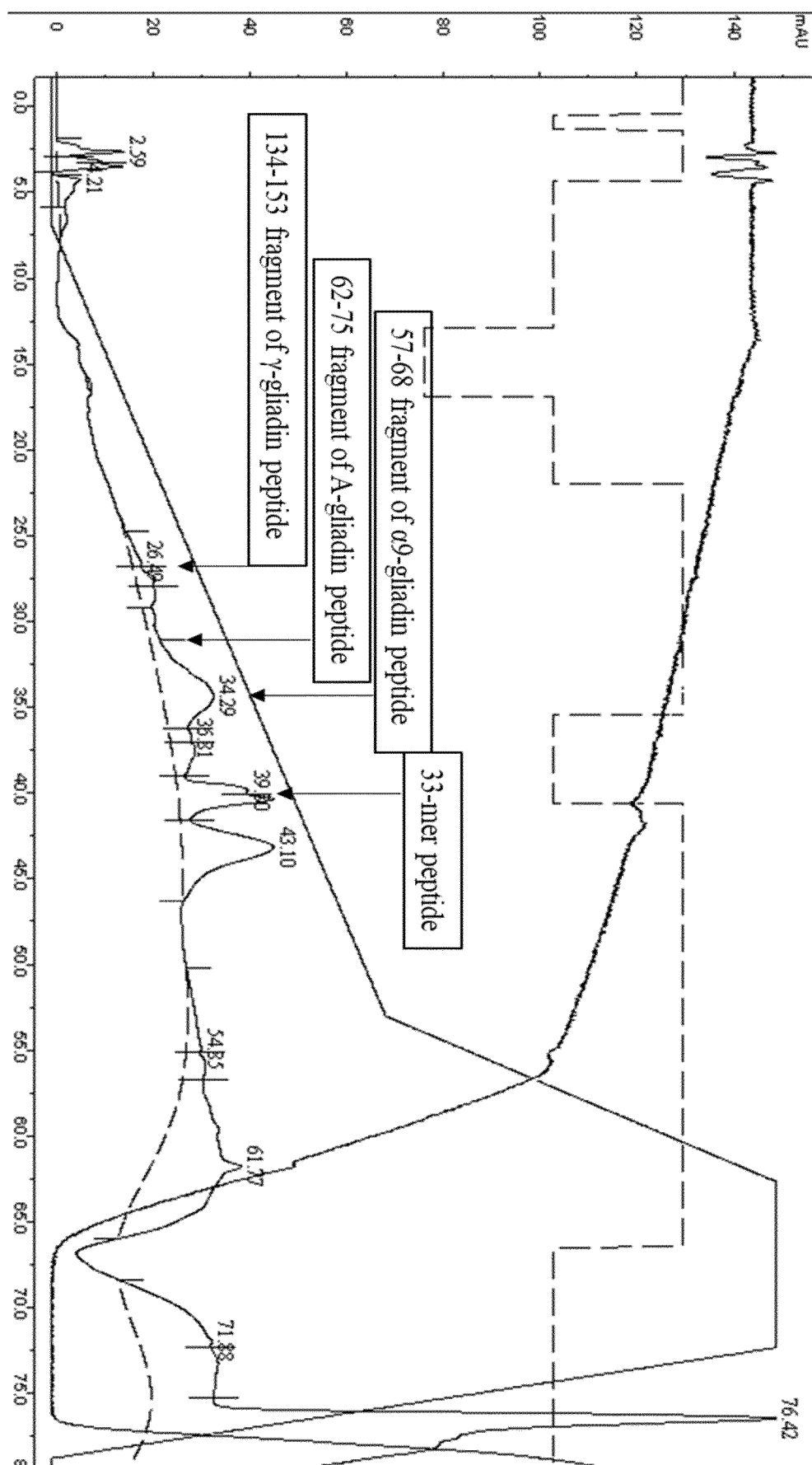


Figure 6 A: RP-HPLC peptide profiles of control digested wheat bread samples. M4 and M5 were added of E1, E2, Veron PS, Veron HPP commercial enzymes.

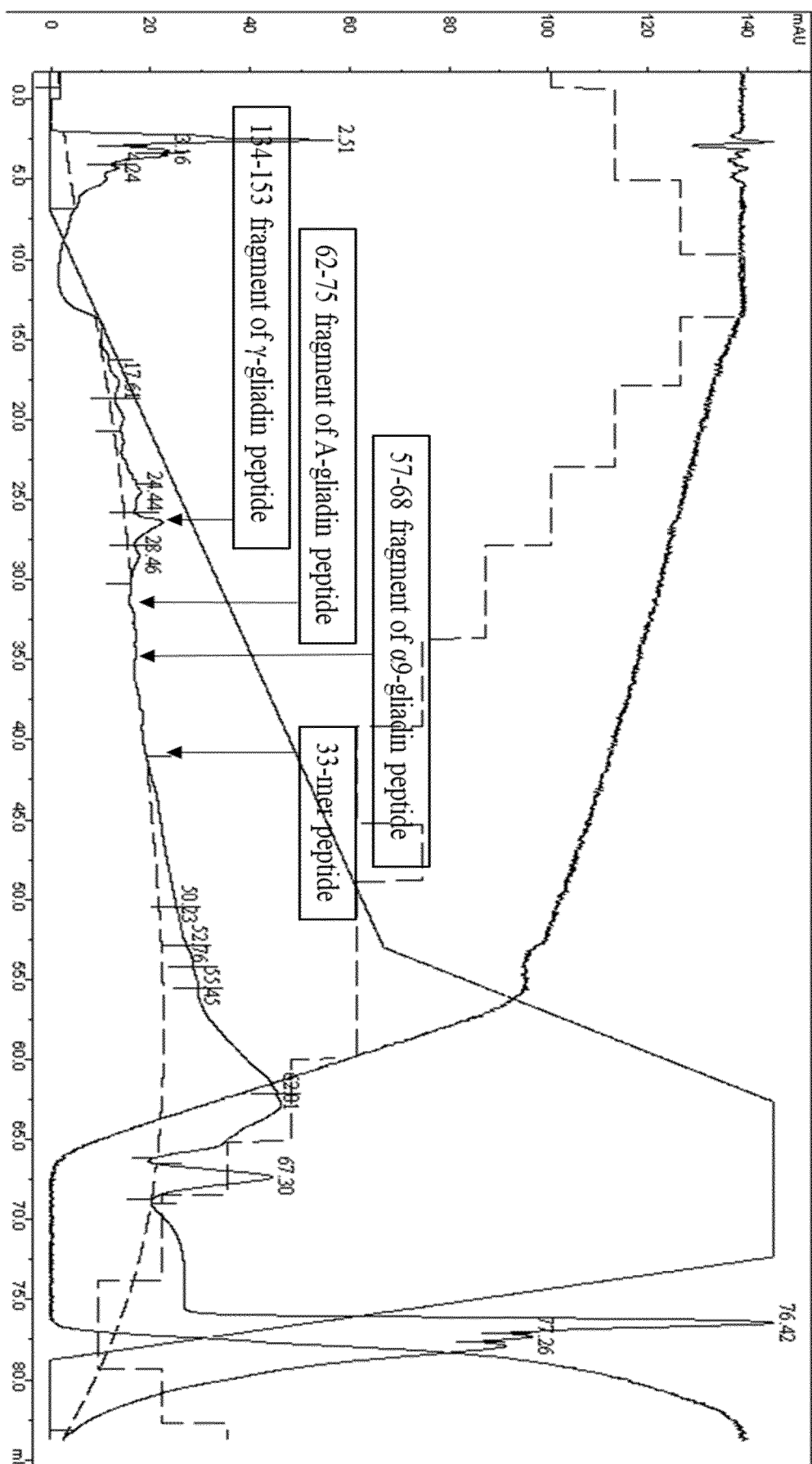


Figure 6 B: RP-HPLC peptide profiles of Mixture 4 digested wheat bread samples. M4 and M5 were added of E1, E2, Veron PS, Veron HPP commercial enzymes.

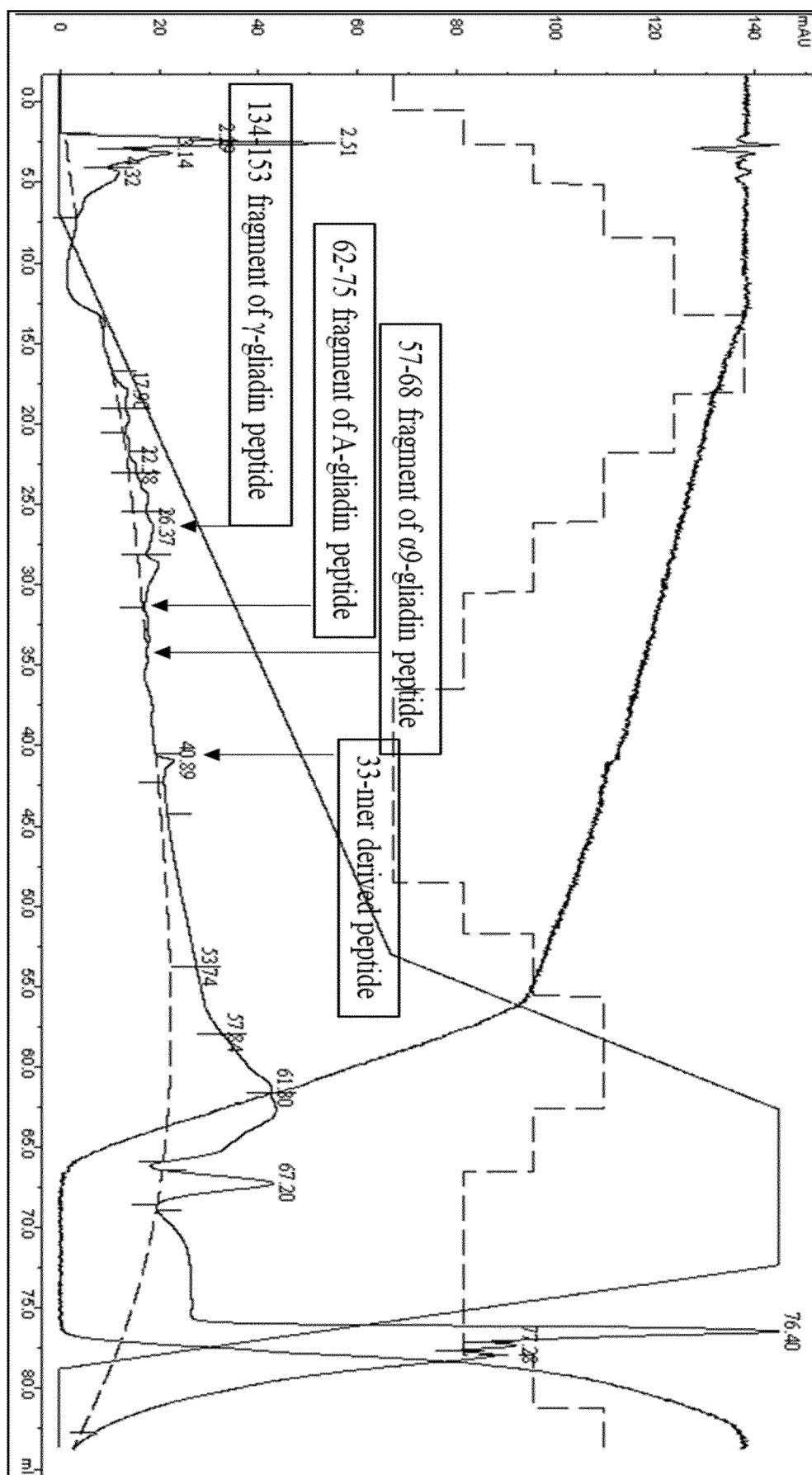


Figure 6 C: RP-HPLC peptide profiles of Mixture 7 digested wheat bread samples. M4 and M5 were added of E1, E2, Veron PS, Veron HPP commercial enzymes.

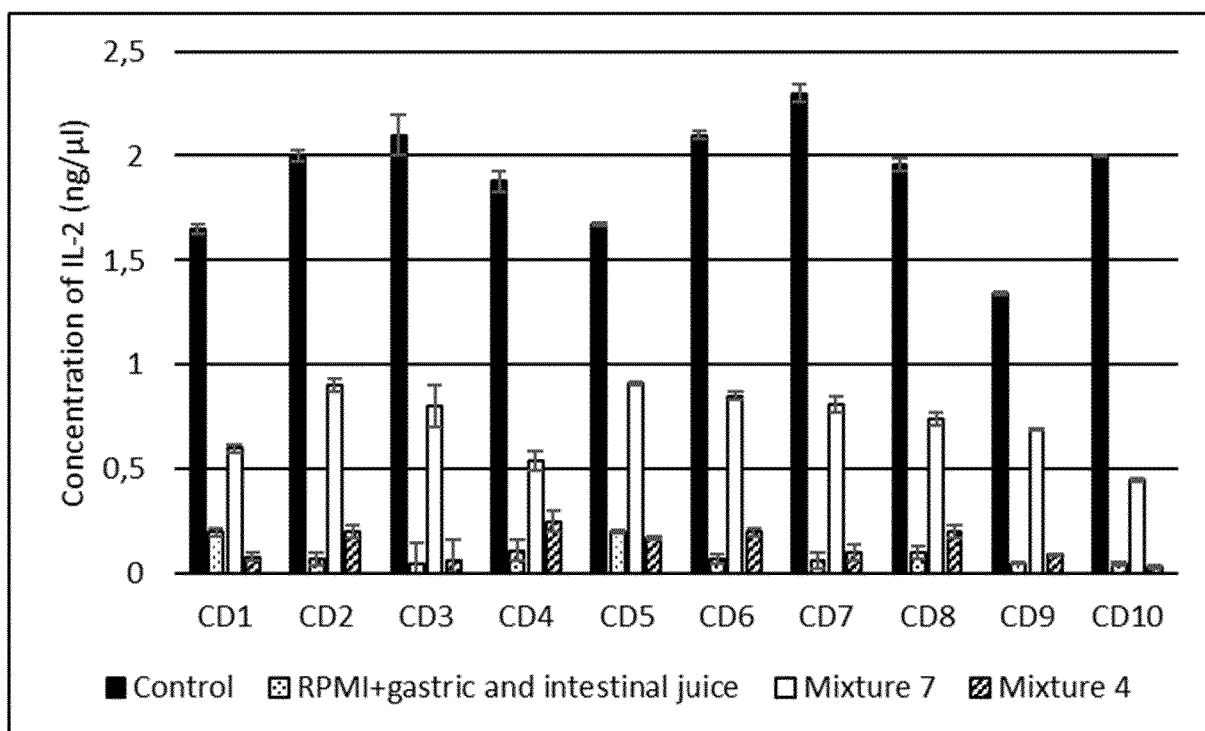


Figure 7 A: Concentration (ng/μl) of interleukin 2 (IL-2) in duodenal biopsy specimens from patients with CD.

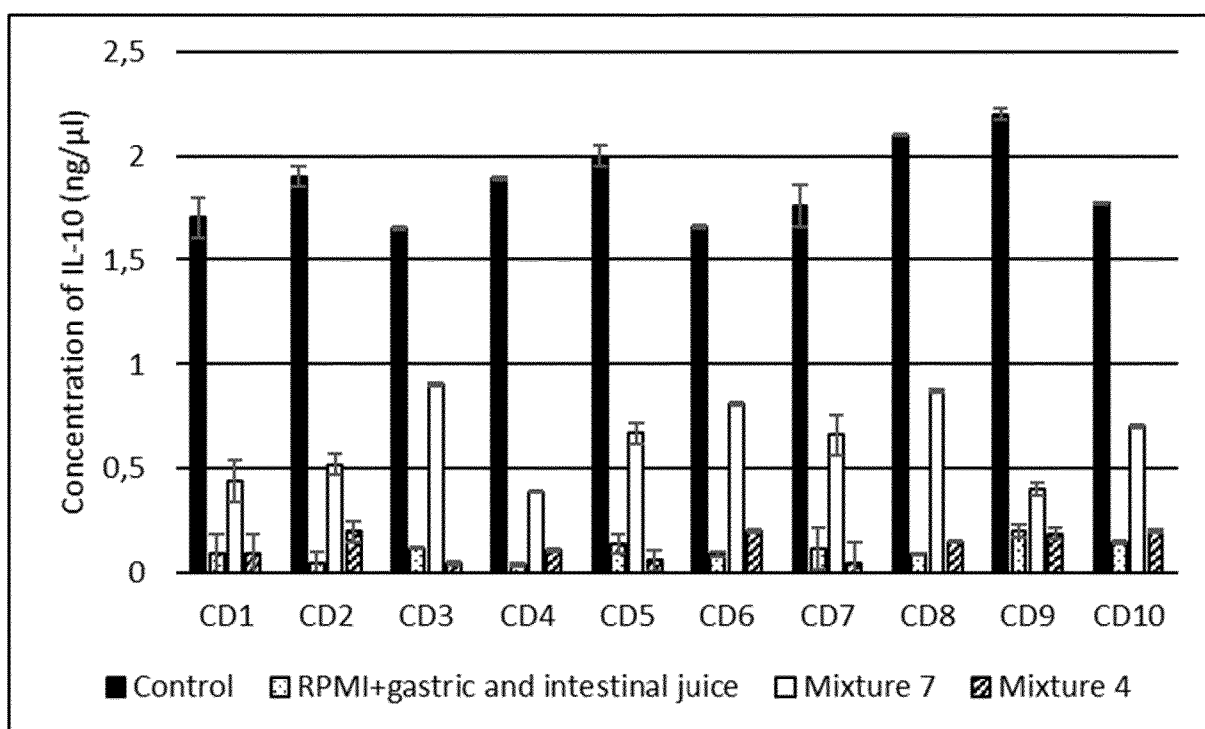


Figure 7 B: Concentration (ng/μl) of interleukin 10 (IL-10) in duodenal biopsy specimens from patients with CD.

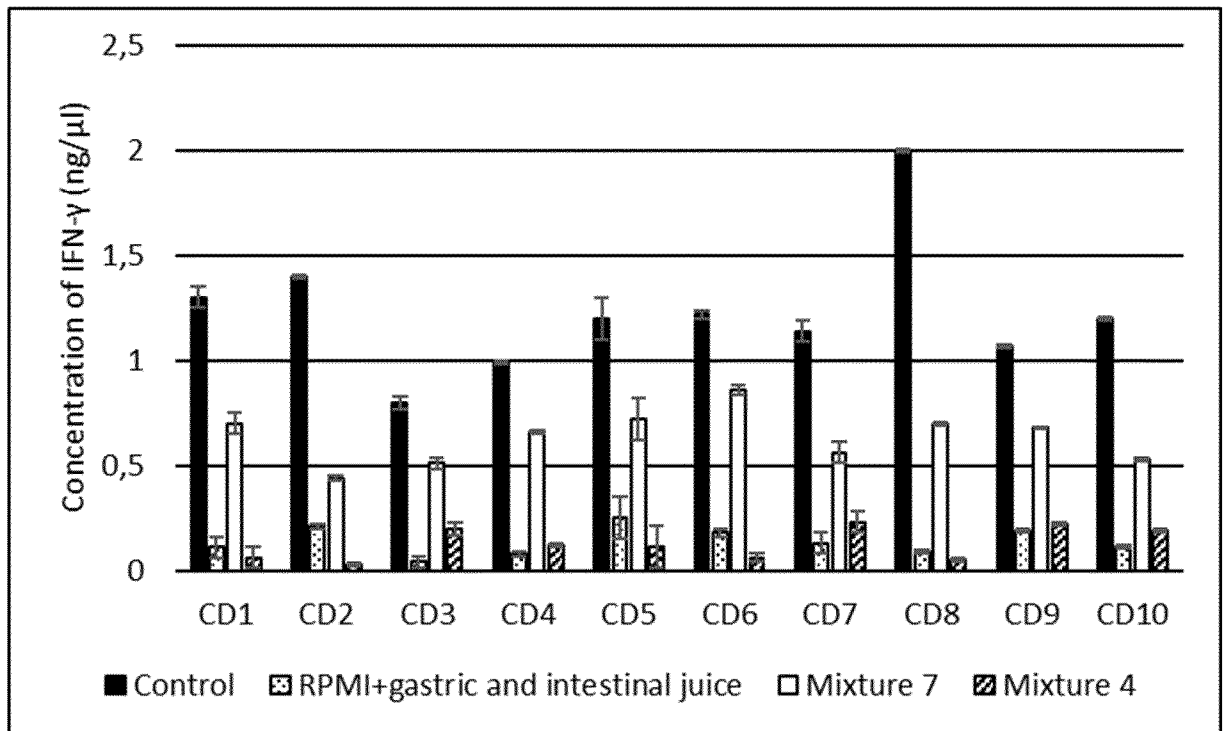


Figure 7 C: Concentration (ng/μl) of interferon gamma (IFN-γ) in duodenal biopsy specimens from patients with CD. Control, wheat bread digested without the addition of bacterial cells and microbial enzymes; RPMI+gastric and intestinal juice, negative control; Mixture 4, wheat bread digested with the addition of live and lysed cells of *L. plantarum* DSM 33363 and DSM 33364, DSM 33365, *L. paracasei* DSM 33373, *Bacillus subtilis* DSM 33298 and *Bacillus pumilus* DSM 33301 and E1, E2, Veron PS, Veron HPP commercial enzymes) and Mixture 7, wheat bread digested with the addition of live and lysed cells of *L. plantarum* DSM 33362, DSM 33366 and DSM 33370, *L. reuteri* DSM 33374, *Bacillus megaterium* DSM 33336, and *Bacillus subtilis* DSM 33353 and E1, E2, Veron PS, Veron HPP commercial enzymes. CD1 to CD10, duodenal biopsy specimens from celiac patients.