EVALUATION OF INDIGENOUS PROBIOTIC LACTIC ACID BACTERIA TO OVERCOME GLUTEN INDUCED CELIAC DISEASE

Dissertation submitted in partial fulfillment of the requirement of the degree of

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By

KRITIKA SHARMA– 143801

SARITA BHAWANANI– 141833

UNDER THE GUIDANCE OF DR. GUNJAN GOEL

JAYPEE UNIVERSITY OF INFORMATION TECHNOLOGY, WAKNAGHAT

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CERTIFICATE

We hereby declare that the work presented in this dissertation entitled **"evaluation of indigenous probiotic lactic acid bacteria to overcome gluten induced celiac disease"**was carried out by Ms. Kritika Sharma and Ms. Sarita Bhawanani at Department Of Biotechnology And Bioinformatics, **Jaypee University of Information Technology, Waknaghat (Solan) India,**and is an authentic record of our work carried out under the supervision of **Dr. Gunjan Goel.**We have not submitted this work elsewhere for any other degree or diploma. It is also certified that above statement made by the candidate is true to the best of our knowledge.

Dr. Gunjan Goel

Associate Professor

Deptt. of BT/BI

JUIT, Waknaghat

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Date:

Kritika Sharma -143801

Sarita Bhawanani -141833

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ABBREVIATIONS

CD	**Celiac Disease**
GFD	**Gluten Free Diet**
GDB	**Gluten Degrading Bacteria**
LAB	**Lactic Acid Bacteria**
GBM	**Gluten Based Medium**
MRS	**De Man, Rogosa and Sharpe agar**
tTg	**Tissue Transglutaminase**
℃	**Degree Celsius**
µl.	**Microlitres**
ml.	**Millilitres**
gm.	**Grams**
rpm	**Revolution per minute**

ABSTRACT

The current study has an objective of identifying probiotic indigenous lactic cultures which are able to degrade dietary gluten to produce safer fermented products. The gluten degrading activity was assessed qualitatively and quantitatively in gluten based media. A total of four lactic cultures, *Lactobacillus paracasei CD4, L. gastricus BTM7, L. plantarum K 90* and *L. rhamnosus GG* were analysed. The gluten degrading ability was visualized in all the lactic cultures as a clear zone around colonies in gluten containing agar plates after staining with Coomassie brilliant blue R250. Further, the growth and metabolic action of lactobacilli strains in a gluten base medium (GBM) was evaluated for appropriate selection of strains efficient to degrade gluten to be used furtherin SDS-PAGE which indicated that the gluten was hydrolysed into higher fraction of high and lower levels of low molecular weight protein fragments. The preliminary results acquired in this study indicated the potential of indigenous probiotic lactic cultures isolated from fermented foods to degrade wheat gluten and can be further used to develop probiotic based foods to prevent immunogenic reactions related to celiac disease.

CHAPTER 1

INTRODUCTION

1. INTRODUCTION

Celiac disease

Celiac disease (CD) is an autoimmune inflammatory condition that predominantly distressing the intestinal part proximally small intestine, the main causative agent for this is gluten proteins present in most of the food stuffs such as wheat, barley and rye and surprisingly it is observed in both men and women across the globe irrespective of age and race.

The prevalence of this disease is 0.7-2% among universal population i.e. 1 in every 100individual is affected by this allergy. Most people with celiac disease never know they have it and researchers think that around 80% cases of Celiac disease remain undiagnosed. There are genetic studies, saying that it could be inherited or the person suffering from this health issue carry some kind of receptors HLA-DQ2 and HLA-DQ8 MHC-class molecules on their antigen presenting cells. This can be easily identified by gastrointestinal uneasiness and the incompetent absorption of nutrients and many more digestion related symptoms due to duodenal inflammation and villous atrophy. (Helmerhorst Eva J. 2014).

A possible therapeutic way to neutralize the gluten-derived immunostimulatory responses could be the gluten digestion by enzymes. (Bethune MT et al 2007, Cerf-Bensussan N et al 2012). These degrading enzymes are isolated from numerous of microbe such as prolyl endopeptidases from *Sphingomonascapsulata* and *Aspergillus niger* and by the barley species itself EP-B2, but the main limitation of these enzymes, these are not stable and cannot tolerate the acidic pH of the human stomach and are still on clinical trials (Tack GJ et al 2013, Lahdeaho ML et al 2014). The only treatment known so far is the strict gluten free diet which is however challenging since, gluten is a type of protein found in the grains wheat, barley, rye, and triticale (a wheat-rye cross) which are the staple food of many countries. Alternatively, probiotics with proteolytic activity may help people having Celiac disease to consume food containing gluten. There would be a distinct advantage if probiotics used for therapy in case of CD are naturally associated with the human body then there would have an expected lower incidence of allergy or other intolerances associated with the introduction of foreign substances. In many researches, it has been detailed that probiotics, especially those found in the gastrointestinal tract (GI) have many benefits other than gluten indigestion or intolerance, these may have other wellbeing benefits for the patients suffering with other digestive related diseases or problems.(Petschow B et al 2013, Serban DE et al 2013).

Gluten

One of the most common causative agents of food allergy is wheat flour. Celiac disease is a problem some people have with gluten rich food products. Wheat gluten comprises of plant storage proteins called prolamins that are deposited in the endosperm of the cereal grain. It consists of gliadin and glutenin, and complex molecular network is formed when water is added to it that offers elastic properties of flour dough. Gluten proteins are divided into two main fractions according to the solubility in aqueous alcohols: the soluble gliadins and the insoluble glutenins. Both the gliadins and glutenins have protein components glutamine and proline. Gliadins are the monomeric proteins having molecular weights (MWs) around 28,000–55,000 while the glutenin have a different size ranging from 500,000 to more than 10 million. And, glutenin subunits have been divided into the high-molecular-weight (HMW) proteins MW 67,000– 88,000 and low-molecular-weight (LMW) proteins MW 32,000–35,000. (Kucek et al 2015)

Upon continuous consumption to gluten via CD patient it may lead to increase the severity of the disease that produces several different auto antibodies which will majorly affect the epithelial layer and as well as cells of small intestine.

Lactic Acid Bacteria

LAB are generally non- pathogenic microorganisms and may act as probiotics if given in ample and adequate amount that brings positive health benefits. Lactobacillus is one of the probiotic bacterial genera having positive health benefits other than *Bifidobacterium, Streptococcus* etc. Dairy products fermented or non- fermented are rich in LAB so it's easy to isolate these cultures from dairy food products obtained from different regions of Himalayas. (Sharma K et.al, 2017)

Our laboratory has reported the efficacy of different probiotic lactic cultures from these foods under simulated gut environmentexhibiting maximum gastric tolerance. Therefore, these cultures were further evaluated for their potential as probiotic therapy to combat increasing trends of celiac diseases in Indian population. As these cultures have been derived from fermented foods therefore these are considered under GRAS (Generally Recognised as Safe).Therefore, the objective of our study is theevaluation of indigenous probiotic lactic acid bacteria to overcome gluten induced celiac disease.

CHAPTER 2

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

2.1 Celiac disease

Celiac disease (CD) is an autoimmune inflammatory condition mainly distressing the intestinal part proximally small intestine, it is caused by gluten proteins having in wheat, barley and rye and is observed in both men and women across the globe irrespective of age and race.The symptoms of CD may appear with the ingestion of gluten rich food products mainly cereals such as wheat, rye, barley. Or could be genetically inherited disorder passed from generation to generation. Although the signs and symptoms not very clear but classical symptoms are related to the gastrointestinal tract, indigestion problem, other extraintestinal problems may include anemia, infertility, psychiatric syndromes or various neurological disorders also. The diagnostic study till now is only based on serological testing i.e. antiendomysial, antigliadin and antitransglutaminase antibodies inspecting the titer of these enzymes in the patient the other way is classical one, observing the histopathological changes (villous atrophy, inflammation of the intestine, irritable bowel syndrome as well) on distal duodenal biopsy. A gluten-free diet (GFD) rapidly reverses the mucosal lesion and corrects the gluten indigestion problem (José Ibiapina S.N. et al 2004).

Associated diseases and prevalence in celiac disease patients

Table 2.1: Prevalence of other autoimmune disorder in CD patients

Diagnosis of age groups	Chances of developing another autoimmune disorders
Group 1 **1- 2 years**	**10.5%**
Group 2**2- 14 years**	**16.7%**
Group 3**14- 20 years**	**27%**
Group 4**Over 20 years**	**34%**

Table 2.2: Other associated diseases

Autoimmune disorders	Prevalence in CD Patients
Anemia	12-69%
Autoimmune Hepatitis	2%
Autoimmune Thyroid Disease	26%
Chronic Fatigue Syndrome	2%
Dermatitis Herpatiformis	25%
Down's Syndrome	12%
Gluten Ataxia	10-12%
Liver Disease	10%
Lymphocytic Colitis	15-27%
Type 1 Diabetes	8-10%
Peripheral Neuropathy	10- 12%
Unexplained infertility	12%

World gastroenterology organization

2.2 Prevalence in North India:

In recent times, there has been a sudden rise with the number of cases with celiac disease conjointly has been reported from numerous pivots in India. The first community based study including both children and adults from an Asian region, it was observed that the prevalence of celiac disease in the northern part of India to be 1.04% (1 in 96) (Makharia GK et al). In another study, on a questionnaire-based survey including 4347 school children (aged 3–17 years) the prevalence of celiac disease was to be 1 in 310 study from Chandigarh (northern part of India) On the basis of the above respective community based study the pervasiveness of this CD is increasing exponentially and approximately 5–8 million people are expected to have celiac disease in India. The prevalence of CD in India is more or less same as reported worldwide, such as European nations and United States. CD is not a new disease which is coming up in the image rather, it is known to have occurred in India for a long time but, lack of awareness was the main reason that people didn't recognize it as a major issue; there was a long hush approximately the occurrence of celiac disease in India. Most of the ensuring

reports on CD have reported from the northern portion of the country India; Punjab, Delhi, Rajasthan, and Uttar Pradesh where wheat is the staple nourishment. These studies also observed that many of these patients were symptomatic for long and did not seek medical care and proper treatment. In fact, most of the time patients refused initiation of gluten free diet. In the future time, this is possibly being a challenge for the physicians dealing with celiac disease in India and worldwide too. At present, the commercially available gluten free food products are not reliable and even not having good market in INDIA. (Gupta R et.al 2009)

This increase in prevalence can be partially caused to the improvement in diagnostic techniques and disease awareness and therefore now spreading in new regions like Asian countries. The cases of the epidemiology of CD in the Asia Pacific region is limited and confined to India mainly. In the northern part of the country the frequency of CD seems to be higher, hence also called as "celiac belt." (Catassi. C et.al 2014). Other than, genetic factor there might be some other factors that are responsible for CD prevalence, regional differences can be explained by dietary geographical and genetic factors, in INDIA the consumption of wheat is very high mainly in north region and this may contribute to high prevalence of CD in respective region.

2.3 Tissue Transglutaminase with celiac disease:

Transglutaminase 2 or tTg is a protein binder that links two proteins together. The enzyme then binds a glutamine on one protein with a lysine on the other and covalently joins the two side chains. The resulting covalent bond is similar to the peptide bond found in proteins. When this binding function is not required, several allosteric mechanisms that alter the activity of the enzyme through a conformational change by inducing a different molecule in its active site are used to inactivate transglutaminase.

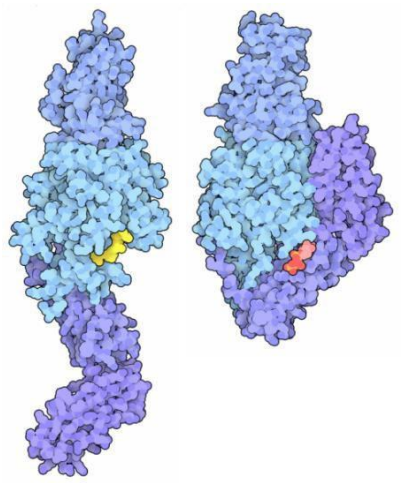


Figure 2.1: tTg in the active form (left) bound to the gluten molecule and GTP-bound inactive (right) form.

(Costanzo L.D., et.al, 2017) [PDB-101]

2.3.1 Mechanism of occurring of celiac disease:

Tissue transglutaminase removes a molecule of ammonia from glutamine to form glutamic acid present in gluten rich food. This deamination reaction is the main cause of celiac disease, damages the small intestine and causes a variety of symptoms related to digestion such as irritable bowel syndrome, iron deficiency (anemia), nutritional deficiency and many other digestive problems. Celiac disease is more prevalent in Western countries that consume foods containing gluten. Gluten is rich in glutamine, and tissue transglutaminase present in our digestive system converts small amount of this glutamine to glutamic acid i.e. removal of ammonia. In the mucosa of the small intestine the patients with celiac disease have special MHC proteins (known as HLA-DQ2 or DQ8) these MHC molecules recognize the deaminated gluten and identify it as foreign antigen and hence create an immunogenic response in the body. This complex is recognized by T-cell receptor of the Th1 type cells that produce inflammatory cytokines. This abnormal mucosal immune response results in the enteropathy and that is how, CD is considered as an autoimmune disorder because of the presence of autoantibodies in both the serum and the intestinal mucosa of the celiac disease patients. (Costanzo L.D et.al,MAY 2017) [PDB-101]

As the gluten eliminate from their diet simultaneously there is decrease of the titer of these antibodies, immunoglobulin IgA and IgG class to gliadin have also been used in the past to diagnose CD, tTg is also involved in both the humoral and cellular response. In initial stage

of CD the diagnosis is mainly done while checking the concentration of tTg in the patient's body. After diagnosis an intestinalbiopsy is carried out for confirmation. Finally, several potential interventional strategies might be applied or used to combat this respective problem of CD. (Reif S et.al 2004). Moreover, in recent studies it was found that cell surface tTg present on macrophages and dendritic cells that are responsible for the pathogenesis of celiac disease. The Cystamine, a tTg inhibitor used mostly in organ culture experimentations that represses the proliferation of gluten-responsive T-cells after gluten challenge of small intestinal tract biopsies. (Sabatino A et.al 2012)

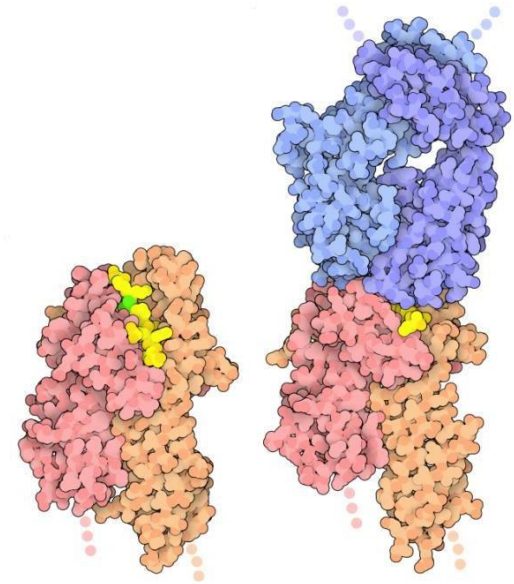


Figure 2.2: Deaminated gluten peptides that are represented with yellow, bind with deaminated glutamine (green) are identified by HLA molecules (orange and pink) and he whole complex is recognized by T-cell receptors (blue).

(Costanzo L.D., et.al, 2017) [PDB-101]

2.4 Emerging approaches to combat celiac disease:

Presently, the only effective treatment for celiac disease is strict and lifelong gluten-free diet (GFD) and surprisingly the small amounts of gluten (50 mg/day) can be harmful to the CD patients hence the food that contain gluten as it is or the derivatives must be eliminated completely from the diet. Although, it's not possible to completely remove the gluten from the food products. So, in 2012, it was turned up that the level of gluten in foods that could be considered gluten-free should be 20 ppm (mg/kg), with "very low gluten" foods defined as

those with less than 100 ppm gluten, more than his amount of gluten will become toxic for the patients. (Makharia GK, May, 2011). However, it has been researched that recovery of mucosal layer of damaged small intestine does not occur immediately, this also establish an aspect of social burden, nutritional deficiencies and many more issues.

2.4.1 Enzymatic therapies:

The principal of assisted digestion of gluten is to eradicate the toxicity of gluten by either of the two ways:

1. Digestion of the gluten in the in the gastrointestinal lumen using endopeptidases, intra-luminal enzymatic digestion occurs.
2. Another is, using predigested gluten where gluten is broken down into small peptides and partially digested before ingestion by using some probiotic strains of *lactobacillus* spp.or others.

Since, gluten is the causative agent of food allergy in people prone to celiac disease. Therefore, the possible solution to reduce the immunogenic response of gluten could be breaking down into smaller peptides and generating small chains of amino acids. Prolyl oligopeptidases, enzymes have been reported from different bacterial species that can cut after a proline residue in peptides, have been examined (Shan et al., 2002, 2005). However, the only limitation is, the enzymes are unstable and non-functional under acidic conditions of the stomach and cannot degrade gluten epitopes before they reach the small intestine. Till now, numerous dietary digestive enzyme supplements can be found in the market that claim to degrade gluten (Shan et al., 2002). Some of the supplements contain the fungal enzyme DPPIV, that are ineffective at low pH (Yoshimoto et al.,1977).A recent study on another enzyme that is prolyl endoprotease isolated from *Aspergillus niger* , this enzyme effectively cut the epitopes into smaller fragments that are non-immunogenic. (Janssen et al., 2015; König Julia et al., 2017). A 50% gluten degradation compared to placebo in the duodenum identified with the higTh dose of AN-PEP (König Julia et al., 2017). Another studies by Zamakhchari et al., 2011; FTernandez-Feo et al., 2013 reported the presence of gliadin degradation activity in bacteria from oral cavity. They reported *R. dentocariosa* and *S. salivarius* from the saliva having a proteolytic potential for the degradation of gluten and development of an oral probiotic or enzyme therapy. The role of lactic acid bacteria *L. plantarum* KKP 593/p and W37/54, *L. helveticus* Lh10, *L. rhamnosus* Lr23, *L. sakei* 750, *L. curvatus* 750, *L. coryniformis* pA, *W.cibariaEKO31*, *P.pentosaceus EKO23* and *1850* and *P.*

acidilactici EKO26 has also been reported, have ability to reduce albumin/globulin and gliadin concentrations in sourdoughs prepared from wheat flour in bakeries and fermented with the selected starter cultures. Other bacterial strains belonging to *Bifidobacterium* and *Lactobacillusspecies* that mainly play a key role in probiotic development have been proposed as potential probiotics also having proteolytic activity to degrade gluten from the wheat in CD patients. (McCarville JL et.al, 2015)

India has a rich culture of production of traditional fermented foods, whereby the people since long have been dependent on fermentation for preparation of various delicacies where food prepared from different traditional food processing technologies from available raw material is governed by ethnic preference, socio-cultural ethos and religion. The health benefits and the role of microorganisms in these fermentations have not been investigated for many foods.

2.4.2 Blocking the epitopes:

As discussed above, celiac disease occur in genetically predisposed people having HLA-DQ2 and –DQ8 genes, present on the surface of antigen presenting cells (APCs). These APCs activate the T-lymphocytes. Therefore while blocking the epitopes of these receptors HLA-DQ2 and HLA-DQ8 does not activatethe – lymphocytes and hence could prevent this problem.(Makharia GK, 2014)

2.4.3 Inhibiting the activity of TG2 or tTg enzyme:

In the mucosal layer within the small intestine of CD patients, TG2/ tTg deaminates glutamine residues into glutamic acid and create potent T cell receptors, it also induces crosslinking of gluten peptides with matrix proteins, thereby generate complexes of gluten that induce an auto immune response. Therefore, TG2 tTg inhibitors can be used to tackle this emerging problem and might have promising avenues for celiac disease therapy. (Caputo I et.al, 2009).

2.4.4 Inducing tolerance against gluten:

Peptide-based vaccination by intra-dermal injection is a protein-based insensitive therapy is frequently used to treat allergic reactions of the body. NexVax2 is a desensitizing or therapeutic vaccine and is still under development by an "ImmuSanT company" a biotechnology based company, this vaccine uses three gluten peptides inducing a tolerogenic response in patients. NexVax2 has showed efficacy in HLA-DQ2 transgenic mice having gluten-sensitive T cells. Vaccinated subjects revealed the presence of Interferon-y that produce anti-gluten T cells. The real efficacy of such a strategy is still to be established and hence clinical trials are still ongoing. One of the limitations is, this synthetic vaccine is developed only for a defined number of long chain of immunogenic gluten peptides. Another limitation is related to the efficacy of the vaccine and its long term effects before commercializing into the market. (Makharia GK, 2014)

2.4.5 Consumption Ancient wheat

Over the past many decades, there has been observed an increase trend in wheat consumption per capita, as well as increased gluten consumption in food processing and the consumption of processed foods that contain gluten because of the puffiness it gives to the dough and hence people like it more such as in bakeries has also been increased from last five decades. It might be possible that due to these changes in the wheat consumption pattern and increase in T cells stimulatory epitopes in wheat, incidence of celiac disease over the globe has been increased. Due to evolutionary changes the wheat genome has changed from diploid to hexaploid. Earlier, the wheat that was cultivated *Triticum monococcum* having very less gluten content whereas, the wheat which we are consuming nowadays *Triticum aestivum* is having large amount of gluten in it so therefore; the overall set of evidences tends to suggest that the ancient wheat variety is less immunogenic or harmful to the CD patients and can be easily consumable by those. (Makharia GK, 2014)

2.4.6 Probiotics and Celiac Disease:

It has been hypothesized that the enhanced permeability of intestinal area is one of the major risk factor responsible for causing CD, sometimes it my found out that due to the alterations in gut microflora that are mainly caused by gluten itself, there is change in the permeability

of intestine and hence people in such cases are more prone of developing such disease. Hence, instead of following GFD this is a very harsh challenge, recently pathophysiological advancement have contributed the development of novel and promising therapeutic solutions that is a “probiotics”. It is defined as “live organisms” that are beneficial for our gut microflora and enhances their growth as well, when administered in adequate amount to the host. Nowadays, people are consuming probiotics such as Yakult or Probiotic Yoghurt in their day to day life and therefore, use of this probiotic has come up as a promising alternative to the CD patients. (de Sousa Moraes LF et.al,2014). The bacterial diversity of the intestinal microbiota gets changed with the diet by influencing its composition and function and in consequence the health of the host, especially in case of patients suffering from foodborne illness. A probiotic food addressed towards celiac patients represents a favourable and encouraging alternative to improve the quality of life of people suffering from CD. (Lorenzo PisarelloMJ et.al, 2015). Strains that should be used in probiotics must have the property to degrade gliadin peptides before consuming the food rich in gluten protein and secondly, the probiotics should also induce anti-inflammatory response in CD patients.

Currently, in Phase II testing different gluten degrading enzymes and modulators of intestinal permeability have been analysed and reported and are prior to introduce in Phase III clinical trials and possible to come up within the next 5-10 years. However, the initial goal of these non-dietary therapies is to serve as adjunctive agents (additional treatment that increases the efficacy of primary treatment i.e. GFD) in combination with a GFD and to alleviate the effects of gluten ingested. (Veeraraghavan G et.al, 2015). In some population based studies it has been elucidated that the people suffering from CD found to have more gram negative microbes in their gut because of dysbiosis. (Losurdo G et.al, 2016)

CHAPTER 3

MATERIALS AND METHODS

MATERIALS AND METHODS

Materials

3.1 Chemical Requirements:

Gluten, MRS agar, ethanol, distilled water, Coomassie brilliant blue R250, Methanol, Glacial Acetic acid, Glucose, KH2PO4, K2HPO4, Tween-80, Tri-chloro acetic acid, NaCl, KCl, Na2HPO4, Bradford reagent, Bovine serum albumin (BSA), Acrylamide, Bisacrylamide, tris HCl, SDS, Ammonium per sulphate, TEMED, Glycerol, Bromophenol blue, 2-þmercaptoethanol, Tris Base, Glycine, Acetate, Ninhydrin reagent.

3.1.1 Other Requirements:

Petriplates, Centrifuge tubes, Eppendorf tubes, test tubes, inoculating loop, Bunsen burner, micropipette, centrifuge, spectrophotometer, water bath, microwave, spreader, 96 well plate, a tank, lid with power cables, casting stand, casting frames, combs, glass plates, electrode assembly, gel rocker, gel box

3.1.2 Gluten

Wheat gluten was procured from Urban Platter Vital Wheat Gluten (500g),the protein found in the endosperm of wheat berry, and having about 75% to 80% protein. After mixing this gluten with water it will make a complex structure with the help of water and thus becomes highly elastic. The elasticity of dough is majorly because of this gluten and makes the bread or chapatti puffier.

3.1.3 Lactic Cultures

A total of four lactic cultures already isolated in our laboratory for their probiotic attributes were taken for the study. The cultures were maintained on MRS broth at 4C. The cultures used were:

Lactobacillus paracasei CD4

Lactobacillus gastricus BTM7

Lactobacillus plantarum K90

Lactobacillus rhamnosus GG

All these cultures were isolated from various fermented and non-fermented dairy products like milk (from Buffalo, cow, goat), curd, buttermilk, were collected from different regions of Himachal Pradesh.

3.2Methods:

Screening of Lactic Acid Bacteria for Gluten Degradation

3.2.1 Qualitative screening:

The ability of lactic culture to utilize gluten was assessed by plate assay as discussed below:

3.2.1.1 Preparation of Gluten Agar plates:

The gluten was prepared by adding 1 gm of wheat gluten in 22 ml of 60% ethanol. The mixture was kept overnight for incubation in a shaker at temperature of at 37°C at 100 rpm. The mixture was centrifuged at 10000 rpm at 4°C. From the supernatant, 850 µl of gluten was added to the 20 ml of MRS agar media as a gluten containing media. (Berger et al 2015)

3.2.1.2 Screening of LAB for gluten degrading activity:

After preparation of plates, different lactic cultures, *Lactobacillus paracasei CD4, Lactobacillus gastricus BTM7, Lactobacillus species L90,* LRGG - *Lactobacillus rhamnosus GG were* streaked on the plates. The plates were incubated overnight at 37°C temperature. And, after incubation, the plates were simultaneously stained with coomassie brilliant blue R250 (0.5%) staining solution, followed by destaining with destaining solution (Ethanol: acetic acid: water, 5:1:14). The gluten degrading ability was assessed by formation of clear zones against dark blue background.

Composition of staining and destaining solutions are as follows:

Table 3.1: Staining solution composition**:**

INGTREDIENTS	VOLUME(200ml)
Coomassie brilliant blue R250	1gram
Methanol	100 ml
Acetic acid	18.5 ml
Distilled water	81.5ml

Table 3.2: Destaining solution composition:

INGREDIENTS	VOLUME(200ml)
Ethanol	50ml
Acetic acid	10ml
Distilled water	140ml

3.2.2 Quantitative Analysis

The Gluten Base Media (GBM) was prepared in distilled water and after mixing it was sterilized at 121℃. The quantitative estimation of gluten degradation was done in Gluten Base Media (GBM). The overnight grown cell suspensions of each LAB strains were inoculated in GBM and incubated at 37°C. Aliquots were taken out in vials at different time intervals of 12 h upto 72 h. Then, the microcentrifuge tubes were centrifuged at 10000 rpm for 15 min. A1 ml supernatant of different time intervals was suspended with trichloroacetic acid (TCA) in equal amounts for protein precipitation. The samples were again centrifuged and pellets obtained were now suspended in PBS (pH 7).The Protein concentration of the

extracts was determined with the **Bradford method** using bovine serum albumin (BSA) as a standard.

Table 3.3: The GBM was prepared as follows: (Gerez C.L. et.al 2005)

INGREDIENS	PERCENTAGE (%)
Gluten	9%
Glucose	2%
KH_2PO_4	1%
K_2HPO_4	1%
Tween-80	0.1%

Table 3.4: Bradford Standard

BSA(mg/ml)	Volume of BSA (µl)	Water (µl)	Bradford Reagent (µl)	O.D. (595nm)
0	0	100	100	0
0.1	10	90	100	0.13
0.2	20	80	100	0.25
0.4	40	60	100	0.45
0.6	60	40	100	0.50
0.7	70	30	100	0.55
0.8	80	20	100	0.58
0.9	90	10	100	0.63
1	100	0	100	0.64

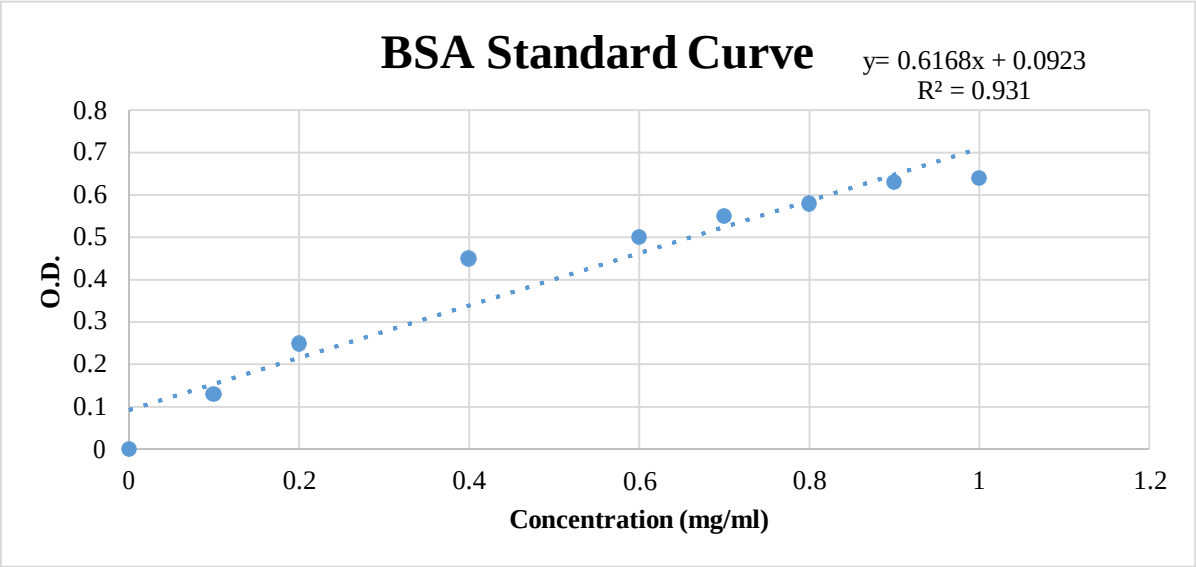


Figure 3.1: Graphical representation of BSA Standard

3.2.3 Gluten degradation kinetics

The gluten degradation kinetics was studied using SDS-PAGE analysis where the aliquots of LAB strains grown in GBM were taken at different time interval were used as samples.

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE)

Table 3.5: Resolving gel (12%)

Components	For 10 ml
Distilled water	3.3 ml
30% Acrylamide	t4 ml
2M Tris (pH=8.8)	2.5ml
10% SDS	0.1 ml
10% APS	0.1 ml
TEMED	0.004ml

Table 3.6: Stacking Gel (5%)

Components	**For 5 ml**
Distilled water	3.4 ml
30% Acrylamide	830 µl
1M Tris(pH= 6.8)	630 µl
10% SDS	50 µl
10% APS	50 µl
TEMED	2 µl

Table 3.7: Sample Buffer

Components	**For 10 ml**
Glycerol (50% v/v)	5 ml
SDS (10%)	2ml
1 M Tris HCl (pH= 6.8)	600 µl
2-þmercaptoethanol	500 µl
Bromophenol blue (1%)	900 µl
Distilled water	1 ml

Table 3.8: Coomassie Blue Stain

Components	For 1 litre
Distilled water	500 ml
Coomassie R250	1g
Glacial Acetic Acid	100ml
Methanol	400ml

Table 3.9: Destaining solution

Components	For 1 litre
Distilled water	700 ml
Glacial Acetic Acid	100 ml
Methanol	200 ml

Table 3.10: Running Buffer (For 1000ml)

Components	10X	1X
Tris Base	30 gm	3gm
Glycine	144gm	14.4gm
SDS	10gm	1gm

Procedure:

Polyacrylamide gel was prepared according to standard protocol i.e. 12% resolving gel was prepared and about 5-6ml was pipetted between the gel casting plates. It was left for solidification and after that 5% stacking gel was pipetted over it, immediately comb was inserted and left till the gelation of the well. Protein samples were diluted to same concentrations and were loaded (8 µl each) in wells along with a **ladder of 13-97 kDa** of geneiin 1x SDS Running Buffer. Once the dye reached the foot of the glass plates the SDS-PAGE was stopped. Now the gel was put in gel box having staining solution and was kept on gel rocker for about 60 minutes. Coomassie blue staining solution was poured off. Gel was covered with destaining solution and was kept on gel rocker for overnight. After overnight destaining, different bands were observed under protein scanner.

3.2.2 Ninhydrin Test

For ninhydrin test following reagents were prepared:
Standard amino acid stock solution of Asparagine (1mg/ml), stock of Ninhydrin reagent (4mg/ml) (24 mg in 6ml of absolute ethanol)

Procedure:

Standard amino acid solution was pipetted out different concentrations (0.2-1 mg/ml) to the respective labelledmicrocentrifuge tubes. Distilled water was added in all the test tubes to make up the volume to 100µ l. To the Eppendorf labelled blank 100µl of distilled water was added. Now all the test tubes were filled with 100µl of ninhydrin reagent. Contents of tubes were properly mixed and lids of the tubes wereclosed and were incubated in boiling water for 15 minutes. Test tubes were cooled down and pipetted into 96 wells plates. O.D was measured at 570nm by using spectrophotometer.

Table 3.11: Ninhyrdrin standard

Concentration of Standard Amino Acid (1mg/ml)	Volume of Standard Amino acid (µl)	Water (µl)	Ninhydrin reagent (µl)		O.D. (570 nm)
Blank	-	100	100	Incubation in boiling water For 15 min	0.054
0.2	20	80	100		0.099
0.4	40	60	100		0.12
0.6	60	40	100		0.156
0.8	80	20	100		0.181
1	100	-	100		0.346

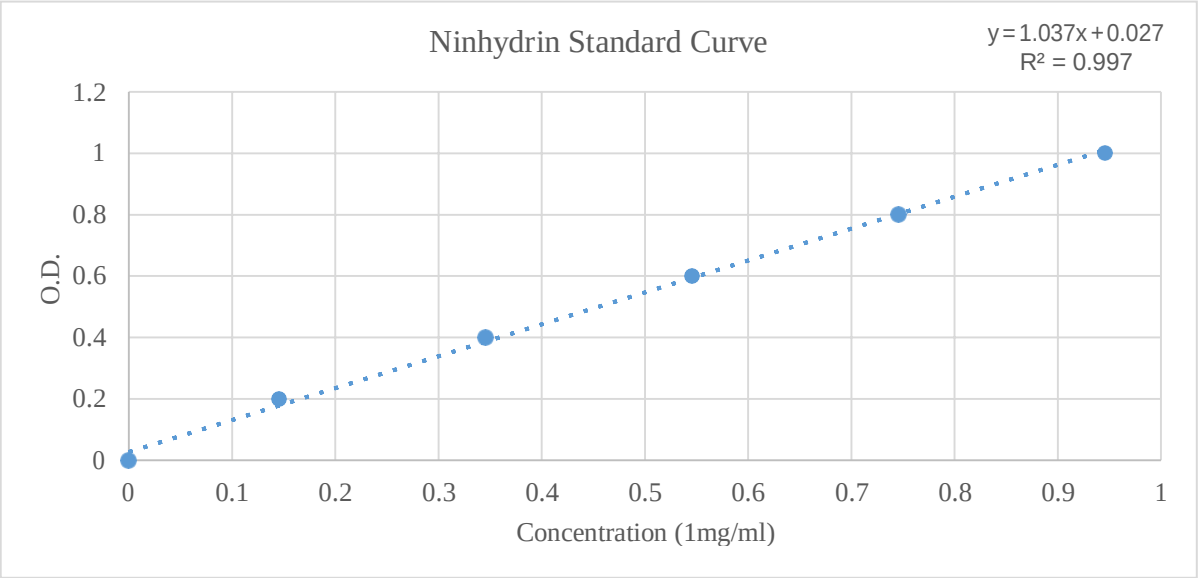


Figure 3.2: Graphical representation of Ninhydrin Standard curve

CHAPTER 4

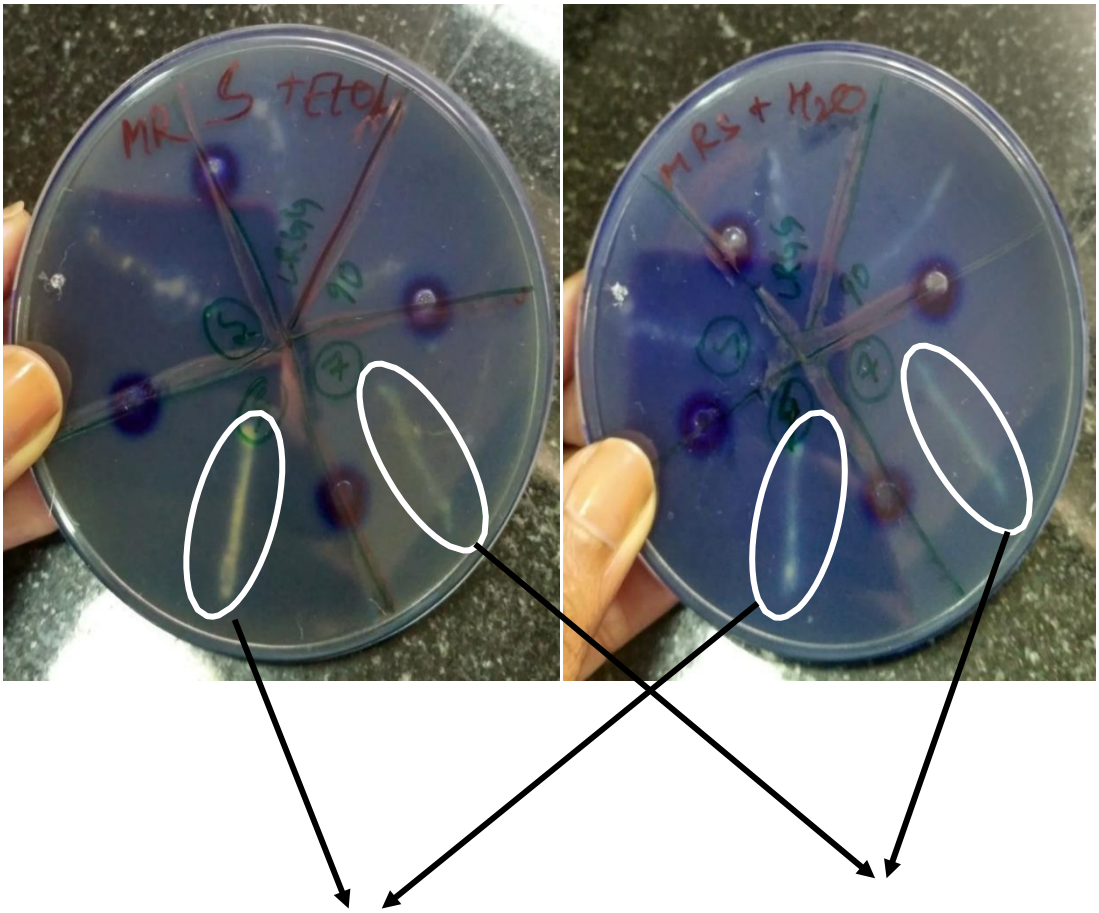
RESULTS AND DISCUSSION

3. RESULTS AND DISCUSSION

4.1 Screening of LAB for gluten degradation

A total of four lactic strains - *Lactobacillus paracasei CD4, Lactobacillus gastricus BTM7, Lactobacillus species L90,* LRGG - *Lactobacillus rhamnosus GG*were tested for their ability to degrade gluten on MRS agar plates supplemented with gluten. After staining and destaining reactions, a clear zone of hydrolysis around the colonies against dark blue background was observed as indication of degradation of gluten.

All the four lactic cultures tested indicated that these had the ability to degrade gluten.However, for detailed kinetics of gluten degradation, *Lactobacillus paracasei CD4, Lactobacillus gastricus BTM7* were used.



Lactobacillus paracasei CD4 **showing clear zones of hydrolysis of gluten**

Lactobacillus gastricus BTM7 **showing clear zones of hydrolysisof gluten**

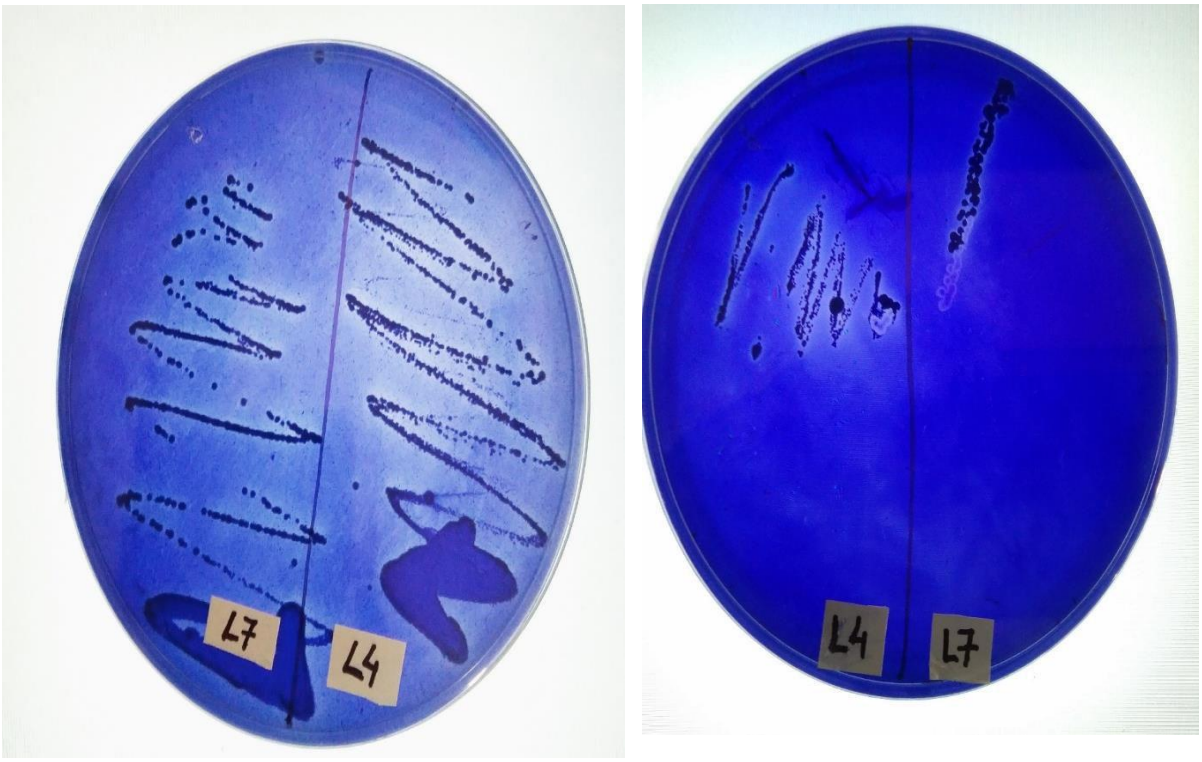


Figure 4.1: Clear zones of hydrolysis of gluten by LAB on gluten based media

4.2 Quantitative assay for degradation of gluten

All the lactic cultures were inoculated in minimal media (GBM) containing gluten as only Nitrogen source. For studying the gluten degradation kinetics, a sample aliquot was withdrawn at regular intervals up to 72h. The samples were subjected for protein estimation (Bradford assay) and further for SDS –PAGE analysis.

4.3 Bradford assay (Protein content)

The protein content after TCA precipitation in sample aliquots was conducted using Bradford assay with Bovine serum albumin (BSA) as standard.

The observation is as follows:

Table 4.1: Observation table

Time of incubation	***Lactobacillus paracasei CD4*** **OD**	**mg/ protein content**	***Lactobacillus gastricus BTM7*** **OD**	**mg/ protein content**
GBM	0.265	0.28	0.441	0.565
0^{th}	0.185	0.15	0.125	0.053
12^{th}	0.126	0.054	0.107	0.024
24^{th}	0.129	0.059	0.149	0.092
36^{th}	0.225	0.0215	0.312	0.35
72th	0.145	0.085	0.134	0.068

From the table, a trend can be followed where the protein content is gradually decreasing (from 0^{th} to 24^{th} hr) then increasing in 36^{th} hour and then again decreasing in 72th hr. Possible reason for increment and decrement in the protein content might be due to the degradation of gluten by different strains of Lactic Acid Bacteria and the increment may be because of the presence of the microbial protein.

Graphical representation between BSA concentration and Absorbance:

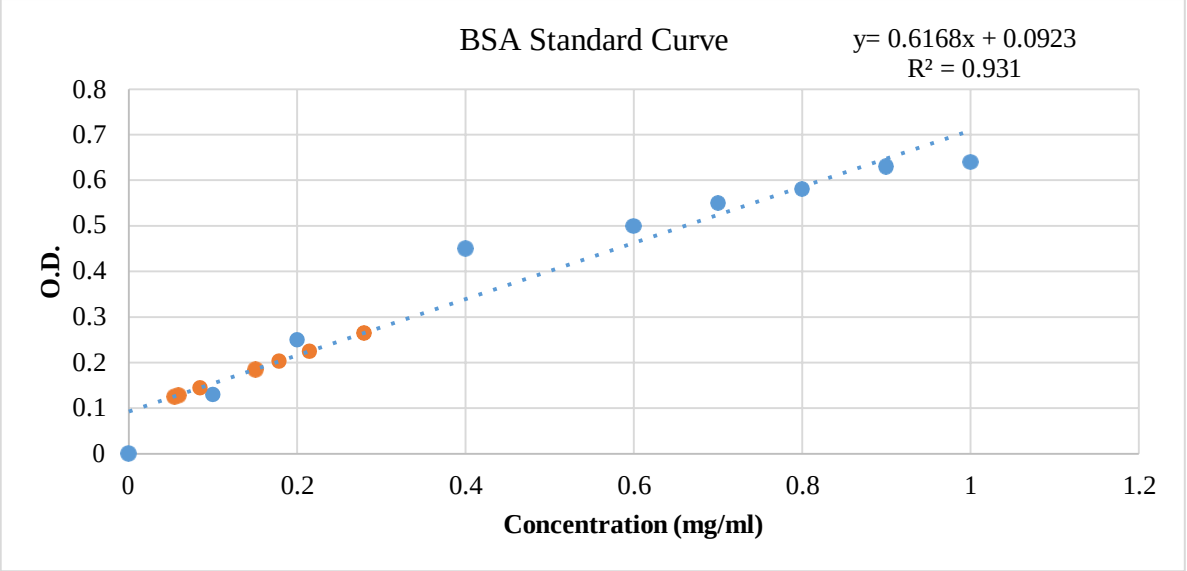


Figure 4.2.: Protein estimation of *Lactobacillus paracasei CD4*with respect to standard BSA.

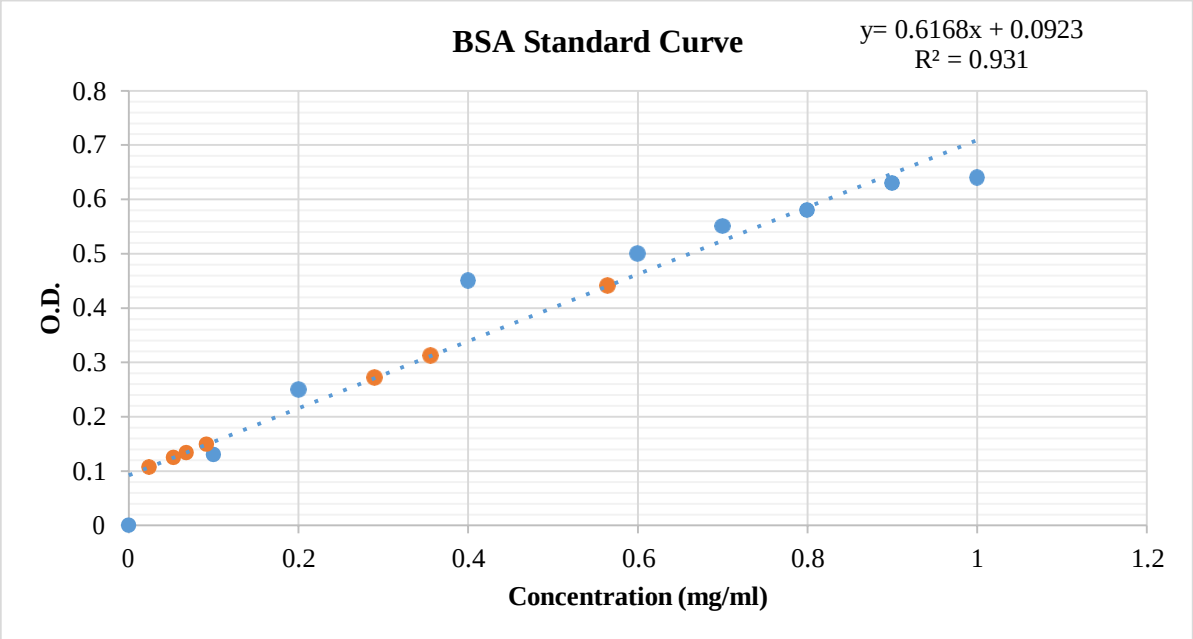


Figure 4.3: Protein estimation of *Lactobacillus gastricus BTM7*with respect to standard BSA.

Red: Sample; Blue: Standard

4.4.1 SDS –PAGE observation

As samples were taken at different intervals of time to check the gluten degradation with respect to time. In this image we can cleraly see different bands in different intervals of time as:

- High molecular weight protein which are few in number in the upper part of the gel.
- Low molecular weight protein are also visible at the bottom of the gel
- Omega proteins, these are the dominating proteins present in the middle of the gel

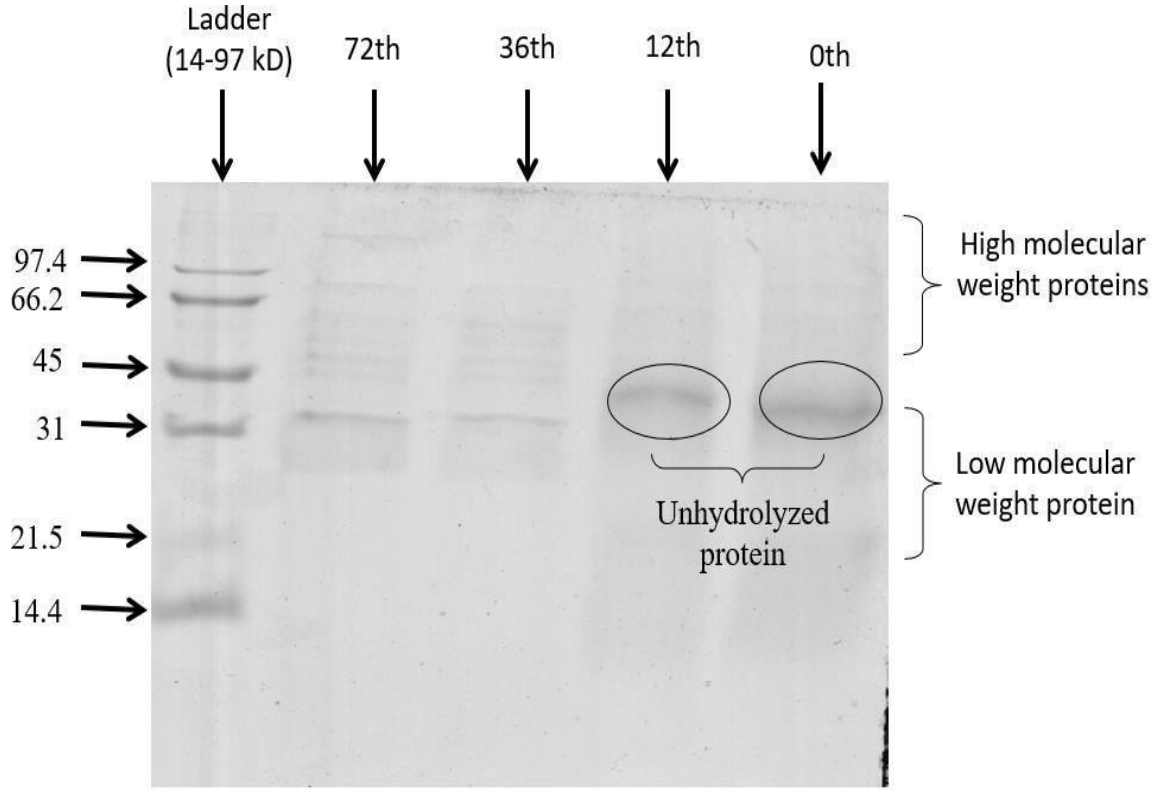


Figure 4.4: SDS PAGE analysis for degradation of gluten by lactic cultures at different incubation time

The SDS-PAGE analysis clearly revealed that the gluten was hydrolyzed in different protein fragments of high and low molecular weight protiens which needs to be identified. There are some additional bands which are observed above unhydrolyzed bands in 36^{th} hr and 72^{th}hr lane having high molecular weight, these bands were perceived may be because of presence of microbial proteins in sample aliquots.

4.4.2 Comparison between SDS-PAGE analysis of *Lactobacillus paracasei CD4* in GBM where gluten is added after autoclaving the media (Non-autoclaved Gluten) v/s *Lactobacillus paracasei CD4* in GBM where gluten is added before autoclaving the media (Autoclaved Gluten)

SDS PAGE: Non-autoclaved Gluten in GBM

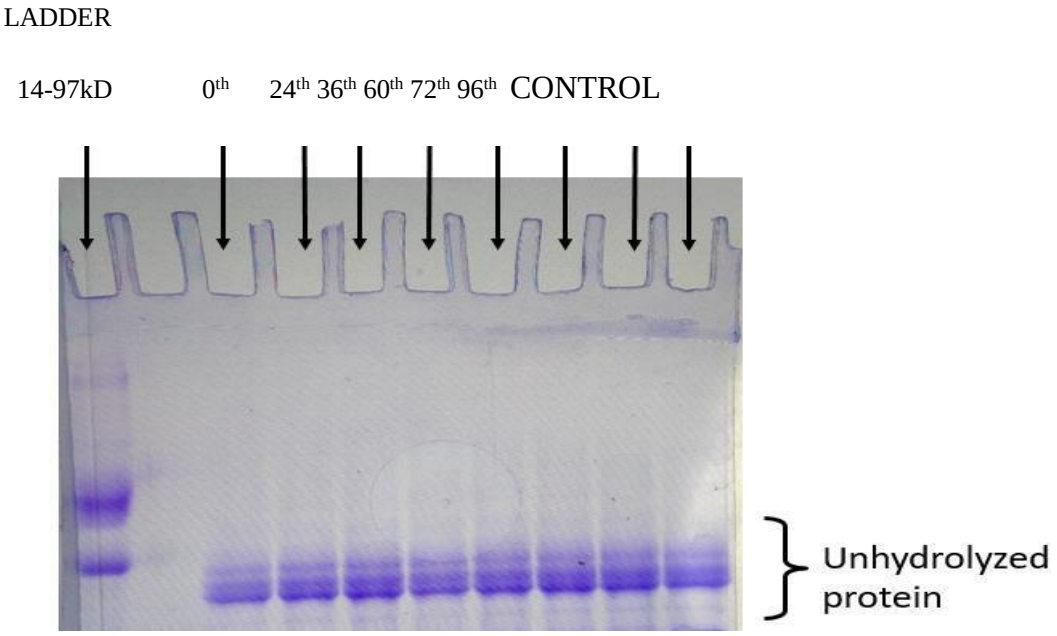


Figure 4.5 SDS PAGE analysis for degradation of non-autoclaved gluten by ***Lactobacillus paracasei CD4*** at different incubation time

The SDS PAGE analysis in the case of non-autoclaved gluten in GBM clearly revealed that no gluten degradation took place by *Lactobacillus paracasei CD4.*

SDS-PAGE: Autoclaved gluten in GBM

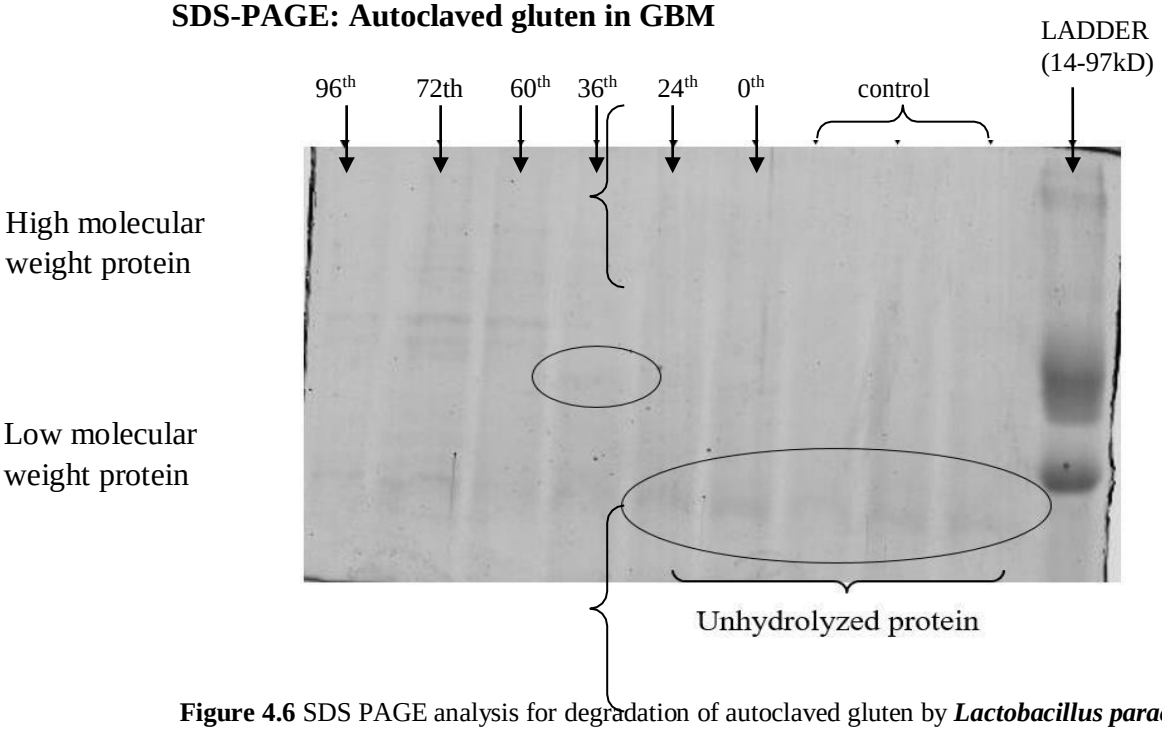


Figure 4.6 SDS PAGE analysis for degradation of autoclaved gluten by ***Lactobacillus paracasei CD4*** at different incubation time

The SDS PAGE analysis of autoclaved gluten in GBM having *Lactobacillus paracaseiCD4* revealed that degradation of gluten had taken place. Clearly, in 0th and 24th hour samples only one unhydrolyzed lower molecular weight protein band was observed. But in 36th hour sample total two protein bands can be detected. Gradually in 72th hour and in 96th hour samples, many high molecular weight protein bands were observed. This justifies the degradation of gluten and gives a possibility to use this *Lactobacillus paracasei CD4* for the gluten degradation.

4.5 Quantitative estimation of Amino Acids by Ninhydrin test

The amino acid concentration was determined by Ninhydrin test using Asparagine as standard. It also gave the confirmatory test for the presence of protein by changing the colour to Ruhemann's purple. The colour was dominantly found to be yellowish purple which infers the majority of Proline amino acids.

Table 4.2: Observation table

Time of incubation	***Lactobacillus paracasei CD4* OD**	**Amino Acid concentration (mg/ml)**	***Lactobacillus gastricus BTM7* OD**	**Amino Acid concentration (mg/ml)**
0th	0.053	0.024	0.042	0.013
12th	0.05	0.021	0.051	0.022
24th	0.042	0.013	0.029	0.001
36th	0.066	0.036	0.032	0.004
60th	0.365	0.324	0.042	0.013
84th	0.64	0.589	0.045	0.016
96th	0.274	0.237	0.056	0.027

Graphical representation between Asparagine concentration and Absorbance:

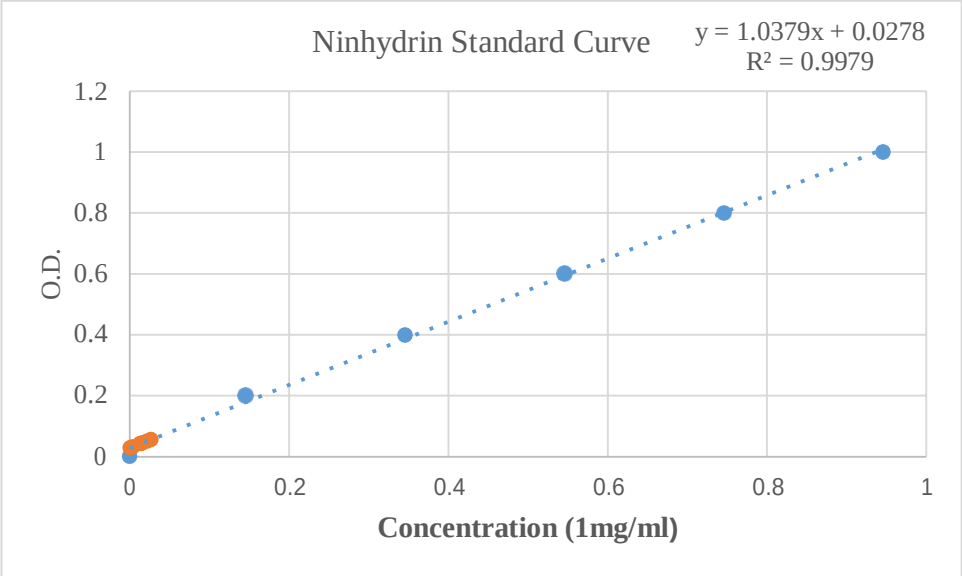


Figure 4.7: Amino Acids estimation of *Lactobacillus gastricus BTM7*with respect to standard Asparagine

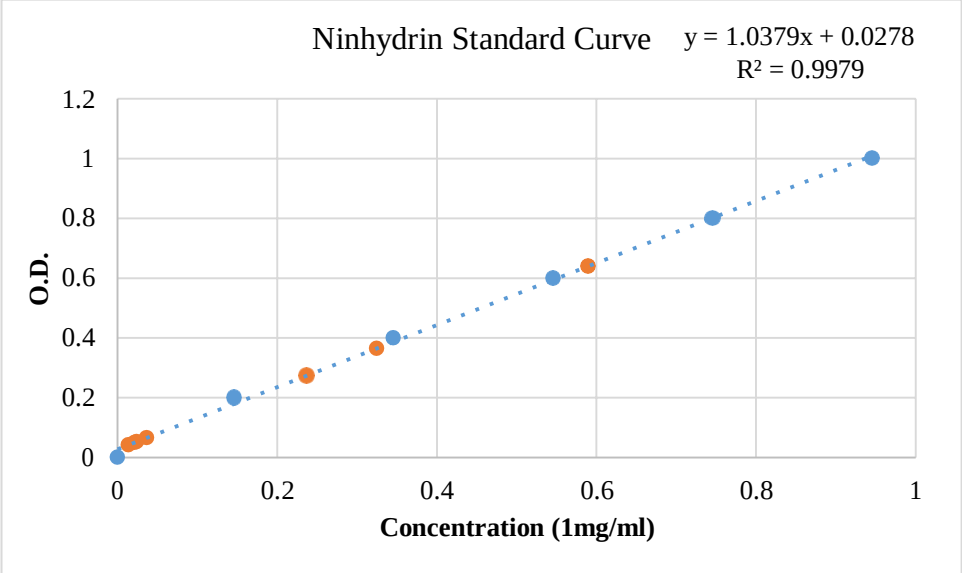


Figure 4.8: Amino Acids estimation of *Lactobacillus paracasei CD4*with respect to standard Asparagine

Red: Sample

Blue: Standard

CHAPTER 5

SUMMARY AND CONCLUSION

4. SUMMARY AND CONCLUSION

In today's era, Celiac disease is one of the foremost pervasive autoimmune malady that might arise in genetically predisposed people or could be inherited from the parents or other family members where the ingestion of gluten leads to harm within the small intestine in gastrointestinal tract causes the inflammation because of villous atrophy. The cases of gluten intolerance have escalated abruptly over the last few years. Till date, the only therapy that is usually prescribed by any physician for gluten intolerant people is the complete avoidance of dietary gluten commonly known as "Gluten Free Diet". However, to endure this gluten free diet is very arduous hence, there is an entail for a non-dietary treatment that involves consumption of probiotics, researchers are trying to produce a non- dietary therapy that could be given to the CD patients along with the gluten free diet or as such. Consuming probiotics or using oral enzyme therapies are the new emerging approaches to combat this issue.

The probiotic lactic cultures*Lactobacillus paracasei CD4*, *Lactobacillus gastricus BTM7*,*Lactobacillus plantarum K90* and *Lactobacillus rhamnosus GG*possess the ability to degrade gltuen into different fragments and these indigenous lactic cutltures can be used as a therapeutics against rising cases of celiac diseases after critical assessment. These LAB can be added to the fermented food which is largely consumed by the people in day to day life, so that these bacteria having probiotic attributes could reside in the gut and start degrading gluten which enters the body, hence breaking the immunogenic gluten to its non immunogenic form. The results so far achieved in the study demonstrate the degradation activity of the gluten by the selected LAB strains, further studies concerning the issue of gluten intolerance will be carried out, because the selected LAB strains may be applied as unique probiotics as a boon for gluten intolerant people. In conclusion so far the isolated lactic acid bacterias have shown the possible therapeutic potential against the gluten induced celiac disease and can give a good market and public response.

REFERENCES

[1] Berger, M., Sarantopoulos, C., Ongchangco, D., Sry, J. and Cesario, T., "*Rapid isolation of gluten-digesting bacteria from human stool and saliva by using gliadin-containing plates"*, Experimental Biology and Medicine, vol. 240, pp. 917-924, Jul.2015.

[2] Bethune, M.T. and Khosla, C., "*Oral enzyme therapy for celiac sprue*." Methods in enzymology, Academic Press Vol. 502, pp. 241-271, 2012

[3] Cerf-Bensussan, N., Matysiak-Budnik, T., Cellier, C. and Heyman, M."*Oral proteases: a new approach to managing coeliac disease*". *Gut*, *56*(2), pp.157-160. 2007

[4] Catassi, C., Gatti, S. and Fasano, A., *"The new epidemiology of celiac disease*". Journal of pediatric gastroenterology and nutrition, vol. 59, pp. S7-S9, Jul. 2014.

[5] Caputo, I., Barone, M.V., Martucciello, S., Lepretti, M. and Esposito, C., "*Tissue transglutaminase in celiac disease: role of autoantibodies*". Amino acids, vol. 36, pp. 693-699, Apr. 2009.

[6] de Sousa Moraes, L.F., Grzeskowiak, L.M., de Sales Teixeira, T.F. and Peluzio, M.D.C.G.,"*Intestinal microbiota and probiotics in celiac disease*". Clinical microbiology reviews, vol. 27, pp. 482-489, Jul. 2014.

[7] Di Sabatino, A., Vanoli, A., Giuffrida, P., Luinetti, O., Solcia, E. and Corazza, G.R., "*The function of tissue transglutaminase in celiac disease*". Autoimmunity reviews, vol. 11, pp. 746-753, Aug. 2012.

[8] Fernandez-Feo M, Wei G, Blumenkranz G, Dewhirst FE, Schuppan D, Oppenheim FG, Helmerhorst EJ. "*The cultivable human oral glutendegrading microbiome and its potential implications in coeliac disease and gluten sensitivity*."ClinMicrobiol Infect; 19:e386–94; 2013

[9] Gass, J., Ehren, J., Strohmeier, G., Isaacs, I. and Khosla, C., "*Fermentation, purification, formulation, and pharmacological evaluation of a prolyl endopeptidase from Myxococcus Xanthus"*implications for Celiac Sprue therapy,Biotechnology and Bioengineering, vol.92,pp.674-684, Dec.2005.

[10] Gerez, C.L., Rollan, G.C. and Valdez, G.D., "*Gluten breakdown by lactobacilli and pediococci strains isolated from sourdough".* Letters in Applied Microbiology, vol. 42, pp. 459-464, May 2006.

[11] Gupta, R., Reddy, D.N., Makharia, G.K., Sood, A., Ramakrishna, B.S., Yachha, S.K., Thapa, B.R., Banerjee, R., Anuradha, S., Dutta, U. and Puri, A.S.,"*Indian task force for celiac disease"*current status. World Journal of Gastroenterology:WJG, vol. 15, p. 6028, Dec. 2009.

[12] Helmerhorst, E.J. and Wei, G. "*Experimental strategy to discover microbes with gluten-degrading enzyme activities".* In Proceedings of SPIE--the International Society for Optical Engineering, vol. 9112, p.91120D, Jun 2014.

[13] Helmerhorst, E.J., Zamakhchari, M., Schuppan, D. and Oppenheim, F.G., "*Discovery of a novel and rich source of gluten-degrading microbial enzymes in the oral cavity*". PloSone,vol. 5, p.e13264, Oct 2010. .

[14] Janssen, G. et al. *"Ineffective degradation of immunogenic gluten epitopes by currently available digestive enzyme supplements."*PLoS One 10, e0128065; 2015

[15] Kim, N., "*Production of wheat gluten hydrolyzates by enzymatic process at high pressure*". Food Science and Biotechnology, vol. 26, pp. 1587-1593, Dec. 2017.

[16] König, J., Holster, S., Bruins, M.J. and Brummer, R.J., "*Randomized clinical trial: Effective gluten degradation by Aspergillus niger-derived enzyme in a complex meal setting".* Scientific reports, vol. 7, p.13100, Oct 2017.

[17] Kucek, L.K., Veenstra, L.D., Amnuaycheewa, P. and Sorrells, M.E., "*A grounded guide to gluten: how modern genotypes and processing impact wheat sensitivity"*. Comprehensive Reviews in Food Science and Food Safety, vol. 14, pp. 285-302, May 2015.

[18] Lähdeaho, M.L., Kaukinen, K., Laurila, K., Vuotikka, P., Koivurova, O.P., Kärjä-Lahdensuu, T., Marcantonio, A., Adelman, D.C. and Mäki, M.,"*Glutenase ALV003 attenuates gluten-induced mucosal injury in patients with celiac disease."* Gastroenterology, *146*(7), pp.1649-1658. 2014

[19] LorenzoPisarello, M.J., Vintiñi, E.O., González, S.N., Pagani, F. and Medina, M.S., "*Decrease in lactobacilli in the intestinal microbiota of celiac children with a gluten-free diet, and selection of potentially probiotic strains*". Canadian journal of microbiology, vol 61, pp. 32-37, Dec. 2014.

[20] Losurdo, G., Principi, M., Iannone, A., Ierardi, E. and Di Leo, A., "*The interaction between celiac disease and intestinal microbiota"*. Journal of clinical gastroenterology, vol. 50, pp. S145-S147, Nov. 2016.

[21] Lu, S.H.A.N., Marti, T., Sollid, L.M. and KHOSLA, C., "*Comparative biochemical analysis of three bacterial prolyl endopeptidases: implications for coeliac sprue"*. Biochemical Journal, vol. 383, pp.311-318, Oct 2004.

[22] Makharia, G.K., Verma, A.K., Amarchand, R., Bhatnagar, S., Das, P., Goswami, A., Bhatia, V., Ahuja, V., Datta Gupta, S. and Anand, K., 2011. "*Prevalence of celiac disease in the northern part of India: a community based study".*Journal of gastroenterology and hepatology, vol. 26, pp.894-900, May 2011.

[23] Makharia, G.K.D., "*Current and emerging therapy for celiac disease*". Frontiers in medicine, vol. 1, p. 6, Mar. 2014.

[24] McCarville, J.L., Caminero, A. and Verdu, E.F., "*Pharmacological approaches in celiac disease"*. Current opinion in pharmacology, vol. 25, pp. 7-12, Dec. 2015.

[25] Mitea, C., Havenaar, R., Drijfhout, J.W., Edens, L., Dekking, L. and Koning, F.,"*Efficient degradation of gluten by a prolyl endoprotease in a gastrointestinal model: implications for coeliac disease*". Gut, vol. 57, pp.25-32, Jan 2008.

[26] Petschow, B., Doré, J., Hibberd, P., Dinan, T., Reid, G., Blaser, M., Cani, P.D., Degnan, F.H., Foster, J., Gibson, G. and Hutton, J., "*Probiotics, prebiotics, and the host microbiome: the science of translation"*. Annals of the New York Academy of Sciences, vol. 1306, pp.1-17, Dec. 2013.

[27] Qiao, S.W., Bergseng, E., Molberg, Ø., Xia, J., Fleckenstein, B., Khosla, C. and Sollid, L.M.,"*Antigen presentation to celiac lesion-derived T cells of a 33-mer gliadin*

peptide naturally formed by gastrointestinal digestion". The Journal of Immunology, vol. 173, pp.1757-1762, Aug. 2004.

[28] Reif, S. and Lerner, A., "*Tissue transglutaminase—the key player in celiac disease: a review"*. Autoimmunity reviews, vol. 3, pp. 40-45, Jan. 2004.

[29] Rose, P.W., Prlić, A., Altunkaya, A., Bi, C., Bradley, A.R., Christie, C.H., Costanzo, L.D., Duarte, J.M., Dutta, S., Feng, Z. and Green, R.K., "*The RCSB protein data bank: integrative view of protein, gene and 3D structural information".* Nucleic acids research, p.gkw1000, Oct 2016.

[30] SiqueiraNeto, J.I., Costa, A.C.L.V., Magalhães, F.G. and Silva, G.S., *"Neurological manifestations of celiac disease*". Arquivos de neuro-psiquiatria, vol. 62, pp. 969-972, Dec. 2004

[31] Shan, L., Molberg, Ø. Parrot, I., Hausch, F., Filiz, F., Gray, G.M., Sollid, L.M. and Khosla, C., "*Structural basis for gluten intolerance in celiac sprue*". Science, vol. 297, pp.2275-2279, Sep. 2002

[32] Shan L, Qiao SW, Arentz-Hansen H, Molberg Ø, Gray GM, Sollid LM, Khosla C. *"Identification and analysis of multivalent proteolytically resistant peptides from gluten: implications for celiac sprue."* J Proteome Res; 4:1732–41; 2005

[33] Sharma, K., Mahajan, R., Attri, S. and Goel, G., "*Selection of indigenous Lactobacillus paracasei CD4 and Lactobacillus gastricus BTM 7 as probiotic: assessment of traits combined with principal component analysis"*. Journal of applied microbiology, vol. 122, pp. 1310-1320, May. 2017.

[34] Stefańska, I., Piasecka- Jóźwiak, K., Kotyrba, D., Kolenda, M. and Stecka, K.M., "*Selection of lactic acid bacteria strains for the hydrolysis of allergenic proteins of wheat flour*". Journal of the Science of Food and Agriculture, vol. 96, pp.3897-3905. Aug. 2016.

[35] Stepniak, D., Spaenij-Dekking, L., Mitea, C., Moester, M., de Ru, A., Baak-Pablo, R., van Veelen, P., Edens, L. and Koning, F., "*Highly efficient gluten degradation with a newly identified prolyl endoprotease: implications for celiac disease".* American

Journal of Physiology-Gastrointestinal and Liver Physiology, vol. 291, pp.G621-G629, Oct. 2006.

[36] Tack, G.J., Van de Water, J.M., Bruins, M.J., Kooy-Winkelaar, E.M., van Bergen, J., Bonnet, P., Vreugdenhil, A.C., Korponay-Szabo, I., Edens, L., von Blomberg, B.M.E. and Schreurs, M.W., "*Consumption of gluten with gluten-degrading enzyme by celiac patients: a pilot-study*". World journal of gastroenterology: WJG, vol. 19, p.5837, Sep. 2013.

[37] Tye-Din, J.A., Stewart, J.A., Dromey, J.A., Beissbarth, T., van Heel, D.A., Tatham, A., Henderson, K., Mannering, S.I., Gianfrani, C., Jewell, D.P. and Hill, A.V., "*Comprehensive, quantitative mapping of T cell epitopes in gluten in celiac disease"*. Science translational medicine, vol. 2, pp.41ra51-41ra51, Jul. 2010.

[38] Veeraraghavan, G., Leffler, D.A., Kaswala, D.H. and Mukherjee, R., "*Celiac disease 2015 update: new therapies*". Expert review of gastroenterology & hepatology, vol. 9,pp. 913-927, Jul. 2015.

[39] Yoshimoto, T. & Walter, R. *"Post-proline dipeptidyl aminopeptidase (dipeptidyl aminopeptidase IV) from lamb kidney. Purification and some enzymatic properties."*BiochimBiophysActa 485, 391–401; 1977.

[40] Zamakhchari M, Wei G, Dewhirst F, Lee J, Schuppan D, Oppenheim FG, Helmerhorst EJ. "*Identification of Rothia bacteria as gluten-degrading natural colonizers of the upper gastro-intestinal tract*". PLoS One;6:e24455–65, 2011