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Use of alternative protein sources in the bakery industry

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1. SUMMARY

Through the production of gluten free products, stakeholders of the food industry are striving to serve the needs of celiac patients as widely as possible with a continuously increasing product range, and to ensure the right nutrient intake. Our working group would like to contribute to this goal by developing bakery products that provide the body with a valuable source of protein and essential amino acids in addition to the required amount of carbohydrates. Our goal was to create a flour mixture that is gluten free, has a higher protein content than bread cereals, and is also suitable for use in the bakery industry, primarily for making wafers. Millet flour was chosen as the basis of the flour mixture, to which hemp, alfalfa and lupine flour were mixed as additional sources of protein.

After performing analytical studies regarding the amino acid composition, protein qualification values (Amino Acid Score – AAS, Protein Digestibility Corrected Amino Acid Score – PDCAAS) were determined, and these were used to optimize the flour mixtures.

Rheological measurements were carried out to examine the crunchiness of the wafers. Compared to the control samples, hardness data were not significantly altered by the addition of lupine or hemp flour, however, the addition of alfalfa softened the dough.

In addition to achieving a more favorable protein content, naturally, it was also our goal to manufacture a product with the right organoleptic properties for consumers. Compared to the control sample, the bitter taste of millet-based doughs was reduced and the hardness of the wafers improved by the addition of lupine. Mixing 35% of hemp seed flour with the millet flour also resulted in a bakery product with the right texture and and taste. Despite its good amino acid profile, the addition of alfalfa resulted in the deterioration of the rheological and organoleptic properties of the wafers.

2. Introduction

Cereals and bakery products made from them make up a significant part of our diet. These products satisfy a significant portion of our daily carbohydrate and protein needs. The most important raw material of the bakery industry is wheat, which, in addition to its beneficial physiological effects, is one of the most common sources of allergens among celiac patients. Celiac disease is a genetically determined autoimmune disease that affects the whole body, characterized by, in addition to antibodies to gluten, malabsorption, an abnormal intestinal villus structure and gastrointestinal symptoms of varying severity **[1]**. Celiac disease is one of the most common chronic diseases of the European population, affecting at least 1% of the population, and its incidence has been on the rise in recent decades **[2]**. Celiac disease is incurable, so the only solution for patients to avoid symptoms is a lifelong gluten free diet, meaning the

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exclusion of wheat, barley, rye and, in the case of certain patients, oats, as well as foods containing any form of these or made from them **[3]**.

The plant protein sources used by our working group in the course of this research are already used in the feed industry, so we are confident that our research results can also contribute to their introduction for human use. The amino acid compositions of the different flour mixtures are adjusted on the basis of the amino acid composition of the FAO/WHO reference protein by mixing the raw materials, promoting the utilization of the protein in the body **[4]**. Since plant protein sources are not complete, the purpose of flour blending is to supply the limiting essential amino acids by adjusting the proper ratio of the individual raw materials. For example, millet is rich in the essential amino acids methionine, cysteine, leucine and isoleucine, while lupine possesses outstanding amounts of lysine, leucine and threonine, so that the amino acid content of the mixture can be supplemented by adding these.

The aim of the research was to find the flour mixtures that have optimal nutrition values, are suitable for use in the bakery industry, more specifically, in the preparation of wafers, meet nutritional needs and have satisfactory organoleptic properties.

3. Literature review

3.1. Millet (Figure 1)

In terms of botany and physiology, millet is a monocotyledonous plant belonging to the order of Poales and family of Poaceae. The subfamily Panicoideae of millet includes several genera, but it is the species Panicum miliaceum L., i.e., proso millet that is most abundant worldwide.

Within this, several varieties are distinguished. Based on the differences between the panicle and the palea covering the grains, we can talk about variants with branched or squarrose, flag or packed panicles, as well as white (P. miliaceum album), red (P. miliaceum rubrum), gray (P. miliaceum griseum) or yellow (P. miliaceum luteum) millet, which also have different growing seasons **[5]**.

In terms of morphology, its roots are deep-penetrating tufted roots, the stem is a characteristic straw stalk that can grow up to 100 cm in height. Its panicle and the cover consisting of two smooth glumas encapsulating the grains are characteristic of the species and have distinctive colors. Its flower has a complex, clustered panicle that is self-pollinating and has a distinctive color. Its leaves are 1-2 cm wide and up to 50 cm long, the leaf sheaths are hairy. For germination, it needs half as much precipitation as cereals, but its heat demand at this time is higher **[6]**.

3.2. Lupine (Figure 2)

Lupine is a widely used legume that has been cultivated since ancient times and is a protein-rich fodder plant of outstanding biological value due to plant breeding.

From a botanical point of view, lupine belongs to the order of Fabales, the family of Fabaceae (Leguminosae), and within this to the genus Lupinus. We can find among them both perennial and annual species. Their bitterness or sweetness is determined by the amount of alkaloids contained in them. Their allergenic effect is due to the reserve proteins in the seed.

Lupine is a herbaceous plant with a taproot. The seeds germinate at low temperatures (2-6 °C). The number and width of the finger-shaped lanceolate leaves are different for the yellow, blue and white-flowered varieties. Their flowers differ not only in their color, but also in their smell. The fruit is an upright pod in which the seeds (depending on the species) are bone white or pink in color. In addition, the shape of the seeds, as well as the thickness and color of the fruit wall differ from species to species. The time and manner of harvesting of lupine species is determined by the goal of cultivation. As a result of plant breeding, today there are more than 450 varieties, and the protein content of the bred varieties is exceptionally high **[7]**.

3.3. Alfalfa (Figure 3)

Alfalfa has been an important crop of ours since ancient times and it belongs to the family of Fabaceae. Here it forms an independent genus, and its further taxonomical classification is complicated by a number of factors. Most of the species are wild alfalfa species, but from an industrial point of view, forage alfalfa is of the greatest importance. In Hungary and in Europe, the species with blue and variegated flowers (Medicago sativa L. and Medicago varia Martyn) are called forage alfalfa. They have very similar forage values, but their cultivation characteristics are slightly different, as alfalfa with the variegated flowers is a well-established mixture species that is less demanding than common alfalfa and can therefore be grown on weaker, sandy soils **[9]**.

In terms of morphology, its pealike flowers are arranged in a cluster, and their color may vary in color and shade from species to species. Most of the flowers are not self-pollinating, but are pollinated by wild bees and insects. Its fruit is a multi-seed pod with a varied (twisted or even sickle-like) shape. The ripeness of the seeds is indicated by a bright yellow or reddish-brown color. They have triply complex leaves, with a leaf-to-stem ratio characteristic of the species. (It is more favorable for the species with variegated flowers than in the case of species with

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blue flowers, but they have less fruit.) The stem is a characteristic hollow stem, and the root consists of main and secondary roots. Its high drought tolerance is due to the fact that the main root of the plant can penetrate to a depth of 16 to 20 meters in some cases, so it can absorb calcium, potassium and phosphorus together with the water. Its roots enrich the soil with nitrogen thanks to the Rhizobium bacteria living in symbiosis with it. For this reason, alfalfa is also used by many crop farmers as soil improvers **[9]**.

3.4. Hemp (Figure 4)

Hemp belongs to the family of Cannabinaceae. Previously, the genus Cannabis L. was classified in the family of Uritcaceae or Moraceae, but today they form a separate family with its related species, hops (Humulus L.).

Industrial hemp (subsp. culta) is clasified into four different races: Northern hemp race, Central Russian and Southern (Mediterranean) hemp group, and Asian hemp. Depending on the race, not only the place of cultivation, but also the fiber and tetrahydrocannabinol (THC) content, growing time and stem height of the plant differ.

Hemp is a dicotyledonous plant, the stems of some species can reach a height of up to 4-5 meters. The size of the branched taproots and lateral roots is different for male and female specimens, which can be explained by the different growing seasons. From an economic point of view, in addition to the seed, the woody, square-shaped, baggy stalk of the hemp covered with glandular hairs is also considered to be its product. The thickness of the stem is significantly determined by the nutrient supply of the area, as well as the gender of the plant. Varieties sown specifically as fiber hemp have a dense and unbranched stem. 24-25% of the total weight of hemp is made up by the leaves that consist of 7 to 11 smaller leaves. The number of small leaves that make up the finger-like composition of the leaves can avry depending on the species and the growth stage of the hemp. Its flower is unisexual and cross-fertilizing. The male flowers (stamens) are yellowish green in color and star-shaped when opened. In contrast, female (fruiting) flowers are simple and barely noticeable even from a short distance **[10]**.

4. Materials and methods

4.1. Test samples (Figure 5)

For the preparation of the wafers, in order to be gluten free, Dénes Natura millet flour was used as the base flour. To increase the protein content, Aby Bio Perfect Day hemp seed protein powder, Raab Vitalfood GmbH organic lupine protein flour and Zöldvér 100% alfalfa tablets were used, with the latter being pulverized before use in a crusher.

4.2. Methods and equipment used

Amino acid analysis was performed with an AAA 400 (Ingos Kft., Czech Republic) Automated Amino Acid Analyzer. During the separation, gradient elution was used with lithium citrate based buffers. The column was an OSTION LG ANB cation exchange resin (200x3.7mm). Chromatograms were evaluated using the CHROMULAN V 0.82 (PIKRON, Czech Republic) program.

Amino acid analysis was performed for both the flour raw materials and the finished wafers. Flour samples and the wafers prepared, after homogenization with a crusher, were hydrolyzed with a 6 M HCl solution at 110 °C for 24 hours in a dry block thermostat, followed by neutralization with a 4 M NaOH solution. After filing to mark and homogenization, the samples were filtered through fluted filter paper and then through a 0.22 μm syringe filter. The samples thus prepared were analyzed at the appropriate dilution. Due to acid hydrolysis, the indole group of tryptophan decomposes, therefore it cannot be determined using this sample preparation.

The rheological measurements of the wafers were carried out using a Stable Micro Systems TA.XT2i Texture Analyzer. The probe necessary for the 3-point-bend method and the sample holder were installed on the device, and verification of the force on the pressure head (19.61 N) and the distance (40 mm) was performed using a 2 kg weight (***Figure 6***).

At the start of the measurement, the probe moves downward at a speed of 1.0 mm/s until it reaches the surface of the sample. After reaching the surface of the product, the head travels downwards 5 mm at a speed of 3 mm/s. The pressure head then starts to move upwards at a speed of 10 mm/s. The measured data were evaluated using a computer connected to the instrument and the Exponent program. Fifteen parallel measurements were performed on each wafer at each measurement time point.

The water activity of the samples was measured using a Novasina MS1 type device, and their moisture content using a SARTORIUS MA 50 type rapid moisture analyzer.

4.3. Wafer preparation

During the preparation of the wafers, in order to be gluten free, millet flour was used as the base flour. The protein content of the flour mixes was increased by the addition of hemp seed, lupine and alfalfa flour. The preparation of the recipes was preceded by the amino acid analysis of the basic flours, the results of which were used to prepare the flour mixtures. The composition of the doughs is shown in ***Table 1***.

Based on ***Table 1***, the appropriate amounts of dry and wet raw materials were weighed and then homogenized in a mixing bowl. Following this, 33 g of the prepared dough was weighed into the preheated Trisa 734070 type wafer oven (***Figure 7***) and it was baked for 2 minutes.

The cooled wafers were stored at 24 °C and 42% relative humidity for 8 weeks and any changes in their moisture content and water activity were monitored by measurements.

5. Test results

5.1. Amino acid composition and protein content

The objective of our research was to produce wafers that are gluten free and have an increased and complete protein content. Being gluten free was ensured by the choice of raw materials. To verify that the protein content is complete and increased, after performing analytical tests to ascertain the amino acid composition, protein classification values (AAS, PDCAAS) were also determined.

In the first step, amino acid scores (AAS) were calculated based on essential amino acids **[12]**. This quotient determines the relative deficiencies of the amino acids that make up the given protein. During its calculation, the amino acid content (g/100 g protein) of the protein source tested is divided by the amino acid content of the reference protein (g/100 g protein). The theoretical reference protein, which contains the essential amino acids in an ideal proportion, was developed by the experts of FAO/WHO **[4]**. Since amino acid requirements vary with the individual's age, compositions for infants (less than 0.5 years of age), little children (between 1-2 and 3-10 years of age), adolescents (between 11-14 and 15-18 years of age) and adults (over 18 years of age) were determined by the experts **[13]**. When evaluating our samples, the composition of the reference protein for adults was taken into consideration.

Figure 9 shows the essential amino acid composition of the flour samples tested and the reference protein recommended by the FAO/WHO for adults.

In the case of millet flour, lower results were obtained for lysine, valine and isoleucine compared to the reference sample. Lysine was contained in the smallest amounts in millet and hemp seed flour, so it can be considered the limiting amino acid in the case of these samples. The threonine content exceeded the threonine content of the reference sample, but it was the lowest in millet flour, compared to the other flour samples. The ground millet tested contained leucine the highest amount (12.9 g/100 g protein), which is also supported by the measurement results of Kalinova and Moudry (2006) **[14]**. The amounts of sulfur-containing compounds in the sample are also remarkably high, thus it is able to supplement the limiting cysteine and methionine content of legumes when consumed together with those.

In the case of lupine and alfalfa, sulfur-containing amino acids have been shown to have limiting properties. In addition, the lysine, isoleucine and valine contents of lupine are lower than the reference values. However, the amount of lysine is still almost twice as high (4.24 g/100 g protein) as the value obtained for millet, and it is also richer in threonine, so their combined consumption can result in a more favorable essential amino acid ratio.

Of the samples examined, the most lysine was found in ground alfalfa (6 g/100 g protein), which is also important, because this amino acid is essential for the proper growth of children. In addition, it has significantly higher contents of tryptophan, valine and threonine compared to the reference values and the flours tested.

Hemp seed flour is deficient in isoleucine, lysine and valine compared to the composition of the reference protein. Lysine can be considered its limiting amino acid, which is probably due to the inadequate nitrogen supply of the plant **[15]**. Its methionine and cysteine contents exceed the values measured for the reference sample and the ground legumes, so their combined use provides an opportunity to manufacture a product containing a complete protein.

The utilization of the protein content of foods is influenced by a number of factors. The amount and amino acid composition of proteins actually only allows a comparison, as utilization occurs from food as a complex system. Digestion and absorption do not affect only proteins, but a system consisting of proteins, carbohydrates, fats and other macro- and microelements consumed as food. The utilization, digestion and absorption of plant proteins can be a lengthy process due to the presence of fibers and antinutritive components, while the access to animal proteins can be hampered by fats. Even with animal experiments, we can only estimate what happens to food in the gastrointestinal tract. Nutrition science studies are now facilitated by digestive models that mimic the functioning of the human gastrointestinal tract and that study the digestibility and absorption of complex foods. With the help of such models, experts can determine the digestibility coefficient (%) for certain purified proteins, foods of plant and animal origin (e.g., whey protein, soy protein isolate, green peas, cow's milk, wheat flour), or even ready-to-eat meals. The digestibility coefficients of the flours studied were collected from the literature (***Table 2***) in order to be able to perform further evaluations.

The method adopted by the FAO/WHO for the determination of the biological value of proteins is the Protein Digestibility Corrected Amino Acid Score (PDCAAS). The PDCAAS value is determined by correcting the amino acid score (AAS) of the given protein with the digestibility of the test sample **[12]**. The PDCAAS value cannot be higher than 1.00.

The flour samples examined did not reach 1.00 on the PDCAAS scale (***Table 2***), the PDCAAS value of alfalfa was 0.74, the value of hemp seed flour was 0.67, while in the case of lupine and millet flour, low values were obtained (0.30-0.51).S

Based on this, flour mixtures were prepared to supplement the limiting essential amino acids by adjusting the ratio of the individual raw materials. Of the essential amino acids, millet is rich in methionine, cysteine, leucine and isoleucine, lupine has an outstanding content of lysine, leucine and threonine, so the protein content of the mixture can be made complete by adding these. To facilitate the compilation of the mixtures, an algorithm was developed using the measurement data; by changing the proportion of flours in the algorithm, the PDCAAS valule of the mixture can be calculated (***Table 3***).

The Daily Reference Values (DRV%) for nutrients were developed by the U.S. Food and Drug Administration; this allows us to estimate the percentage of our daily protein needs that is covered by the protein in the food we intend to consume. For the calculation, the protein content and PDCAAS value of the given food and the daily protein requirement, which is uniformly interpreted as 50 g, are taken into account. The formula for the calculation:

If the DRV% value of the food is below 10%, it is considered an "*inadequate protein source*", if it is between 10 and 19.9%, it is classified as a *„good protein source"*, while above 20% it is considered an *„excellent protein source"* **[20]**. The DRV% values of the assembled flour mixtures were calculated and the corresponding classifications were determined for food portions of 100 g (***Table 3***).

The data in ***Table 3*** show what the classification of the raw materials as protein sources will be when mixing millet flour with lupine, hemp seed and alfalfa flour in different proportions. The classification indices of additional mixing ratios that met the requirements for both dough preparation and wafer baking were also selected and calculated.

The flour ratios used after the test dough production and test baking are shown in ***Table 4***.

After optimizing the raw materials, the dough was prepared, followed by a test baking and the baking of the wafers to the proper color. The protein content of the finished products was measured by the Kjeldahl method (***Figure 10***).

5.2. Rheological, organoleptic, moisture, water activity investigations

The results of the rheological measurements are illustrated in ***Figure 11***.

In the case of wafers, crunchiness is an important organoleptic property, therefore the hardness of the wafers made from the four different flour mixtures was examined. There was no significant difference between the hardness of wafers containing lupine (CSK) and hemp (KK) and the millet-based control wafer (Control). However, a significant difference could be found between the samples containing alfalfa (LK) and lupine (CSK), as well as alfalfa (LK) and hemp (KK) using the t-test. The hardness of the wafer containing alfalfa flour (LK) could be slightly improved by the addition of lupine (KEV). In this way, the rheological and nutrition properties of the wafer made from mixed flour (millet:lupine:alfalfa) also proved to be better.

The moisture content of the wafers significantly affects their shelf life. If the moisture content of the product is below 12%, the wafers can be stored for 60 days without any special preservation process. The prepared samples were stored for 8 weeks at 24 °C and 42% relative humidity. The moisture content did not reach 12% even by week 8, the highest value (8.39%) was obtained in the case of wafers made from flour mixed with alfalfa. This sample had the highest initial moisture content, and the result of the hardness measurement also corresponded to this. Water activity values remained below 0.6 at week 8 as well (***Figure 12***).

In addition to achieving a more favorable protein content, it was important that the wafers have favorable organoleptic properties. Millet flour alone gives the product a bitter aftertaste, which limits its use in the bakery industry. By adding lupine, the bitter taste can be reduced and the hardness and crunchiness of the wafers can also be improved. Sensory judges unanimously rated wafers containing lupine the best, with their bright yellow color, hardness and taste ranking them first among the samples. The combined use of hemp and millet flour also resulted in a product with better organoleptic properties compared to the control millet wafer as it tasted more favorable and less bitter. Alfalfa gave the product a green color that is foreign to the wafer and a grassy taste, and its texture did not exceed our expectations either. Adding lupine to this mixture improved the organoleptic properties.

Based on the results of analytical, rheological and organoleptic tests, we believe that we have been able to compile wafer recipes from the selected ingredients that meet gluten free requirements and have a higher protein content than similar wheat-based products. The amino acid content of the wafers was supplemented by mixing different plant-based flours, thus creating a protein source that can be utilized well. Due to the texture suitable for the wafers and the favorable organoleptic properties, the products can be popular, especially among children. Their storability and the preservation of their crunchiness ensures the relatively long shelf life of the products. We plan to continue our research by testing additional flavors and modified flour ratios, and by compiling recipes for sweet and salty wafers.

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