Universidade de Lisboa

Faculdade de Farmácia

Expression of modified gliadin proteins in *Lactococcus lactis*

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Trabalho de Campo Orientado pela Professora Doutora Tina Vida Plavec, Professora Assistente na Univerza v Ljubljana (Jožef Stefan Institute), e coorientado pela Professora Doutora Ana Paula Leandro, Professora Associada com Agregação, da Faculdade de Farmácia da Universidade de Lisboa.

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Trabalho Final de Mestrado Integrado em Ciências Farmacêuticas apresentado à Universidade de Lisboa através da Faculdade de Farmácia

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RESUMO

A doença celíaca afeta 1% da população, que sofre de inflamação intestinal desencadeada pela ingestão de glúten, em particular pelo seu elevado conteúdo de péptidos imunogénicos, onde está incluída a gliadina. (1) O glúten é formado por variadas proteínas estruturais presentes em alguns cereais. Ele pode ser utilizado para cozer pão e também como um aditivo alimentar para melhorar as características reológicas, por exemplo, da carne.

Indivíduos com doença celíaca, doença inflamatória intestinal e distúrbios relacionados com a ingestão de glúten têm dificuldade em evitar o consumo deste componente e manter uma dieta saudável. Os substitutos do glúten não possuem as mesmas características que o original, o que afeta o bem-estar social e psicológico dos indivíduos que o devem evitar.

As bactérias ácido-láticas, como o *Lactococcus lactis*, são microrganismos bem conhecidos, utilizados como fábricas celulares na indústria alimentar. Também são utilizadas na indústria farmacêutica para tratar e prevenir doenças – *Lactococcus lactis* geneticamente modificado pode expressar e libertar proteínas de interesse para alvos terapêuticos. Além disso, esta bactéria tem benefícios para a saúde humana, sendo por isso considerada "geralmente reconhecida como segura".

O objetivo deste trabalho foi produzir gliadina menos imunogénica, para agir como substituto ao glúten convencional na indústria alimentar. O gene da gliadina, ao qual foram adicionadas sequências de restrição, foi amplificado por PCR, para depois ser clonado no plasmídeo pNZ814, através da restrição e ligação. As bactérias *Lactococcus lactis* foram transformadas com o plasmídeo recombinante e as proteínas foram expressas a nível intracelular com o sistema NICE.

O gel desnaturante de poliacrilamida (SDS-PAGE) e análise por "western blotting" mostraram que as bactérias transformadas expressaram a gliadina mutada. As proteínas foram purificadas por cromatografia de afinidade com iões imobilizados (IMAC) e analisadas num SDS-PAGE, em que a gliadina foi identificada, embora uma parte estivesse ou degradada, ou formou oligopeptídeos. Também observamos que as bactérias transformadas para expressar a gliadina mutada cresceram mais rapidamente e secretaram mais proteína, em comparação com o controlo negativo e o "wild type".

Neste trabalho foram dados os primeiros passos para a produção de um potencial substituto do glúten que pode vir a ser utilizado na indústria alimentar, como alternativa aos produtos sem-glúten convencionais.

Palavras-chave: *Lactococcus lactis*, proteína recombinante, gliadina, sem-glúten

ABSTRACT

Celiac disease affects 1% of the population, and those affected suffer from chronic intestinal inflammation triggered by the consumption of gluten, and its high content in immunogenic peptides, including gliadin. (1) Gluten is made of variable structural proteins present in some cereals, such as wheat. It is used in bread making and as a food additive to enhance rheological properties, for example in meat products.

Patients with celiac disease, inflammatory bowel disease and gluten-related neurologic disorders struggle to avoid wheat and maintain a healthy varied diet. Gluten substitutes do not provide the desired qualities that gluten provides, which affects the social and psychological well-being of those who must avoid it.

Lactic acid bacteria, such as *Lactococcus lactis*, are well-known as safe microorganisms used as cell-factories in the food industry. They are also used in the pharmaceutical industry to treat and prevent diseases – genetically engineered *Lactococcus lactis* can express or deliver desired proteins to therapeutic targets. This bacterium has beneficial properties for human health, which earned it the status of "generally recognized as safe".

The aim of this work was to produce less-immunogenic gliadin as a substitute for conventional gluten in the food industry. The gliadin gene construct was amplified by PCR, and restriction sites were added to allow molecular cloning into plasmid pNZ8148, using ligation and restriction. *Lactococcus lactis* bacteria were transformed with the recombinant plasmid and proteins were expressed intracellularly using the NICE system.

Further analysis with denaturant polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting analysis showed that the transformed bacteria expressed mutated gliadin. Proteins were purified by immobilized metal affinity chromatography (IMAC) and analysed by SDS-PAGE, and gliadin was identified, although some was either degraded or in oligomer form. We also observed that bacteria transformed to express the mutated gliadin grew faster and expressed more protein in comparison to the negative control and the wild-type gliadin.

Altogether, in this work an expression system was developed that have the potential to allow the production of a potential substitute for gluten that could be used in the food industry and help people on a gluten-free diet.

Keywords: *Lactococcus lactis*, recombinant protein, gliadin, gluten-free

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To all my family, friends who are like family and colleagues: thank you for letting me be a part of your life.

ABREVIATIONS

A_{280} Absorption at 280 nm

AGE Agarose gel electrophoresis

APS Ammonium persulfate

CeD Celiac disease

CD Chron's disease

Cm Chloramphenicol

CRISPR Clustered regularly interspaced short palindromic repeats

DMSO Dimethyl sulfoxide

GF Gluten-free

GIP Gluten immunogenic peptides

GRAS Generally recognized as safe

IBD Inflammatory bowel disease

IMAC Immobilized metal affinity chromatography

LAB Lactic acid bacteria

MT Mutated type

MWs Molecular weights

NICE Nisin-controlled expression system

OD_{600} Optical density at 600 nm

PCR Polymerase chain reaction

UC Ulcerative colitis

WT Wild type

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1. Introduction

1.1.Gluten

1.1.1 Overview on Gluten: Nutrition, History and Relevance

Gluten comprehends a group of storage proteins found in wheat and some other grains such as rye, barley, and triticale. It is sometimes found in products that do not naturally contain gluten, including oats, when these are processed alongside foods containing gluten. (2,3)

The wheat plant is a species of the genus *Triticeae*, which also includes barley and rye. The domestication of this genus can be traced back to the Fertile Crescent during the Neolithic period, where it later spread through ancient emigration routes. Over the past 8500 to 2300 years, wheat proliferated through Europe and Asia, where these species adapted to different environments, and became landraces. (4)

Gluten has played a historically significant role in the human diet. Health authorities in different countries have been developing food graphics - such as food pyramids and food wheels - to represent a balanced, healthy diet, where gluten is frequently mentioned.

Bread wheat (*Triticum aestivum*) is an important food crop accounting for 20% of the calories consumed by humans and 40 - 50 % of the grain mix for cattle. It is an important source of protein, vitamin A, vitamin B, calcium, iron, and zinc. For that reason, a gluten-free (GF) diet can lead to low mineral bone density and micronutrient deficiencies. (3,5)

Wheat production influences the modern economy as it is an important source of calories, vitamins, and minerals for humans and animals - its deficiency is an indicator of famine and can lead to calls for humanitarian assistance. (6)

The wheat crop outlook may have geopolitical implications, as evidenced by Russia's attack on Ukraine, which raises concerns, because the two countries accounted for 29% of the global wheat exports in 2022. (7) By July 2022, Ukraine had already harvested 27 million tons of wheat, more than expected for that year, considering the war. (8)

1.1.2 Gluten: Glutenin and gliadin

Gluten is a gummy mass obtained from washed wheat dough. Their proteins, mainly prolamins and glutenins, have a rich and unique amino acid composition, showing a high content of glutamine and proline and a low content of amino acids with charged side groups. Prolamins are a family of storage polypeptides formed in the endosperm of the grain and serve as a major source of nitrogen and cysteine in wheat (gliadin), barley (hordein), and corn (zein) (Figure 1). (9–11)

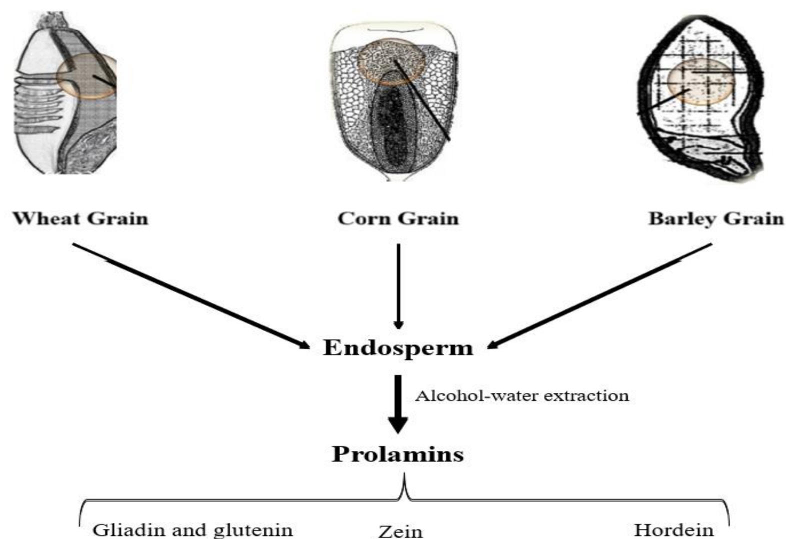

Figure 1 - Location of endosperm in cereal grains to obtain prolamins. Adapted from (66)

Gluten can be described as a "two-component glue", considering the different ways in which two factions contribute to the rheological properties of the dough, and form a three-dimensional protein network after hydration and mixing. There are two fractions of gluten prolamins, distinguished according to their solubility in aqueous alcohols (e.g., 60% ethanol): the insoluble glutenin and the soluble gliadin. (2,9) Glutenins are filamentous proteins with their conformation stabilized by disulphide bonds (Figure 2) that, after reduction, acquire a solubility in aqueous alcohols similar to that of gliadins. (9,10,12). The glutenin fraction acts as a "solvent" being responsible for the cohesive and strong elasticity of the dough when hydrated. The main component of gliadins are monomeric glomerulus proteins, which are

classified into three categories based on their different primary structures which confers different electrophoretic mobility: α/β-, γ- and ω-type. Disulphide bonds might be absent or present as intrachain crosslinks. (9,10,13).

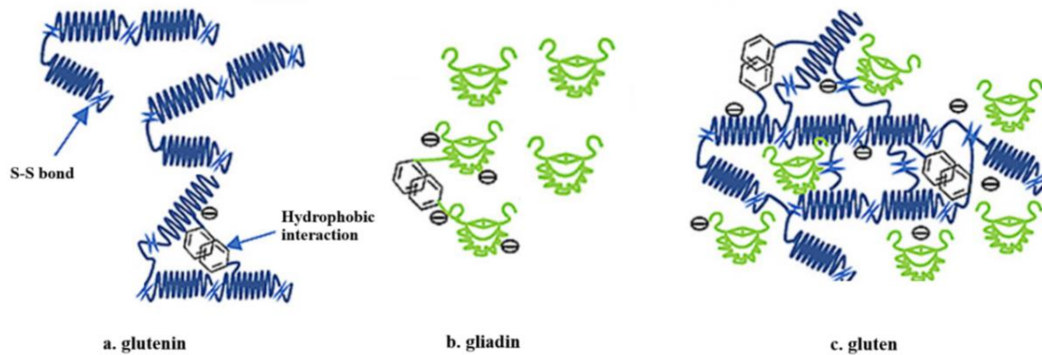

Figure 2 - Schematic representation of glutenin (a), gliadin (b) and gluten (c). Adapted from (67)

The gliadin fraction is responsible for the dough's viscosity and extensibility when hydrated, thus acting as a "plasticizer". Gluten is not only found in bread and cereals but is also used as a food additive for meats, sauces, and condiments because of its water-binding and viscosity-increasing properties, that improve rheological properties. (13) Baking gluten-free bread is challenging because the non-gluten proteins dramatically alter dough rheology, and final bread characteristics. (2)

1.1.3 Immunogenicity of Gliadin

Research on gluten's interference with auto-immune diseases has shifted the way the public perceives gluten - it is not a concern exclusive for patients with celiac disease (CeD), but for individuals with variable sensitivity to gluten immunogenic peptides (GIP).

Different cereals have distinct compositions of gluten proteins, inducing a variable spectrum of immunogenic or toxic responses in susceptible individuals. The mechanisms by which different GIP are processed in the body are not yet fully understood. (10,14) It is known that ingested proteins undergo partial enzymatic digestion through the gastrointestinal tract to

become peptides and later be absorbed by intestinal epithelial cells, in the form of amino acids or small peptides (dipeptides and tripeptides). (10) It is not fully understood how GIP go from the epithelial barrier into the lamina propria to trigger immune responses. CeD patients with lesions typically present loss of small bowel intestinal villi with an infiltration of leukocytes, both in the epithelium and in the lamina propria. (10,15)

Briefly, when GIP reach the intestine they may bind directly to the cell surface receptors HLA-DQ2.5 or -DQ8 and induce T-cell responses that trigger the release of inflammatory cytokines and generate local inflammation. Also, tissue transglutaminase 2 (TG2) can deamidate some glutamine residues to glutamate in gluten peptides, thus increasing their binding affinity to HLA-DQ2.5 or -DQ8, therefore amplifying the immune response (Figure 3). (10,15)

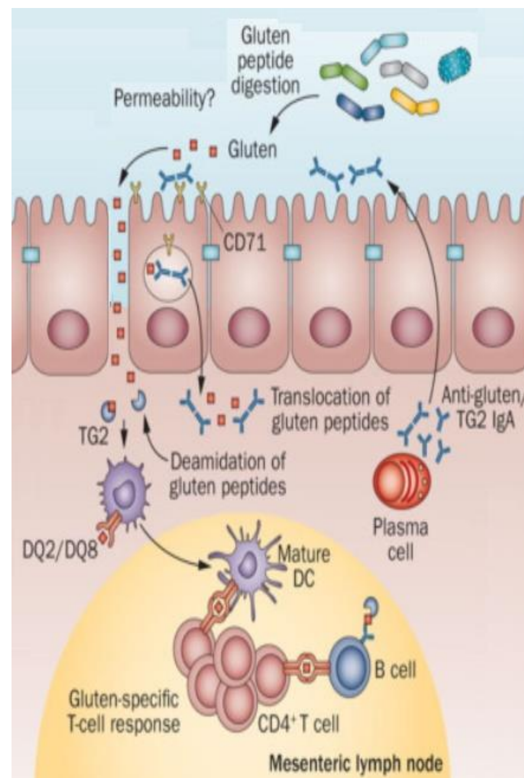

Figure 3 – Celiac disease pathogenesis. Adapted from (68)

In vivo toxicity studies performed on organ cultures from jejunal biopsies have demonstrated that α/β-, γ- and ω-type gliadins have a respectively decreasing toxicity. (10,14)

Recent studies used urine samples to detect the presence of GIP in CeD patients to monitor GF diet adherence. Obtained data indicate that GIP are absorbed to the blood stream

due to intestinal mucosa damage and permeability changes. Some healthy individuals showed some level of renal excretion of GIP which might be due to diet or immunity fluctuations. (10,15)

1.2. Pathologies

1.2.1. Inflammatory Bowel Diseases and Celiac Disease

Immune mediated diseases share a common genetic background and consequently a similar pathogenesis. Inflammatory bowel disease (IBD) and CeD are both characterized by chronic intestinal inflammation, although their causes are not fully understood. (16)

Both diseases are characterized by dysregulation of the innate and adaptive immune responses triggered by genetics and the environment. CeD is a specific disease because it is mainly set off by known triggers. On the other hand, IBD has two clinical forms: Crohn's disease (CD) and ulcerative colitis (UC), in which there appears to be a complex interplay between the microbiota and other environmental factors. (16) IBD can be debilitating, and psychological problems are common in patients. (17)

CeD is a common enteropathy triggered by gluten in genetically predisposed individuals, including the ones seropositive for HLA-DQ2, HLA-DQ8 or, rarely, HLA- DQ7. It is not fully understood how gluten tolerance is lost and tissue destruction starts. CeD affects ~1% of the world population and has shown an increasing incidence. (18,19)

The gold-standard therapy for CeD is a strict GF diet, that is socially challenging, and results in inadequate symptom control. Some patients do not respond to a GF diet and develop refractory CeD. (16) Research shows that patients are not able to maintain a strict GF diet because gluten is ubiquitous in food e.g., as an additive. Alternative therapies are not disease specific. (18,19)

As mentioned before, in CeD, GIP are deamidated by the TG2 enzyme, increasing the GIP affinity for HLA DQ2/DQ8 on antigen-presenting cells. CD4+ T cells are then activated and release pro-inflammatory cytokines, resulting in cell infiltration and cytotoxic activity of CD8+ T cell (18,20). The resulting lesions are characterized by an increase in intraepithelial lymphocytes, crypt hyperplasia and shortening of the villi. Studies suggest that this enteropathy is caused by a complex interplay between adaptive and innate immunity. (21)

Within hours of gluten exposure, CeD patients can present upper gastrointestinal symptoms (e.g., nausea and abdominal pain) and systemic markers of immune activation, whereas in children, diarrhea is the most common symptom. (16,18,22) Symptoms of ulcerative colitis include bloody, mucous diarrhea. For CD, the symptoms include lesions that have diffuse anatomical locations, leading to nonspecific symptoms such as abdominal pain, and other symptoms such as diarrhea, dyspepsia, constipation, asthenia, flatulence, loss of appetite and weight loss. Extraintestinal manifestations may occur in IBD and CeD. (16)

Patients feel that science does not care about their needs - there is no effective (and direct) treatment for inflammatory diseases, including IBD. Therapeutic approaches include the administration of immunosuppressants, such as azathioprine, mercaptopurine and methotrexate. The anti-inflammatory drugs infliximab and adalimumab have become standard biologic therapies. Infliximab has higher efficiency, but some patients do not respond, so adalimumab is the alternative TNF antibody. (16)

1.2.2. Gluten Sensitivity: Neuroinflammation

CeD can have systemic manifestations – consumption of gluten can lead to anaemia, osteoporosis, dermatitis herpetiformis, fatigue, infertility, neurologic and neuropsychiatric manifestations that may precede the typical picture. (10,22)

Nervous system symptoms can precede the typical manifestations of CeD, as indicated by data reported in the literature. Souahyah and co-workers reported two cases of symptoms of small-fiber neuropathy including progressive numbness, tingling and electric-like pain, in patients later diagnosed with CeD. (23) De Sousa and colleagues characterized 62 patients with sensory neuropathy: in 50% of the cases the neuropathy had an unknown cause - eleven of these patients had high CeD-related antibody titers. (24) Brannagan reported 8 cases with CeD and small-fiber neuropathy - in seven of these cases neurological symptoms preceding the CeD diagnosis. (25)

Encephalopathy is characterized by global brain dysfunction, with symptoms ranging from headaches, confusion, disorientation, cognitive deficits and altered level of consciousness. Keller and Dimberg presented 2 cases of refractory CeD and encephalopathy. *Post-mortem* neuropathological findings included loss of Purkinje cells, neuronal loss of the dentate nucleus and perivascular cuffing of lymphocytes. (26,27)

Overall, the most prevalent neurological manifestations in CeD are: (1) cerebellar ataxia, also known as "gluten ataxia", with progressive gait ataxia, dysphonia, dysarthria, pyramidal signs, and abnormal eye movements; (2) peripheral neuropathy, which may precede gastrointestinal manifestations; (3) epilepsy, with a prevalence of 3.5% to 7.2%; (4) headaches, that improve following a GF diet; (5) mild cognitive symptoms, that include concentration struggles, episodic memory deficits and world-retrieval difficulties, and can improve with a GF diet; (6) psychiatric disorders, such as apathy, depression, bipolar disorder, anxiety, schizophrenia, attention-deficit/hyperactivity disorder (ADHD), autism, and sleep complaints. (22)

The risk of developing autoimmune reactions increases in CeD patients later in life. Whether or not this risk is due to years of gluten exposure is still controversial. In most studies, adherence to a strict gluten-free diet was unmonitored. Thus, it is unknown whether patients were exposed to gluten when they developed a neurological dysfunction. (26)

Gluten-related neurological disorders (GRND) are a spectrum of neurological manifestations triggered by gluten. The pathological mechanism of the neurological dysfunction is unclear. (26) Patients with neurological manifestations of gluten sensitivity were also malnourished and were deficient in several vitamins (B1, B6, B12, E) and micronutrients. (22)

1.2.3. Gluten Free Diet: Feasibility, disadvantages, and opportunities in other disease

Research along years suggests that gluten can contribute to the course of immune mediated disorders. (13) However, there is no satisfactory replacement for gluten in the food industry, and despite the benefits of a GF diet, it is difficult to maintain.

To replace gluten, it is expected the same sensory properties (such as colour and flavour) and good nutritional values, since the main concern with a GF diet is nutritional deficiencies. Ziobro's studies claim that pea and lupine proteins are preferred to soy sensory-wise, while albumin is the best for volume increase. Overall, legume proteins, eggs, dairy and non-gluten cereals have a good amino acid profile, and are involved in Maillard browning reactions, which is why they are preferred as substitutes. (28)

Enzymes are used to improve rheological properties by forming crosslinks in between polymers present in the formulation to obtain a network similar to gluten. Regular bread can have glutathione that depolymerizes its network and allows proteins to crosslink through disulphide bonds. This improves rheological properties and gas retention during baking. The same process occurs in rice flour baked in the presence of peptidases. (2)

A GF diet is essential for CeD patients, but it also has disadvantages: lower intake of fibre, calcium, zinc, iron, magnesium, vitamin D and E; higher intake of fatty acids and carbs; higher costs; likelihood of cross-contamination; and social barriers. Overall, both CeD patients and individuals following a GF diet should receive nutritional guidance to avoid nutritional deficits, especially because the GF diet is becoming more popular. (19,29,30)

In vitro studies have shown that gliadin alters gastrointestinal barrier function almost immediately by decreasing transepithelial resistance and increasing permeability (31,32). *Ex vivo* studies on human biopsies of CeD patients and healthy controls also showed barrier disruption. (31,33) Therefore, researchers questioned what other benefits a GF diet can have on other diseases. While evaluating the effect of a GF diet on obesity, metabolic syndrome, and cardiovascular risk in non–celiac patients, Kin and colleagues found that a GF diet was correlated with weight-loss, lower waist circumference, and higher HDL levels. However, a GF diet showed no impact on the prevalence of metabolic syndrome and cardiovascular risk score. (34)

In addition, gluten avoidance can be beneficial for gastrointestinal symptoms (for example irritable bowel syndrome). However, there is no robust evidence suggesting that a GF diet helps to treat or prevent diseases, other than the autoimmune ones triggered by gluten. In fact, gluten avoidance may be associated with adverse effects in patients without gluten-related diseases as a GF diet can reduce gut microbiota variety. (19,20)

Current evidence is insufficient to recommend a GF diet to patients with multiple sclerosis (MS), psoriasis, type 1 diabetes, or autoimmune thyroid diseases. However, large epidemiological studies and meta-analyses of systematic reviews support that these diseases are all associated with CeD.

The correlation between MS and a GF diet was investigated in a single trial, with many limitations in group allocation. Despite that, the GF group showed lower disability and lower

activity on magnetic resonance imaging compared to the regular diet group. (35) It is debated whether CeD and MS are related, since some MS patients express anti-gliadin antibodies. (13) However, recent population-based and case-control studies did not find any association between MS and CeD. (13)

A study from 2000 showed the potential of a GF diet in psoriasis as 73% of patients who lived gluten-free for three months improved symptoms; all those patients were positive for gluten-related antibodies. Despite these optimistic results, larger studies should be conducted. (36)

A cohort study in Denmark suggested that high gluten consumption during pregnancy could increase the risk of children developing type 1 diabetes. However, these findings have yet to be confirmed. (37)

1.3. Lactic Acid Bacteria (LAB)

1.3.1. General Characteristics

Lactic acid bacteria (LAB) are a group of non-sporagenic, anaerobic gram-positive bacteria, that include the genera: *Lactococcus*, *Lactobacillus*, *Oenococcus*, *Enterococcus*, *Streptococcus*, *Pediococcus*, *Tetragenococcus*, *Vagococcus*, *Leuconostoc*, *Carnobacterium*, *Sporolactobacillus* and *Weissella*. (5–7)

LAB have been widely used in the food industry to produce lactic acid through a fermentation process. They usage is safe thus owning the FDA (food and drug administration) status of GRAS (generally recognized as safe). (38,39) They are used as probiotics in food, such as yogurt, cheese, and sauerkraut. (40,41)

LAB grow in acidic culture mediums (up to pH 4.5) and can resist to high temperatures (up to 45 ºC) without oxygen. Growth medium for LAB requires the presence of vitamins, amino acids, peptides, nucleotides, and monosaccharides. (5,8,9) LAB undergo lactic acid fermentation in one of two different pathways: hetero-lactic acid metabolism pathway; or homo-lactic acid metabolism pathway, depending on the species. (9)

The pharmaceutical industry uses genetically engineered LAB to treat and prevent diseases as *Lactococcus*, or *Lactobacillus* can be hosts, or vector cells (in the case of CRISPR-

Cas technology) that deliver therapeutic molecules. (38) The most known species is *Lactococcus lactis* (*L. lactis*), for its well-known genome, and well-established tools and systems for its genetic engineering. (38)

1.3.2. *Lactococcus lactis*

The genera *Lactococcus lactis* includes three subspecies: *L. lactis* subsp. *lactis*; *L. lactis* subsp. *cremoris*; and *L. lactis* subsp. *Chordonia*. They are useful in the food industry to: ferment foods; prevent biofilm formation; enhance sensory and nutritional properties, produce sweeteners and B vitamins; and produce acid and bacteriocins, that extend a products' shelf life. (42) In cosmetic industry they are used as emulsifiers and moisturizers. (43)

L. lactis has been genetically modified to be used as a cell factory to produce industrial metabolites and enzymes. One common strategy is to delete the gene encoding for lactate dehydrogenase, which converts pyruvate to lactic acid, thereby redirecting fermentation to the synthesis of aromatic compounds. (43)

Genetically engineered *L. lactis* is the most desirable and widely used microbial cell factory in the pharmaceutical industry for several reasons: cloning and expression systems are well-known and optimized and can be customized; it is safe; it has a small and fully sequenced genome. (39,43)

Other bacteria, such as *E. coli*, can also be engineered to express a desired protein. However, *E. coli* faces a few disadvantages, including: the production of endotoxins, that compromises safety; and the formation of inclusion bodies, from partially folded overexpressed proteins that aggregate and accumulate in the cytoplasm. *L. lactis* is distinguished for not sporulating or producing endotoxins, meaning it is safe, forming only up to 50% of the total cellular protein without forming inclusion bodies, and having low protease activity. (44–46)

1.3.3. Modified *L. lactis* in therapies and the food industry

L. lactis survives the passage through the gastrointestinal tract without colonizing it and can be genetically engineered to introduce therapeutic proteins or peptides into the digestive tract to prevent or treat diseases. (43) This technology is being tested in clinical trials for IBD,

autoimmune diseases, cancer, and infectious diseases (such as HIV prevention and *influenza* vaccine). (43,47)

Modified LAB are a promising tool to create naturally fortified foods, by producing and releasing vitamins in the fermentation process. Metabolic engineered *L. lactis* showed higher levels of folic acid (vitamin B11) and riboflavin (vitamin B2) in the extracellular fluid. This multivitamin producing strain could be relevant in the food industry, since vitamin deficiencies are common, and can lead to anaemia and other diseases. (48)

Engineered *L. lactis* ssp. *Cremoris* have shown to enhance the production of vitamin K2 in cheese, during fermentation. This is an important aspect as deficient levels of vitamin K2 can increase the risk of cardiovascular disease and osteoporosis. The use of fortified foods is promising in solving public health problems. (49)

1.3.4. Genetic Engineering Techniques

Therapeutic proteins can be expressed in LAB if appropriate expression vectors are used. The expression rate is influenced by many factors, including vector copy number and promoter characteristics. Promoters can be constitutive or inducible – the latter are preferred, because of providing better control over the expression of recombinant protein. (38)

A vector is composed of different coding sequences that can be manipulated in the laboratory with the desired expression components (such as promoters). The most commonly inducible expression system used in *L. lactis* is the NICE system (nisin-controlled gene expression), in which a subinhibitory concentration of nisin (0.1–5.0 ng/mL) triggers expression of the desired gene. Many plasmids are used with this system, including pNZ8048 and its variants pNZ8148 and pNZ8150. (38) Bicistronic vectors (pNZDual) presenting two nisin promoters controlling two different inserted genes, are also available to express two different proteins simultaneously. (50)

Integration of genes into the bacterial genome is an alternative method for expression of recombinant proteins, but it is more time-consuming and less efficient. New genetic engineering techniques include CRISPR-Cas, which can be adapted to LAB. (38) Cas9 is a DNA endonuclease that uses guide RNA – DNA complementary to the target DNA sequence

- to edit the genome. CRISPR-Cas9 can integrate or delete large DNA segments in the bacterial genome with high specificity. (38)

1.3.5. Expression of Recombinant Proteins

Expressed proteins can either accumulate in the cytoplasm (intracellular expression), anchor to the surface, or be secreted into the culture medium. For proteins to be secreted, they must have a signal peptide at the N-end, and an ideal size. Then they can be easily purified and the interaction with the target is facilitated. (38)

LAB have a thick rigid cell wall that allows therapeutic proteins to anchor to it and still interact with target molecules. Biochemical strategies to achieve this include: a) N-terminal transmembrane and lipoprotein anchoring, in which a hydrophobic domain binds to the phospholipidic membrane; b) anchoring via fusion of the protein with the LPxTG sequence, that forms covalent bonds with the membrane; and c) anchoring via the LysM domains, that bind non-covalently to peptidoglycans in the cell wall. (38)

Intracellular expression of recombinant proteins in *L. lactis* offers several advantages, such as: stability, i.e. protection from degradation and denaturation in the extracellular fluid; lower risk of contamination, which is important for food or pharmaceutical applications; and suitability for oral delivery, as *L. lactis* is GRAS (generally recognized as safe) is a potential vehicle for oral delivery of vaccines or bioactive peptides. (43,51,52)

1.3.6. Nisin-controlled gene expression system

Protein expression systems, such as the NICE system, allow transformed bacteria to grow and express a recombinant protein in the presence of subinhibitory concentrations of nisin, an antimicrobial polycyclic peptide. Nisin binds to lipidII – a precursor of cell-wall synthesis – and forms pores through which ATP and other molecules are released to the outside, leading to cell death. (21)

The genes responsible for signal transduction in the nisin gene cluster (nisK and nisR) have been isolated and inserted into *L. lactis* subsp. *cremoris* thus obtaining the strain NZ9000. This means that when this strain is transformed with a vector that contains the inducible promoter PnisA and the gene of interest, the encoding protein will be express it in the presence of nisin. (45)

When nisin interacts with the cell, it binds to the membrane protein kinase – nisK – and acts as a nisin receptor (Figure 4). Then, the protein kinase nisK phosphorylates the nisR protein and induces nisin transcription by activating the promoter PnisA, encoded in the plasmid. (45) Higher amounts of nisin lead to higher concentrations of the protein of interest. (53)

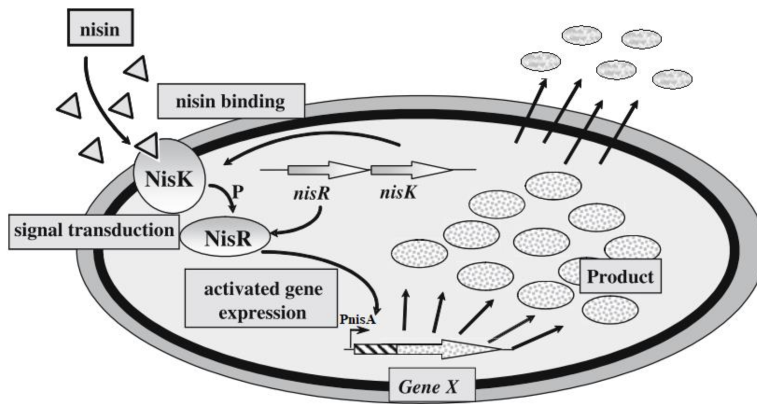

Figure 4 - Nisin-controlled gene expression. (NisK) membrane protein kinase; (NisR) response regulator; (Gene X), gene of interest cloned 3’ to the nisA promoter. Adapted from (45)

2. Hypothesis and Research Objectives

Gluten proteins are widespread in the modern diet, playing a role in nutrition, and psychological and social well-being. CeD is a disease specifically triggered by gluten. IBD, such as CD and UC, are common autoimmune diseases, where gluten can be inflammatory. In addition, it is accepted that gluten proteins have neuroinflammatory effects and are involved in the development of GRND. (26)

GIP are the immunodominant peptides of gluten and are resistant to proteolysis. Modification of these peptides would reduce the immunogenicity of gluten, and thus less harmful to humans. (10) The aim of this thesis was to produce modified gliadin, using biotechnological methods, to replace regular conventional gluten used in the food industry with a less-immunogenic gluten version.

In this work, a LAB (*Lactococcus lactis*) was engineered for intracellular expression of a less-immunogenic gliadin. To attain our objectives two different gene constructs were used corresponding to the wild type (WT) phenotype (gliadin that is found in nature), and a mutated type (MT) previously engineered to be less immunogenic.

The gene constructs were first amplified by PCR method, and restriction sites were introduced in order to allow molecular cloning into the pNZ8148 vector. Competent bacteria were transformed with the developed recombinant expression vectors and the recombinant proteins were then produced using the NICE system. Cell lysates were analysed by denaturing gel electrophoresis (SDS-PAGE) and western blotting. Overexpressed proteins were then purified using immobilized metal affinity chromatography (IMAC) and obtained fractions were analysed by SDS-PAGE.

3. Materials and Methods

3.1. Materials and Reagents

3.1.1. Bacterial Cells

The *L. lactis* NZ9000 (genotype: MG1363*nisRK ΔpepN*) strain was used to express the WT and MT gliadin proteins with the nisin controlled gene expression (NICE system), since they contain the *nisRK* genes.

As an intermediate host, the *Escherichia coli (E. coli) DH5α* strain from Invitrogen (genotype: *F- φ80lacZΔM15 Δ(lacZYA-argF) U169 recA1 endA1 hsdR17 (rk-, mk+) phoA supE44 λ- thi-1 gyrA96 relA1*) was used. DH5α contains mutations, such as the *endA* mutation that inactivates a DNA endonuclease responsible for the degradation of internalised DNA; and the *recA1* mutation that disables the activity of recA, an important recombinase that repairs DNA in a process called homologous recombination. (54)

3.1.2. Plasmid and gene constructs

Two gene constructs, gliadin WT and gliadin MT were used in this work. The gene sequence of gliadin MT will not be disclosed due to confidentiality.

The plasmid used for gene cloning and protein expression was pNZ8148. It is one of the most commonly used plasmids that are derivatives of the bacterial pSH71 plasmid *L. lactis*. (53) The variant pNZ8148 (Figure 5), has more restriction sites, in comparison to other pSH71 derived plasmids like the pNZ8048, where it is only possible to use the *Nco*I restriction site. (45)

Each region of the plasmid (Figure 5) serves a specific purpose on the transformed bacteria. The multiple cloning site (MCS) contains several restriction endonuclease recognition sites, and is under the control of the nisin A promoter sequence (PnisA). A transcription terminator (T) is found at 3' of the MCS. The plasmid also contains a selectable marker, which confers host cells resistance to chloramphenicol (Cm), and the replication genes *repA* and *repC*, which encode essential proteins for plasmid replication. (53)

The pNZ8148 with no gene insertion was used as a negative control for the analysis of gliadin proteins expression.

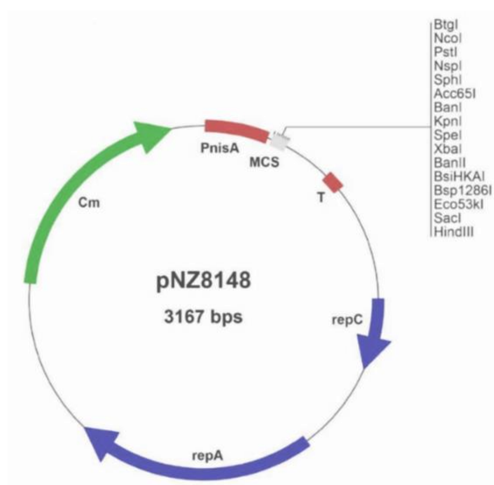

Figure 5 - Vector Map for pNZ8148. (PnisA) nisin A promoter region; (MCS) multiple cloning site; (T) transcription termination sequence; (repA and repC) genes for the replication proteins; (Cm) Chloramphenicol resistance gene. Adapted from (53)

3.1.3. Primers

The genes encoding for the WT and MT gliadins were amplified by PCR using the primers described in Table 1. The PCR primers contained recognition sites for the restriction enzymes NcoI and XbaI (underlined sequences) thus allowing introduction of these sequences in the amplified fragments and further cloning into the MCS of the pNZ8148 vector. Primers were ordered using the OligoAnalyzer™ Tool (IDT, USA), and were dissolved in dH_2O and diluted to a concentration of 5 µM.

Table 1– List of the primers used and their respective melting temperatures (Tm).

	Nucleotide Sequence (5'→3')	**T_m**
GliadWT-F-Nco	TTA ACC ATG GCT AAA ACC TTC TTG ATC CTG GTT	61.4 ºC
GliadWT-R-Xba	AAT TTC TAG ATT AAT GAT GGT GAT GAT GGT GTT TG	57.6 ºC
GliadMut-F-Nco	TTA ACC ATG GCT AAA ACC TTC CTG ATT CTC GTT	61.3 ºC
GliadMut-R-Xba	ATT TTC TAG ATT AAT GAT GAT GAT GGT GAT GCT TAT	57.2 ºC

Note: Underlined sequences correspond to the recognition sequence for NcoI (C↓CATGG) and XbaI (T↓CTAGA)

3.1.4. Reagents, solutions and culture media

The reagents (including enzymes), solutions, buffers and equipment used in this work are presented in Table A to D (Annex). The antibodies used in Western blot assays are listed in Table 2.

Table 2 – List of antibodies used.

Antibodies	**Manufacturer**
Primary Mouse IgG1 Antibodies against His-tag peptide (66005-1-Ig)	Proteintech (USA)
Secondary Goat anti-mouse IgG antibody conjugated with fluorescent dye StartBright Blue 700 (12004158)	Bio-Rad (USA)

The media utilized for bacterial growth is presented in Table E (Annex).

3.2. Methods

3.2.1. Transformation of bacterial cells

Transformation is the uptake of naked DNA from the environment into the host cell. In many bacterial species, it is a mode of parasexual reproduction and increases genetic variability. The ability of bacteria to take up DNA is called competence and can be induced chemically or electrically. (39,55,56)

Hanahan's method to induce *E. coli* competence involves a chemical process followed by a heat-shock which allows transformation. (54,57) Briefly, cells are incubated on ice with different concentrations of cations and dimethyl sulfoxide (DMSO) that bind to negatively charged plasmid DNA and negatively charged lipopolysaccharides (LPS) from the bacteria's inner core, causing the migration of the genetic material to the intracellular space. The following heat shock of 42 ºC for 45 seconds improves DNA uptake. (54,57)

L. lactis cell membrane is thicker, therefore electroporation is necessary to weaken the cell wall and allow the DNA uptake. An electric field flows through the sample, which has to be free of compounds that might interfere with the pulse. In this work acquired cells were already competent free of charged interferants. The high voltage pulse depolarizes the cell membrane and creates pores through which the DNA is then transferred. (58)

3.2.1.1. *E. coli* Transformation

Purchased competent *E. coli* cells (stored at -80 °C) were thawed on ice. 2 µL of thawed plasmid DNA was added to 50 µL of cells, the cells were incubated on ice for 30 minutes following heat-shock at 42 ºC for 45 seconds. The transformed bacteria were incubated on ice for 3 minutes, 450 µL of SOC media was added to bacteria and they were incubated at 37 °C for 45 minutes with shaking to stabilize the cell walls. After incubation, the culture was spread onto LBA solid media agar plates under aseptic conditions and incubated at 37 °C for 1 day.

3.2.1.2. *L. lactis* Transformation

Mixtures of 2 µL of plasmid DNA and 50 µL of purchased competent bacteria were pipetted into electroporation cuvettes. Electroporation was performed at 2 kV, 200 Ω and 25 µF. Thereafter, 1 mL of liquid medium SGM17 + $MgCl_2$ + $CaCl_2$ was added to stabilize cell walls and increase transformation efficacy, and the entire contents were pipetted into microcentrifuges, which were incubated for 1.5 hours at 30 ºC without shaking. The cells were vortexed and spread onto GM17C10 agar plates, under aseptic conditions, and incubated for two days at 30 °C.

3.2.2. Overnight Cultures

Bacterial colonies grown on solid media were transferred to liquid media to isolate and amplify a bacterial clone with identical genetic material. A larger number of bacteria with the desired plasmid, was obtained.

3.2.2.1. *E. coli* Overnight Cultures: experimental work

Three colonies were selected with a specific gene construct. Under aseptic conditions, 5 mL of LBA liquid medium and 5 µL of ampicillin were pipetted into sterile tubes. A single colony was then transferred from the solid culture medium by touching the colony with a sterile toothpick held with tweezers sterilized with flame and dropping it in the tube. The tubes were incubated overnight shaking at 37 ºC. Since the plasmid carries the ampicillin resistance gene, the colonies that are left growing are the ones that were transformed with the plasmid.

3.2.2.2. *L. lactis* Overnight Cultures: experimental work

Under aseptic conditions (Bunsen burner or laminar air flow chamber) 5 mL of GM17 media was added into sterile tubes, with the selection antibiotic (2 µL of Cm). A single colony was transferred from the solid culture medium by touching the colony with a sterile toothpick held with tweezers sterilized by flame and dropping it into the tube. The tubes were mixed with a vortex shaker and incubated overnight at 30 ºC.

3.2.3. Plasmid DNA Isolation

After the replication of colonies in overnight cultures, plasmids were isolated from bacteria according to the protocol from the "NucleoSpin ® Plasmid DNA Purification" kit (59), which was adapted for *L. lactis*. Overnight cultures were poured into centrifuge tubes and centrifuged for 10 minutes at 4.600 rpm. After centrifugation, the sediments were resuspended in 150 µL of buffer A1 and the suspensions were transferred to microtubes. In *L. lactis* cultures 50 µL of a mixture of lysozyme and mutanolysin was then added to each mixture and incubated with shaking (650 rpm) for 30 minutes at 37 ºC to degrade the bacterial cell walls. The assay was then performed according to the supplier protocol with some minor changes namely the replacement of AE buffer for plasmid DNA elution by 50 µL of MiliQ water heated to 70 ºC. After incubation for 5 minutes at room temperature, the tubes were centrifuged for 2.5 minutes (11.000 rpm).

The concentrations of the isolated plasmids were measured spectrophotometrically with the NanoDrop device and the *L. lactis* isolated plasmids were sent for sequencing (Eurofins Genomics, Germany). The purified plasmids were diluted to a final volume of 5 µL in a concentration of 50 – 100 ng/µL. 5 µL of primers in a concentration of 5 µmol/µL were added to samples for sequencing.

The obtained sequences of the constructs were processed with the BioEdit computer software, and they were aligned with the expected sequences on the website "Clustal Omega". The gene constructs with the appropriate nucleotide sequence were later expressed in *L. lactis*.

3.2.4. Polymerase Chain Reaction (PCR)

The PCR technique is used to amplify DNA rapidly and specifically. A thermostable DNA polymerase amplifies a specific segment of a DNA template in repeated cycles of precise temperature changes. First, the reaction mixture is heated to reversibly denature the DNA to a single-stranded DNA chain (denaturation step). The temperature is then lowered to melting temperature (T_m) to allow the oligonucleotide primers align to the single-stranded DNA (annealing step). The mixture is then heated to 72°C to allow complementary chain elongation by thermostable DNA polymerase (elongation step). The reaction mixture requires DNA, two starting oligonucleotides, a thermostable DNA polymerase and four deoxynucleotides (dNTPs) in a reaction buffer containing magnesium ions.

3.2.4.1. KOD Hot Start DNA PCR

Hot Start (Sigma-Aldrich) DNA polymerase (Merck Millipore, Burlington, MA, USA) was used to amplify the pNZ8148 plasmid samples obtained from *E. coli*. The PCR media contains a KOD DNA Polymerase complex and two monoclonal antibodies that inhibit the activities of 3'→5' exonuclease and DNA polymerase at room temperature, reducing the occurrence of non-specific amplification and avoiding mispriming events that might occur in the initial temperature increase. (60)

The PCR assay mixture is presented in Table 3 and was performed in a final volume of 50 μL according to the manufacturer instructions.

Table 3 - KOD Hot Start DNA PCR assay mixture.

	Gliadin WT	**Gliadin MT**	**Final Concentration**
KOD buffer (10X)	5 µL	5 µL	1X
$MgSO_4$ (25 mM)	3 µL	3 µL	1.5 mM
dNTPs (2 mM)	5 µL	5 µL	0.2 mM
KOD Hot Start Polymerase (1 U/µL)	1 µL	1 µL	0.02 U/µL
Forward Gliadin WT Primer (5 µM)	3 µL	-	0.3 µM
Forward Gliadin MT Primer (5 µM)	-	3 µL	0.3 µM
Reverse Gliadin WT Primer (5 µM)	3 µL	-	0.3 µM
Reverse Gliadin MT Primer (5 µM)	-	3 µL	0.3 µM
E. coli Gliadin WT plasmid (4.69 ng/µL)	1 µL	-	0.094 ng/µL
E. coli Gliadin MT plasmid (3.60 ng/µL)	-	1 µL	0.072 ng/µL
PCR grade water	20 µL	20 µL	

Using the OligoAnalyzer™ tool (IDT, USA) the different primers' melting temperatures (T_m) were determined (Table 1) according to their plasmid's undisclosed nucleotide sequence and the respective primers' size in bp. These data were used to design the PCR amplification protocol (presented in Table 4), according to the manufacturer's instructions for plasmids with DNA lengths between 500-1000 bp. Annealing conditions took into consideration the lowest primer T_m (Table 1; 57.5 ºC).

Table 4 – Amplification conditions (temperature, time and number of cycles) using KOD Hot Start DNA PCR.

Step	**Temperature and Time**
1. Polymerase Activation	95 ºC for 2 minutes
2. Denaturation	95 ºC for 20 seconds
3. Annealing	57.5 ºC for 10 seconds
4. Extension	70 ºC for 15 seconds
Steps 2-4	30 cycles

3.2.4.2. Taq Polymerase Colony PCR

PCR for colony analysis is used to determine the presence of a gene insert in the plasmid used to transform the bacteria. In this work after two days of incubation, twenty colonies with a specific gene construct were selected and analysed by PCR. The composition of the reaction mixtures is shown in Table 5.

Table 5 – Taq polymerase colony PCR assay mixture.

	Gliadin WT	**Gliadin MT**
Gliadin WT Forward primer (5 μM)	1 μL	-
Gliadin MT Forward primer (5 μM)	-	1 μL
pNZ8148 reverse primer	1 μL	1 μL
Taq Green Dream buffer	3 μL	3 μL
dNTPs	5 μL	5 μL
Milli-Q water	15 μL	15 μL
Taq solution* (0.1 μL of Taq Polymerase and 4.9 μL of Milli-Q water)	5 μL	5 μL
Final Volume	25 μL	25 μL

Note: *added after colony pick and first step denaturation and annealing (see Table 6).

Once all the components, except the Taq DNA polymerase, were inside the PCR tubes, the selected colonies were picked under sterile conditions and mixed thoroughly (five times pipetting) with the assay reaction. The PCR was then performed as described in Table 6, taking into consideration that the DNA Taq polymerase (5 μL) is added to each PCR tube, after the first denaturation step (step 1.1: 99 ºC) when the PCR system reaches 50 ºC.

Table 6 – Colony amplification conditions (temperature, time and number of cycles) using DreamTaq DNA polymerase.

Step	Temperature and Time
1. Initial step	**1.1 Denaturation:** 99 ºC for 10 minutes, after which 5 µL of Taq mixture is added **1.2 Annealing:** 50 ºC for 2 minutes
2. Denaturation	94 ºC for 30 seconds
3. Annealing	46 ºC for 1 minute
4. Extension	72 ºC for 1 minute
Steps 2-4	**30 cycles**
5. Final Extension	72 ºC for 5 minutes
6. End of Reaction	4 ºC

3.2.5. Agarose Gel Electrophoresis (AGE) and DNA Extraction from the Gel

An agarose gel is a porous matrix in which negatively charged DNA molecules migrate from a negative electrode to a positively charged anode, depending on their size and charge ratio. The nucleic acid separates and moves horizontally on the agarose gel placed in a buffer. Smaller molecules pass through the pores faster and consequently migrate more.

To detect double-stranded DNA molecules, fluorescent dyes are used to intercalate between the base pairs of the nucleic acids, and the fluorescence of the dye is detected by UV radiation. The samples are loaded into the gel's deposition buffer to minimize sample loss. The samples contain a dye that passes through the agarose gel at the same rate as short nucleic acids, allowing electrophoresis to be followed by the naked eye.

AGE was performed to separate PCR products for further isolation and detection of plasmids prepared from colony-based PCR, analyse restriction products for further use, and confirm plasmid composition.

To prepare the agarose gel, 0.5 mg of agarose was weighed in an Erlenmeyer flask, dissolved in 50 mL of TAE (Tris-Acetate EDTA buffer), and heated in a microwave oven. After cooling to 60 ºC, 5 µL of SYBR safe DNA dye was added. The dye binds to the DNA

molecules, which later allows detection of the DNA products under UV light. The solution was poured into the electrophoresis container and the comb was inserted.

After the gel solidified, the container was filled with TAE buffer and each well was filled with 50 µL of PCR products. Prior to loading, 10 µL of Loading dye was added to the products to observe the progression of electrophoresis - the dye moves along with the smallest DNA strands.

12 µL of the previously prepared marker was pipetted into one of the wells (1 µL of 100 bp size marker geneRuler™, 2 µL of Loading Dye, 9 µL of dH_2O). The GeneRuler is a marker that consists of several different sections of DNA with different known lengths that migrate during electrophoresis.

Electrophoresis was performed at 45 minutes at 95 V. The gel was removed, and the DNA strands were visualized under UV light. PCR products were then excised from the gel using a sterile scalpel under the Visiblue transilluminator.

Gene constructs were isolated and purified according to the protocol of the "NucleoSpin® Gel and PCR Clean-up" kit. In the DNA elution step, 20 µL of dH_2O heated to 70 ºC was added, instead of NT3 buffer, followed by incubation at room temperature for 5 minutes and centrifugation (11.000 x *g* for 2.5 minutes). The concentrations of the isolated constructs were measured with a NanoDrop spectrophotometer.

3.2.6. DNA digestion

Restriction endonucleases are enzymes that cleave DNA at a specific site, a nucleotide sequence called a palindrome. They were discovered in bacteria, where they prevent viruses from growing, by cutting their DNA.

Four reaction setups were prepared. Two contained the plasmid pNZ8148 (P1 and P2) as a vector, and the other two contained different PCR constructs, as shown on table 7.

Table 7 – Reaction setup for digestion of plasmid pNZ8148 (P1 and P2), Gliadin WT and Gliadin MT using NcoI and XbaI.

	pNZ8148 (P1 and P2)	**Gliadin WT**	**Gliadin MT**
NcoI	2 µL	2 µL	2 µL
XbaI	2 µL	2 µL	2 µL
plasmid pNZ8148	9 µL	-	-
Gliadin WT	-	14 µL	-
Gliadin MT	-	-	14 µL
FD Buffer (10X)	5 µL	5 µL	5 µL
Milli-Q Water	32 µL	27 µL	27 µL
Final Volume	50 µL	50 µL	50 µL

Microtubes containing the mixtures presented in Table 7 were incubated for 30 minutes at 37 ºC, where the process of hydrolysing the PCR constructs and plasmids in places with the correspondent restriction sequences for NcoI and XbaI happened.

According to the procedure from 3.2.5, the DNA segments obtained from endonuclease restriction assays were separated by size and charge with an AGE and purified from the gel. This step not only confirms that the sequences were digested correctly (expected size), but also allows the isolation of specific DNA sequences from the gel and their purification and further concentration measurement with a NanoDrop spectrophotometer. The appropriate fragments were later used for ligation.

3.2.7. Ligation

In recombinant DNA technology, ligation is the process of joining compatible ends of DNA fragments previously digested with restriction enzymes. Ligation is performed by ATP-dependent DNA ligase enzymes that catalyse the formation of phosphodiester bonds between the adjacent 5'-phosphate of one nucleotide and the 3'-hydroxyl group of another nucleotide. The composition of the reaction assembly was determined using an online ligation calculator (insilico, University of Duesseldorf).

Plasmid pNZ8148 (vector), insert WT and insert MT were previously digested with two enzymes, NcoI and XbaI, resulting in two different sticky ends, single-stranded DNA overhangs, matching to other DNA fragments that were hydrolysed. During ligation, the gene constructs were inserted into the plasmid in a ratio of 3:1 (insert:vector), resulting in the recombinant plasmid.

Ligation reactions were placed on ice in Eppendorf tubes, and T4 ligase was added last, as it is an enzyme, and therefore sensitive to degradation. Insert size (943 bp), vector size (approximately 3600 bp) and vector mass (in ng, calculated from the concentration of the isolated linearized plasmid) were entered into the "Ligation calculator" to predict the needed volumes for ligation mixtures.

Table 8 shows the composition of the reaction mixtures for the two ligation assays.

Table 8 – Reaction setup for the ligation.

	Gliadin WT	**Gliadin MT**
WT Insert (13.5 ng/µL)	8 µL	-
MT Insert (14.2 ng/µL)	-	7 µL
Vector pNZ8148 P1 (8.4 ng/µL)	16 µL	-
Vector pNZ8148 P2 (7.6 ng/µL)	-	16 µL
T4 DNA Ligase Buffer	3 µL	3 µL
T4 Ligase	1 µL	1 µL
Milli-Q Water	2 µL	3 µL
Total Volume	30 µL	30 µL

To obtain cohesive (sticky) ends, samples were incubated overnight at 16 °C to isolate the recombinant plasmid the following day, when 70 µL of NTI solution was added to them and the plasmids were isolated according to procedure 3.2.3. The isolated plasmid solutions were then concentrated from 20 µL to 2 µL (10 minutes at 30 ºC on a centrifugal concentrator).

3.2.8. NICE Protein Expression

Protein expression in *L. lactis* strain NZ9000 was induced using the NICE system, as the recombinant plasmid contains the Pnis promoter that triggers the expression of the insert gene in the presence of a subinhibitory concentration of nisin.

L. lactis were transformed with recombinant plasmids, for which the nucleotide sequences were confirmed, and an empty plasmid was used for negative control (pNZ8148). After two days, overnight cultures were prepared according to the procedure from 3.2.2.2 and incubated at 30 ºC for approximately 24 hours.

Expression was carried out in sterile tubes, each containing 10 mL of GM17 liquid medium, 4 µL of Cm and 200 µL of a bacterial culture (1:50 dilution). Optical density at 600nm (OD_{600}) was measured with a NanoDrop spectrophotometer to monitor growth.

The mixtures were incubated for approximately 4 hours at 30 ºC until bacteria reached an OD_{600} of 0.4-0.5 – even though *L. lactis* usually grows up to higher OD_{600} values in the above period. Then 10 µL of nisin (20 ng/µL) was added to the tubes and incubation proceed for about 3 to 4 hours - until OD_{600} was 3.5–4.0.

The cultures were transferred to 15 mL tubes and centrifuged at 4 ºC (10 minutes, 4.500 x *g*). The obtained pellets were resuspended in 400 µL of PBS buffer and stored overnight at -20 ºC to perform SDS-PAGE in the following day.

For the IMAC purification, four sterile tubes contained 200 mL of GM17, 4 mL of the bacterial culture and 80 µL of Cm, were subject to the expression conditions described above. The cultures were transferred to 50 mL tubes and centrifuged at 4 ºC (20 minutes, 5.000 x *g*). The pellets were resuspended in 15 mL of equilibrium buffer in 50 mL Falcon tubes and stored overnight in at 4 ºC. Gliadin WT and MT were sonicated two times for 15 seconds the next day to obtain the cell lysates. 200 µL of each cell lysate were stores in a microtube at 0-4 ºC for further SDS-PAGE analysis.

3.2.9. IMAC Purification

Recombinant proteins contain an affinity tag fused to the N- or C-terminus to enable affinity purification and detection. The affinity tag used was His-tag, consisting of six or more consecutive histidine (His) residues. This His-tag peptide has usually does not affect folding, and rarely interferes with the structure or function of the recombinant protein. (61)

This tag is used since some amino acids, like histidine, can bind around metals through noncovalent bonds, resulting in a chelating effect. (62) In this technique, proteins with an affinity for metal ions are retained in a gravity flow column, that contains an immobilized resin with metal ions, the chromatographic matrix. (61). Proteins that bind to the resin are then eluted using an elution buffer containing imidazole (Annex, Table C).

Proteins containing the His-tag were isolated with BD Talon metal affinity resin (BD Biosciences), using gravity-flow column purification and imidazole elution according to the manufacturer's instructions.

Samples obtained from procedure 3.2.8. were thawed on ice, and the suspension was centrifuged in a 50 mL centrifuge (round bottom tubes rotor) at 15.000 × *g* for 20 minutes at 4 ºC. 200 µL of each bacterial culture lysate supernatant was stored at 0–4 °C to prevent protein degradation for further SDS-PAGE analysis. The remaining was stored to be mixed with the resin.

Two batches of the BD TALON Resin, one for Gliadin WT, and one for Gliadin MT, were resuspended, and 1.5 mL (2-times the bed volume of 0.75 mL) were immediately transferred to a sterile tube to be centrifuged at 700 x *g* for 2 minutes to pellet the resin.

The supernatant was discarded and 7.5 mL (10 bed volumes) of the IMAC equilibration buffer (Annex, Table C) was briefly mixed to pre-equilibrate the resin, and then re-centrifuged at 700 x *g* for 2 minutes to pellet the resin and discarding the supernatant. This step was performed twice, and the final supernatant was not discarded.

The supernatant lysates were added to the resin tubes, and they were put on a gentle mixer for 20 minutes at 100 rpm, and centrifuged at 700 x *g* for 5 minutes. 100 µL of each of the sample's unbound supernatant was transferred to a tube for further SDS-PAGE analysis.

The remaining supernatant was removed carefully (to avoid disruption of the resin pellet). The resin was washed by adding 11 mL of equilibrium wash buffer (approximately 11 times the bed volume) in a soft mixer for 10 min to promote thorough washing.

The mixtures were centrifuged at 700 x *g* for 5 minutes and 100 µL of the supernatant was stored for SDS-PAGE (first wash) before being discarded. This step was repeated, and the second wash was stored for SDS-PAGE analysis.

One bed volume (0.75 mL) of IMAC equilibrium buffer was added to the resin to resuspend it gently and transfer it to the gravity-flow column with the end-cap in place, until the resin settled. The end-cap was removed so the buffer would drain until it reached the top of the resin bed making sure no air bubbles were trapped.

The columns were washed 6 times with 5 bed volumes (3.75 mL) of the IMAC equilibrium buffer each time and the fractions were saved for SDS-PAGE analysis. (W1-W6) During the washing steps, the UV absorption at 280 nm (A_{280}) was monitored spectrophotometrically to confirm the washes were being successful (A_{280} of approximately 0) and confirming that they did not contain any protein traces.

Similar to the washing step, elution was performed until A_{280} in the obtained fractions was approximately 0, indicating that recombinant protein bound to the resin was completely eluted. To perform the elution 5 bed volumes (3.75 mL) of the IMAC elution buffer (Annex, Table C) were added to the column and the eluate was collected in 375 µl fractions on ice, to prevent protein degradation. For Gliadin WT 10 elution steps (E1-E10) were performed while for Gliadin MT 11 elution steps were necessary (E1-E11), until A_{280} was close to 0.

All collected samples (elution, and wash fractions) were stored at 4 ºC to further SDS-PAGE analysis.

3.2.10. SDS-PAGE

Polyacrylamide gel electrophoresis (PAGE) is the most commonly used method for separating proteins based solely on their size under an electric field, since the charge-to-length ratio of proteins is constant in the presence of sodium dodecyl sulphate (SDS). Separation occurs in the presence of SDS, an anionic surfactant that binds non-specifically to the proteins, that besides denature them also confers a negative charge. (63)

Electrophoretic separation occurs vertically (Figure 6) from the negatively charged cathode at the top to the positively charged anode at the bottom in a discontinuous system consisting of two differently cross-linked gels – the upper stacking gel (concentration) and the lower resolving gel (separation).

The polyacrylamide gel consists of a polymer of acrylamide cross-linked with N,N'-methylenebisacrylamide molecules. The porosity of the gel depends on the degree of cross-linking, with a higher degree of cross-linking resulting in a smaller pore size.

The stacking gel has a lower degree of cross-linking than the resolving gel and does not prevent the molecules from moving, so all the proteins are concentrated in it and do not separate. This prevents band broadening and poor resolution during electrophoresis.

The separated proteins can be detected by appropriate staining (e.g. Coomassie Brilliant Blue dye) or by immunological methods (Western Blot). To avoid staining of the gel, SDS-PAGE can also be performed with "stain-free technology" using a polyacrylamide gel with trihaloalkanes that covalently bind to the protein’s tryptophan residues and enhance their fluorescence under UV light.

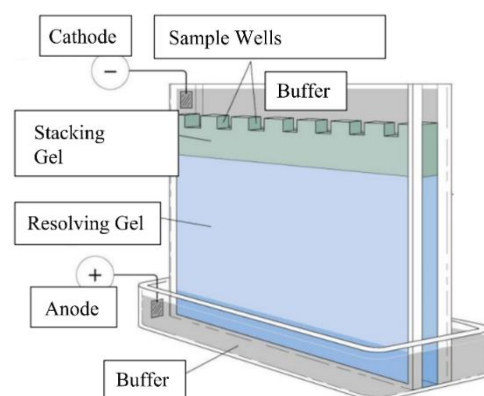

Figure 6 – Illustration of SDS-PAGE. Adapted from (69).

SDS-PAGE was performed according to the protocol from the "TGX and TGX Stain Free Fast Cast acrylamide 12%" kit (64), using the volumes described in Table 9. Ammonium persulfate (APS) and N,N,N′,N′ -tetramethylethylenediamine (TEMED) were added just before casting the gel, as they initiate crosslinking of the gel. The resolving gel was poured into the casting mould, followed immediately by the stacking gel. The comb was then inserted to form the wells. After gel polymerization (30 – 45 minutes), the comb was removed and the slides with the gel were transferred to the SDS-PAGE electrophoretic system, which was filled with 10x SDS buffer.

Table 9 – Composition of stacking and resolving gel for SDS-PAGE (for 1 gel, 1 mm thick).

Stacking Gel		**Resolving Gel**	
Component	**Volume**	**Component**	**Volume**
Stacking A	1 mL	Resolving A	3 mL
Stacking B	1 mL	Resolving B	3 mL
TEMED	2 µL	TEMED	3 µL
10% APS	10 µL	10% APS	30 µL

Note: (TEMED) N,N,N′,N′ -Tetramethylethylenediamine; (APS) Ammonium persulfate.

Samples (cell lysates of pNZ8148, WT and MT) with the highest concentration of cell lysate (highest OD_{600} in 3.2.8.) were analysed by SDS-PAGE. To this end, DTT, sample buffer and Milli-Q water was added according to the volumes indicated in Table 10.

Table 10 – Reaction mixture for sample loading on SDS-PAGE.

	pNZ8148	**WT**	**MT**
Cell lysate	25 µL	45 µL	35 µL
Sample buffer (loading dye)	12.5 µL	12.5 µL	12.5 µL
DTT	2.5 µL	2.5 µL	2.5 µL
Milli-Q Water	5 µL	-	-
Final volume	45 µL	60 µL	50 µL

Before loading, all prepared mixtures were heated for 10 minutes at 100 ºC to denature the proteins. 15 µL of samples and 5 µL of the SDS-PAGE molecular mass marker were loaded into the gel wells. Electrophoresis was performed at 200 V. After 30 min, the gel was removed from the slides and activated under UV light with the ChemiDoc system.

3.2.11. Coomassie Brilliant Blue (CBB)

Coomassie brilliant blue dyes are commonly used to stain proteins in SDS-PAGE gels.

CBB has sulfuric acid groups that have a negative charge (anionic) under acidic conditions and therefore bind (reversibly) to positively charged protein amino acid residues (such as histidine, lysine and arginine) by electrostatic interactions. The acidic conditions also allow to fix the proteins on the gel, to avoid diffusion onto the gel. Uncharged protein moieties (e.g., aromatic rings present in phenylalanine) can also bind to CBB through weaker "Van der Waals" interactions.

The gels are soaked in dye, and the excess dye is eluted with a destaining solution. This procedure allows visualization of the proteins as blue bands on the clear gel.

After SDS-PAGE electrophoresis 40 mL of CBB and 20 mL of acetic acid were mixed in a closed container and gel was emersed in this solution for 1 hour with shaking.

For the destaining step, approximately 60 mL of the 30% destaining solution was added to the gel after the previous staining solution was discarded. The gel was shaken for 30 minutes.

Finally, the gel was left on the shaker overnight with approximately 60 mL of the 10% destaining solution.

3.2.12. Western Blot

Western blot is an immunochemical detection method where antibodies against a specific protein are used to identify proteins previously separated by polyacrylamide gel electrophoresis. However, first the proteins must be transferred from the polyacrylamide gel to a nitrocellulose or nylon membrane (where it is immobilized) to avoid protein diffusion from the gel during antibody-antigen incubation. The transfer takes place either under the influence of electric current by cross electrophoresis, or under the influence of capillarity by passive diffusion.

After transfer, the membrane must be incubated in a blocking agent solution (e.g., milk, bovine serum albumin) to occupy the entire surface of the membrane and prevent nonspecific binding of the antibodies used to label the proteins.

The transfer is followed by an incubation step in a solution of the selected primary antibody directed against a specific protein. The second incubation solution contains a secondary antibody that binds to the Fc region of the primary antibodies and can be detected by imaging techniques.

In this work, a gel-sized nitrocellulose membrane was cut and soaked in Western Blot transfer buffer, as well as two filter pads that served as a buffer reservoir during transfer. The first filter pad was placed in the transfer cassette, followed by the nitrocellulose membrane, the activated polyacrylamide gel and the second filter pad (order from anode to cathode). During this step, all trapped air bubbles were removed to avoid insufficient transfer.

The sealed cassette was placed in a semi-dry protein transfer device (Biorad), and the program was started (2.5 A, 25 V, 3 minutes). To confirm that the transfer was efficient, the membrane was checked with a ChemiDoc device.

After the transfer, the membrane was incubated in 5% skim milk in TBST buffer for 1 hour, followed by incubation in a solution of primary mouse antibodies (against His-tag) in a blocking agent (5% skim milk in TBST), diluted 1:2000 with shaking overnight at 4 ºC.

The next day, the membrane was washed three times with TBST buffer for 10 minutes with shaking, and then incubated with fluorescently labelled secondary goat anti-mouse antibody (1:5000) for 1.5 hours. This was followed by washing again with TBST three times for 10 minutes each, and fluorescence was measured using a ChemiDoc imaging system.

4. Results and discussion

4.1. Confirmation of gene construct's suitability

Gliadin WT and gliadin MT gene constructs were amplified using the KOD Hot Start PCR polymerase. The sizes of the amplification products were confirmed by AGE (Figure 7) after digestion with NcoI and XbaI, since both Gliadin WT and Gliadin MT contain these restriction sites.

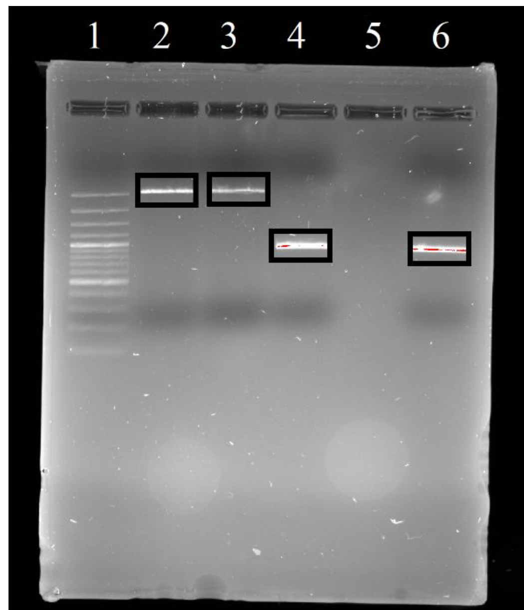

Figure 7 - Agarose gel electrophoresis analysis of PCR amplification of gliadin WT and gliadin MT gene constructs (1) 100 bp size marker GeneRuler™; (2) Plasmid pNZ8148 construct P1; (3) Plasmid pNZ8148 construct P2; (4) Gliadin WT gene construct; (5) Negative Control, containing the mixture for the PCR reaction without bacteria added to it; (6) Gliadin MT gene construct. Constructs (2), (3), (4), and (6) were digested with NcoI and XbaI restriction enzymes. Electrophoretic conditions: 1% agarose, 95 V, 45 minutes.

The comparative analysis of samples with the size marker (Figure 7, lane 1) allowed to confirm the expected size of the products: 3600 bp for plasmid pNZ8148 (Figure 7; lanes 2 and 3); and 942 bp for the gene constructs (Figure 7; lanes 4 and 6). These two PCR products were isolated from the gel as well as plasmid pNZ8148 that was previously digested with the same combination of restriction endonucleases. The purified constructs were then inserted into the corresponding plasmid by ligation.

After ligation, the recombinant plasmids were used to transform the competent *L. lactis* bacteria. Next the bacteria grew on agar plates and some colonies were checked by PCR, where the products obtained were separated with AGE to determine which of the colonies contained the recombinant gene. As shown in Figure 8, several colonies were obtained where the correct plasmid construct were observed (approximately 1260 bp).

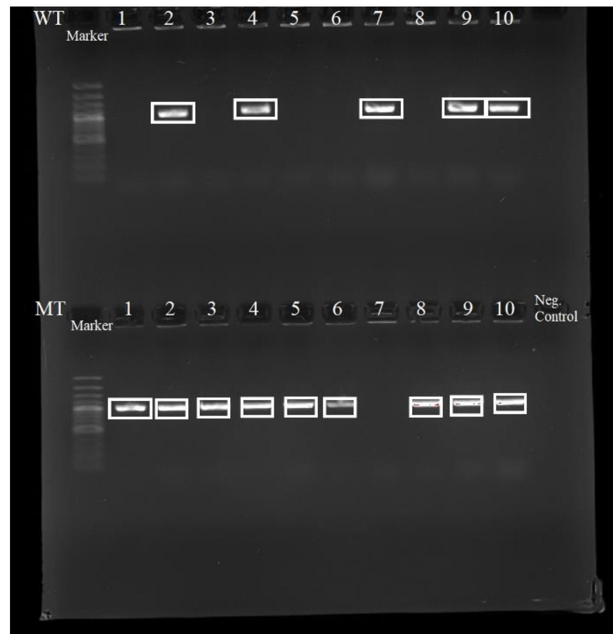

Figure 8 - Agarose gel electrophoresis analysis of *L. lactis* transformed colonies amplification pNZ8148-Gliadin WT recombinant plasmids: lanes 1-10, upper panel; pNZ8148-Gliadin MT recombinant plasmids: lanes 1-10, lower panel. The negative control (PCR reaction mixture without colony) is shown in the last lane on the lower panel. Electrophoresis conditions: 1% agarose, 95 V, 45 minutes.

Plasmids were then isolated from the overnight cultures of the colonies and nucleotide sequencing was performed (Eurofins Genomics, Germany), using the samples with the highest DNA concentration (determined with NanoDrop) namely: pNZ8148-Gliadin WT 4, 7, 9 and pNZ8148-Gliadin MT 1, 3, 4.

Analysis of the DNA sequences was done by the BioEdit software (Manchester, UK), and revealed that sequences pNZ-Gliadin WT 7 and pNZ-Gliadin MT 4 had little background noise. Using Clustal Omega (Cambridge, UK), alignment of the sample's sequences to their respective described sequences showed that all isolated plasmids did not present any mutation, thus corresponding to the expected sequence/construct (Figure 9).

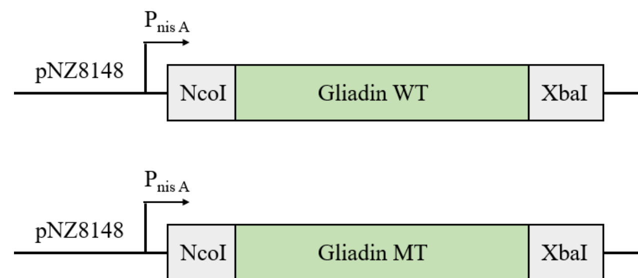

Figure 9 - Schematic representation of gene constructs in the recombinant plasmid. Upper panel pNZ8148-Gliadin WT and lower panel pNZ8148-Gliadin MT.

4.2. Detection of Expressed Gliadin Proteins

L. lactis bacteria were transformed with the isolated plasmids namely: pNZ8148-Gliadin WT, pNZ8148-Gliadin MT, and pNZ8148 (as a negative control). Expression of recombinant proteins was induced with nisin. During expression, bacteria did not reach the desired OD_{600} values. Interestingly, pNZ8148-Gliadin WT transformed bacteria grew slower than pNZ8148-Gliadin MT transformed bacteria.

After expression, cell lysates were prepared from the bacterial cultures and the proteins were separated by SDS-PAGE. The separated proteins were detected under UV light in the ChemiDoc device, to check the success of the expression of Gliadin WT and Gliadin MT in *L. lactis* cells (Figure 10).

The activated SDS-PAGE gel revealed that it was possible to recover the bacterial proteins, and we hypothesise that pNZ8148-Gliadin MT produced more total protein based on the darker bands that we obtained after the analysis. However, and probably due to the low

level of expression it was not possible to detect a higher content of gliadin (expected molecular mass of 35 kDa.

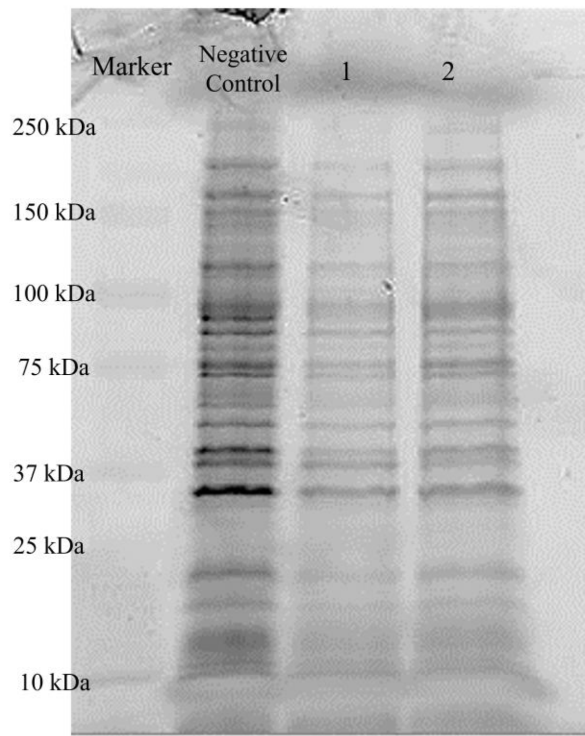

Figure 10 - SDS-PAGE of cell lysates. Marker Precision Plus Protein All Blue Standard. Cell lysates of *L. lactis* bacteria transformed with plasmid pNZ8148 served as a negative control. (1) Cell lysates of *L. lactis* bacteria transformed with recombinant plasmid pNZ8148-Gliadin WT. (2) Cell lysates of *L. lactis* bacteria transformed with recombinant plasmid pNZ8148-Gliadin MT. SDS-PAGE conditions: 200 V, 45 minutes.

Proteins were then transferred from the gel to a nitrocellulose membrane for Western Blot analysis. The membrane was incubated in a solution of primary mouse anti-His-tag antibodies, washed with TBST, and incubated in a solution of secondary goat anti-mouse antibodies conjugated with a fluorescent dye. After washing, the membrane was imaged with the ChemiDoc system and gliadin proteins were detected (Figure 11).

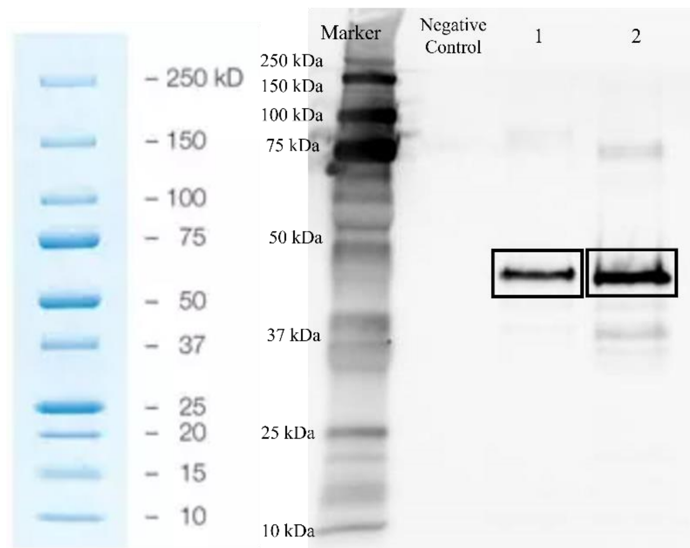

Figure 11 –Western Blot results. Marker Precision Plus Protein All Blue Standards was used. Cell lysates of *L. lactis* bacteria transformed with plasmid pNZ8148 served as a negative control. (1) Cell lysates of *L. lactis* bacteria transformed with recombinant plasmid pNZ8148-Gliadin WT. (2) Cell lysates of *L. lactis* bacteria transformed with recombinant plasmid pNZ8148-Gliadin MT. Marked spots (1 - 2) represent Gliadin WT and Gliadin MT proteins, respectively.

On both cell lysates from plasmid pNZ8148-Gliadin WT and plasmid pNZ8148-Gliadin MT (Figure 11 lane 1 and 2) faded bands with different molecular masses (MM) were detected. For gliadin WT, MM of approximately 90, 48 and 35 kDa were observed, while for the gliadin MT the observed bands presented with MM of approximately 75, 48, 35 and 30 kDa. As gliadin has an expected MM of 35 kDa we can hypothesize that bands with lower MM can result from protein degradation, while bands with higher MM could be the result of the assembly of oligomers (dimers, etc) through disulphide bonds, for example.

Prestained protein molecular weight markers might have variations in protein electromobility that change the resolution of a protein band, leading to incorrect molecular weight determinations. We would have to repeat the experiment using a protein with the same size as our interest protein to rule this hypothesis. (65)

Another SDS-PAGE analysis was performed to analyse staining of the proteins by Coomassie brilliant blue. The gel was imaged with the ChemiDoc (Figure 12).

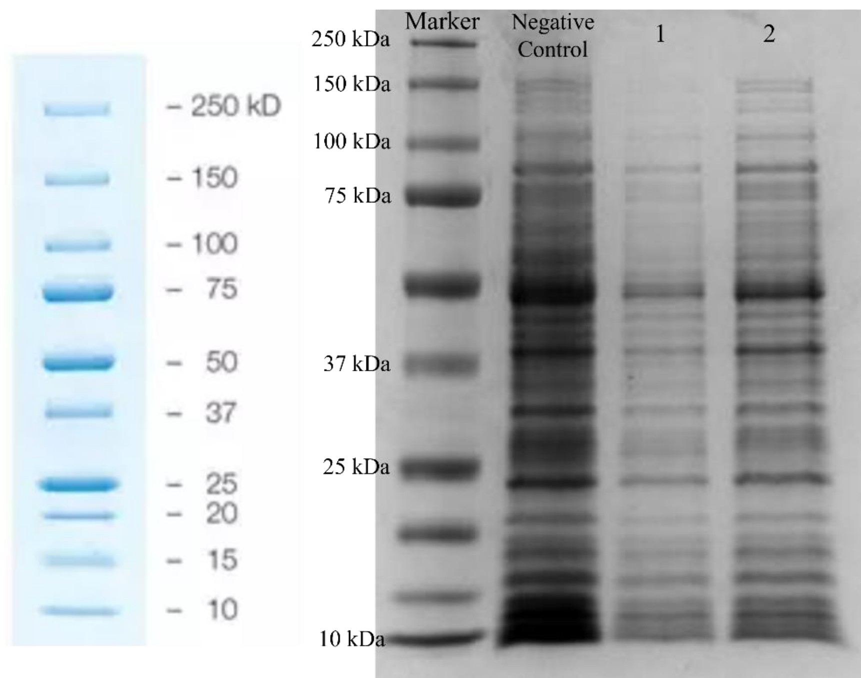

Figure 12 – Polypeptide profile of cell lysates. Marker Precision Plus Protein All Blue Standards was used. Cell lysates of *L. lactis* bacteria transformed with plasmid pNZ8148 served as a negative control. (1) Cell lysates of *L. lactis* bacteria transformed with recombinant plasmid pNZ8148-Gliadin WT. (2) Cell lysates of *L. lactis* bacteria transformed with recombinant plasmid pNZ8148-Gliadin MT. Gels were stained with Coomassie brilliant blue solution.

Gliadin MT proteins for IMAC purification and later SDS-PAGE analysis (Figures 13-A and 14-A).

Elution and wash fractions were monitored for protein concentration by measuring concentration values with Nanodrop, where negative values indicate the absence of protein, as seen in figures 13-B and 14-B (Full table in annex F and G). The fractions were analysed by SDS-PAGE (Figures 13-A and 14-A). We confirmed that pNZ8148-Gliadin MT transformed bacteria produced more recombinant protein.

This experiment was successful in isolating the His-tagged proteins. Total mass of interest protein was calculated for both Gliadin WT and Gliadin MT (Table 11) using the

concentration values measured with Nanodrop (Full table in annex F and G). Bacteria transformed with pNZ8148-Gliadin WT expressed less protein and this was in line with the results obtained during expression.

Table 11 – Total mass of recombinant protein obtained from IMAC.

Interest Protein	Total mass (E1-E10)
Gliadin WT	0.04125 mg
Gliadin MT	0.111375 mg

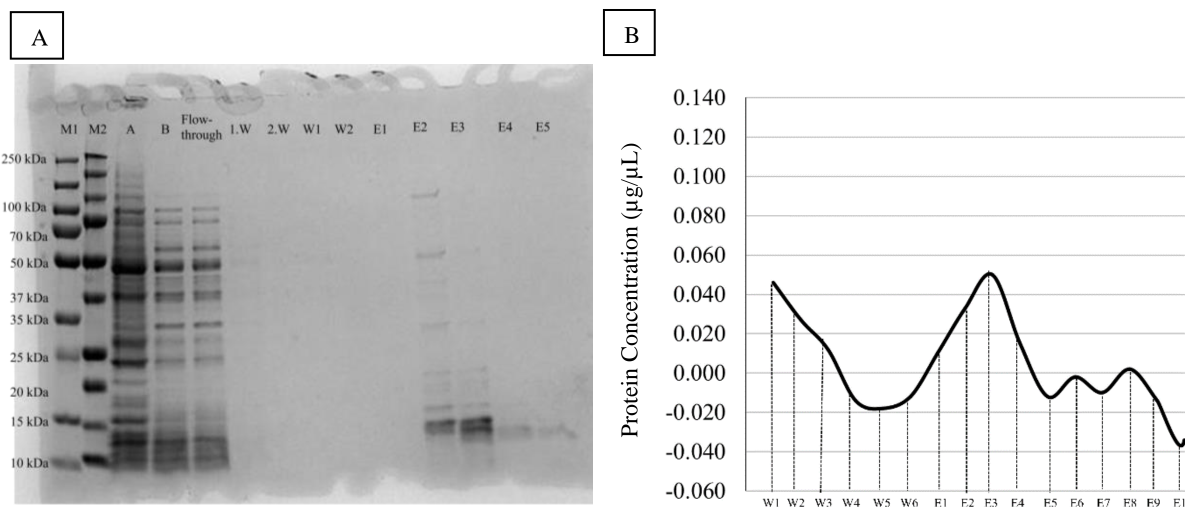

Figure 13 - Purification of pNZ8148-Gliadin WT using IMAC. **(A)** Purification results of the wash and elution fractions; M1 - PageRuler Prestained Protein Ladder Plus marker; M2 - Protein All Blue Standards marker; A- Bacterial culture lysate; B - Bacterial culture lysate supernatant; 1.W and 2.W are the first and second wash; W1-W2 and E1-E5 are wash and elution fractions from the IMAC column. SDS-PAGE conditions: 200 V, 40 minutes. **(B)** Concentration of protein obtained from the purification essay where proteins were monitored using NanoDrop. W1-W6: fractions where the resin was washed until protein concentration was zero. E1-E10: fractions where gliadin WT was eluted. E2 and E3 had the highest concentrations of protein.

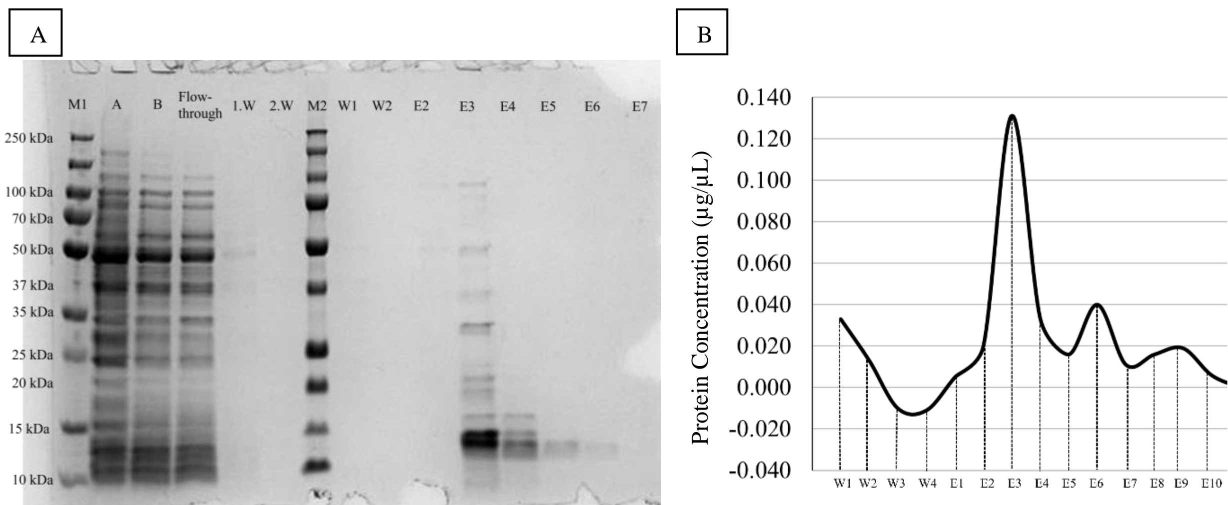

Figure 14 - Purification of pNZ8148-Gliadin MT using IMAC. **(A)** Purification results of the wash and elution fractions; M1 - PageRuler Prestained Protein Ladder Plus marker; M2 - Protein All Blue Standards marker; A- Bacterial culture lysate; B - Bacterial culture lysate supernatant; 1.W and 2.W are the first and second wash; W1-W2 and E2-E7 are wash and elution fractions from the IMAC column. SDS-PAGE conditions: 200 V, 40 minutes. **(B)** Concentration of protein obtained from the purification essay where proteins were monitored using NanoDrop. W1-W4: fractions where the resin was washed until protein concentration was zero. E1-E10: fractions where gliadin MT was eluted. E3 and E4 had the highest concentrations of protein.

The protein of interest should have a molecular weight of 35 KDa, corresponding to its gene length of 942 bp. The multiple bands on the elution fraction of the SDS-PAGE analysis suggest that some of the proteins were not only degraded – represented by the lower kDa bands; but that they might also have formed dimers through disulphide bonds – leading to the higher kDa bands.

5. Conclusions

It is well-known that *Lactococcus lactis* is a safe and well-known bacteria used as a cell factory. In this work, we used it for genetical modification to produce a gliadin protein safe for gluten intolerant patients, where the protein would be less immunogenic.

Bacteria were transformed with the developed recombinant plasmid and the gliadin proteins were successfully expressed using the NICE system. The obtained cell lysates were analysed with SDS-PAGE and western blotting that revealed the presence of the developed protein. Then, the isolated proteins were purified using IMAC, and the fractions were analysed with SDS-PAGE.

The analysis showed that the proteins underwent degradation during the process. Data from the literature suggests that proteins intracellularly expressed in *L. lactis* are prone to degradation. We would have to repeat the analysis to better understand the cause of such degradation.

We noticed that bacteria transformed with the mutated gliadin plasmid grew faster and expressed more proteins in comparison to the negative control and wild type gliadin. This finding still needs confirmation with further analysis of the bacteria growth in more detail. We can only speculate that if the finding would remain, it could mean that the mutated gliadin is more stable.

The data obtained in this work although preliminary, open the doors for the development of a gliadin protein potentially less immunogenic that could constitute an alternative to be used in diet of people suffering from celiac disease, inflammatory bowel disease or gluten-related neurological disorders.

Annex

A. List of enzymes and respective manufacturers.

Enzyme (Function)	Manufacturer
NcoI (restriction of sequence CATGG)	Thermo Fisher (USA)
XbaI (restriction sequence TCTAGA)	Thermo Fisher (USA)
Hot Start DNA polymerase (DNA synthesis)	Thermo Fisher (USA)
Dream Taq Green DNA polymerase (DNA synthesis)	Thermo Fisher (USA)
T4 DNA ligase (Ligation of DNA segments)	New England Biolabs (USA)
Lysozyme (cell wall breakdown in *L. lactis*)	Sigma (USA)
Mutanolysin (cell wall breakdown in *L. lactis*)	Sigma (USA)

B. List of reagents/solvents and respective manufacturers.

Reagent/Solvent	Manufacturer
6x application solution for electrophoresis (Loading Dye)	Fermentas (USA)
Laemmli 2x Sample Buffer	Bio-Rad (USA)
10x DreamTag Green buffer	Fermentas (USA)
10x FastDigest buffer (FD buffer)	Thermo Fisher (USA)
10x T4 ligation buffer	New England Biolabs (USA)
10x KOD Hot Start DNA Polymerase Buffer	Thermo Fisher (USA)
100 bp Size Marker (GeneRuler™ 100 bp Plus DNA Ladder)	Fermentas (USA)
Precision Plus Protein All Blue Standards	Bio-Rad (USA)
PageRuler™ Prestained Protein Ladder Plus	Thermo Fisher (USA)
96% Ethanol	Carlo Erba (Italy)
Agarose	BioWhittaker Applications (USA)
Ammonium Persulfate (APS)	Sigma-Aldrich (USA)
Autoclaved milliQ water	Prepared in the laboratory
Deoxynucleotides (dATP, dCTP, dGTP, dTTP)	Promega (USA)
Dithiothreitol (DTT)	Fermentas (USA)
Glucose monohydrate	Fluka (USA)
Chloramphenicol	Sigma (USA)
Nisin	Fluka (USA)
NucleoSpin® Gel and PCR Clean-up Kit: NTI buffer, NT3 buffer	Macherey-Nagel (Germany)
Destaining Solutions (10% and 30%)	Prepared in the laboratory
Skimmed Milk Powder	Pomurske mlekarne (Slovenia)
NucleoSpin ® Plasmid DNA Purification Kit: buffers A1, A2, A3, AQ	Macherey-Nagel (Germany)
PBS Buffer	Prepared in the laboratory

Western Blot Transfer Buffer	Prepared in the laboratory
Sucrose	Serva (Germany)
SYBR Safe	Invitrogen (USA)
TAE buffer	Prepared in the laboratory
TBS Buffer	Prepared in the laboratory
TBST Buffer	Prepared in the laboratory
TEMED	Serva (Germany)
TGX Stain-Free Fast Cast Acrylamide Kit: Resolvings A and B and Stackers A and B	Bio-Rad (USA)
TRIS	Serva (Germany)
Tween 20	Serva (Germany)
SDS buffer	Prepared in the laboratory
BD Talon metal affinity resin	TALON metal affinity resin, Takara (Japan)
Elution Buffer	Prepared in the laboratory
Equilibrium Buffer	Prepared in the laboratory

C. Composition of buffers and solutions.

Buffer/Solution	Composition	Buffer/Solution	Composition
Coomassie brilliant blue solution	5 tablets of Coomassie brilliant blue 400 mL dH_2O 600 mL ethanol (96%)	Coomassie brilliant blue destaining solution 10%	140 mL 96% ethanol 50 mL acetic acid 810 mL dH_2O
NICE PBS	8 g NaCl (0.137 M) 0.2 g KCl (0.0027 M) 1.78 g Na_2HPO_4 x $2H_2O$ (0.01 M) 0.24 g KH_2PO_4 (0.0018 M)	Coomassie brilliant blue destaining solution 30%	400 mL 96% ethanol 100 mL acetic acid 500 mL dH_2O
Western Blot Transfer Buffer	20 mL 5X Transfer buffer 20 mL ethanol 60 mL dH_2O	AGE TAE Buffer	48.4 g Tris 11.4 mL glacial acetic acid 40 mL EDTA (0.5M) Fill with dH_2O up to 1L
Western Blot TBS Buffer	6.05 g Tris 8.76 NaCl 800mL dH_2O Adjust the pH to 7.5 with HCl (1 M)	Western Blot Milk TBST 0.5 %	50 mL 10x TBS buffer 450 mL dH_2O 250 uL Tween 20
SDS Buffer (SDS-PAGE)	100 mL 10X SDS Buffer Fill with dH_2O up to 1L	IMAC Equilibrium Buffer	3.9 g Na_2HPO_4 8.75 g NaCl 500 mL of MilliQ Water Adjust to pH 7 with HCl and/or NaOH Sterilise with autoclaving
IMAC Elution Buffer (10.2 g/L of imidazole)		100 mL from the previously prepared equilibrium buffer 1.02 g imidazole Adjust to pH 7 with HCl and/or NaOH Sterilise with autoclaving	

D. List of laboratory material and equipment.

Laboratory Equipment	Manufacturer
Freezer (-20 ºC)	Liebherr (Switzerland)
Freezer (-80 ºC)	Sanyo (Japan)
Gene Pulser II electroporation apparatus	Bio-Rad (USA)
Gel electrophoresis apparatus POWER PAC 300	Bio-Rad (USA)
Apparatus for SDS-PAGE	Bio-Rad (USA)
Mastercycler nexus X2 PCR apparatus	Eppendorf (Germany)
Apparatus for the observation of gels	UVItec (Great Britain)
Autoclave	1925X All American
Automatic pipettes	Eppendorf (Germany)
BioRad Trans-Blot® Turbo TM Transfer System	Bio-Rad (USA)
Eppendorf centrifuge	Eppendorf (Germany)
Centrifuge Hettich	Hettich (Germany)
Eppendorf 5301 Centrifugal Concentrator	Eppendorf (Germany)
Electrophoresis Cuvettes	Bio-Rad (USA)
Centrifuge tubes (centrifuges)	Sigma-Aldrich (USA)
Shaking Incubator (Certomat HK)	DJB Lab care (UK)
Minisart 0.2 µm filters	Sartorius Stedim (France)
Refrigerator	Electrolux (Sweden)
Incubator 30 ºC	Memmert (Germany)
0.2 cm GenePulser electroporation cuvettes	VWR (USA)
Laminar Airflow Chamber	Labcaire (USA)
Microwave oven ZM21MS	Zannusi (Italy)
Microcentrifuges	Sarstedt (USA)
PCR microcentrifuges	Lab Logistics Group GmbH (Germany)
Nitrocellulose membrane	GE Healthcare Life Science (USA)
ChemiDoc MP imaging device	Bio-Rad (USA)
Attachments for automatic pipettes	Sarstedt (USA)

NucleoSpin® Gel and PCR Clean-up Kit: columns and collection tubes	Macherey-Nagel (Germany)
NucleoSpin ® Plasmid DNA Purification Kit: columns and collection tubes	Macherey-Nagel (Germany)
NanoDrop ND 1000 spectrophotometer	Thermo Scientific (USA)
Vibromix shaker	Libra (Slovenia)
Scale Genius	Sartorius (Germany)
Scale 200A	Libra (Slovenia)
Thermoblock ThermoShaker	Biometra (Germany)
UPS200S ultrasonic breaker	Hielscher (Germany)
Power source POWER PAC 300	Bio-Rad (USA)
Visiblue Transilluminator	UVP (Germany)
Cooling centrifuge	Domel (Slovenia)
End-over-end shaker	Domel (Slovenia)
Disposable gravity flow columns	Domel (Slovenia)

E. List of culture media and respective composition.

Growth Media (use)	Composition	Growth Media (use)	Composition
M17	5.0 g Pancreatic digest of casein 5.0 g Soy peptone 5.0 g Lactose 5.0 g Beef extract 2.5 g Yeast extract 0.5 g Ascorbic acid 0.25 g Magnesium sulphate 19.0 g Disodium -β-glycerophosphate	**LB** (liquid media for multiplication of *E. coli* cultures)	10 g Peptone 140 (tryptone) 5 g Yeast extract 5 g Sodium chloride
GM17 (liquid growth media for multiplication of *L. lactis* colonies and expression of recombinant proteins)	17 g M17 completed with milliQ water to 400 mL, autoclaved and cooled to 45 ºC. We added: 5 mL of 40% glucose 160 µL 25 mg/mL chloramphenicol	**LBA** (solid growth media for multiplication of transformed *E. coli*)	10 g Peptone 140 (tryptone) 5 g Yeast extract 5 g Sodium chloride 12 g Agar
SGM17 + $MgCl_2$ + $CaCl_2$ (liquid growth media for incubation of *L. lactis* after transformation)	2.125 g M17 0.275 g Glucose (0.5%) 8.56 g Saccharose (0.5 M) 0.0952 g $MgCl_2$ (20 mM) 0.015 g $CaCl_2$ (2 mM) Dissolved in 50 mL of milliQ water and sterilised using a 0.22 µm filter.	**GM17C10** (solid growth media to support the growth of *L. lactis* cultures)	42.5 g M17 15 g of Agar completed with milliQ water to 1L, autoclaved and cooled to 45 ºC. We added: 12.5 mL of 40% glucose 400 µL 25 mg/mL chloramphenicol
SOC (liquid growth media for incubation of *E. coli* after transformation)	2% Tryptone 0.5% Yeast extract 10 mM NaCl 2.5 mM KCl 10 mM $MgCl_2$ 10 mM $MgSO_4$ 20 mM Glucose		

F. Concentration of protein in purified IMAC fractions (Gliadin WT)

Samples	Concentration (µg/µL)	$A_{260/280}$
W1	0.046	-
W2	0.027	-
W3	0.012	-
W4	-0.014	-
W5	-0.018	-
W6	-0.012	-
E1	0.010	3.87
E2	**0.033**	**1.00**
E3	**0.050**	**0.91**
E4	0.015	1.37
E5	-0.012	0.04
E6	-0.002	1.03
E7	-0.010	3.48
E8	0.002	0.69
E9	-0.014	0.65
E10	-0.034	0.85

G. Concentration of protein in purified IMAC fractions (Gliadin MT)

Samples	Concentration (µg/µL)	$A_{260/280}$
W1	0.033	-
W2	0.013	-
W3	-0.010	-
W4	-0.011	-
E1	0.005	1.04
E2	0.020	1.34
E3	**0.131**	**0.77**
E4	**0.033**	**0.88**
E5	0.016	1.04
E6	0.040	1.38
E7	0.011	0.48
E8	0.016	0.59
E9	0.019	1.54
E10	0.006	0.72

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