**Changes in protein profile, bioactive potential and enzymatic activities of gluten-free flours
obtained from hulled and dehulled oat varieties as affected by germination conditions**

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17 **ABSTRACT**

18 There is a growing interest in the development of flours from sprouted cereals due to their enhanced
19 nutritional and bioactive potential, but this research field has been scarcely explored. This study aimed to
20 investigate the influence of germination conditions on protein profile, bioactive potential and enzymatic
21 activities related with quality in oat gluten-free flours. Germination temperatures and duration ranging 12-20
22 °C and 24-216 h, respectively, were studied in hulled and dehulled oat varieties. Germination increased free
23 phenolics, antioxidant capacity, protein hydrolysis and α -amylase activity in oat flours, particularly at high
24 temperatures and longer times. β -glucan content was retained at short germination times (24-96 h) but it was
25 reduced beyond this time period. Lipase activity increased during germination in the hulled variety and
26 declined in the dehulled one. Multi-response optimization of germination conditions allowed to identify the
27 most suitable germination conditions for maximizing nutritional and bioactive quality of sprouted oat flours
28 (16 °C-216 h and 18 °C-156 h for hulled and dehulled varieties, respectively). This study provides useful
29 information regarding the production of gluten-free flours from sprouted oat with improved nutritional and
30 healthy attributes.

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36 *Keywords:* Oat, germination, gluten-free flours, bioactive potential, enzymatic activity.

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1. Introduction

Healthy and sustainable eating is one of the basic conditions needed to prevent the scaling incidence of lifestyle-related diseases such as obesity, diabetes and hypertension. Oat (*Avena sativa* L.) intake has been associated with a strong reduction of markers of cardiovascular disease (Hollnder, Ross, & Kristensen, 2015). This beneficial effect can be attributed to the high content of dietary fiber, in particular β -glucan (Kaur, Bhardwaj, Kapoor, Kaur-Grewal, 2019), and to the presence of phenolic compounds that exhibit antioxidant and anti-inflammatory activities (Tian et al., 2010). Oat has also an excellent nutritional profile due to its high protein (9-11%) and unsaturated fatty acid contents (Chappe et al., 2017).

Despite its nutritive and health benefits, oat is mainly employed for animal feeding and only 14% of the total oat production in Europe is used for human nutrition (Ferguson, Stojanovski, MacDonald-Wicks, & Garg, 2020). Innovations in oat-derived functional products might boost oat breeding and consumption. Although oat does not inherently contain gluten, it is frequently contaminated with gluten-containing cereals during cultivation, transportation, storage or processing. Since increasing acreage for production of oats under gluten-free conditions has been reported (Smulders, et al., 2018), the development of food products derived from gluten-free oats would be of great interest for celiac consumers.

Sprouts are perceived as healthy foods by consumers and meet many needs for a modern trendy lifestyle. Despite they are usually consumed as fresh products, the production of flours from sprouted grains is a promising source of innovation for food industry. Existing scientific evidence have demonstrated the positive effects of germination (sprouting) on cereal nutritional and bioactive properties (Rico et al., 2020). However, the extent of metabolic changes in grains during germination depend on multiple factors, mainly grain genetic diversity, presence/absence of hull and germination conditions (Cceres, Martnez-Villaluenga, Amigo, & Frias, 2014; Swanston & Middlefell-Williams, 2012). Therefore, the selection of the most suitable cultivar and optimization of sprouting conditions deemed crucial for enhancing desirable and reducing detrimental changes affecting the quality of sprouted cereal flours. So far, a comprehensive study aimed at identifying the most appropriate germination conditions for boosting the bioactive and nutritional properties of oat-derived flours has not been performed. The aim of the present study was to investigate the influence of germination period and temperature on protein profile, bioactive potential (β -glucan and phenolic contents),

66 antioxidant capacity, and on the activity of enzymes (α -amylase, protease and lipase) related with quality in
67 flours produced from hulled and de-hulled gluten-free oat varieties. Multi-response optimization approach was
68 used to identify the optimal germination conditions that maximize sprouted oat flour quality.

69 **Materials and Methods**

70 *2.1. Materials.*

71 Hulled (variety Barra) and dehulled (variety Meeri) gluten-free oat grains were kindly provided by Glanbia
72 (Kilkenny, Ireland) and Helsinki Mills (Vaasa, Finland), respectively. Oat grains were carefully cultivated,
73 transported and processed to avoid gluten-contamination. All analytical grade chemicals used were provided
74 by Sigma-Aldrich (Madrid, Spain) unless otherwise specified.

75 *2.2. Grain germination.*

76 Oat germination was conducted as previously reported (Cáceres et al., 2014) with some modifications. Twenty
77 grams of oat seeds were used for germination and soaking (1:6 ratio, w/v) was performed at room temperature
78 ($20\text{ }^{\circ}\text{C} \pm 2\text{ }^{\circ}\text{C}$) for at 4 h. Germination was performed in a cabinet (model G-120, Snijders Scientific,
79 Netherlands) in dark at different temperatures (12, 14, 16, 18, and $20\text{ }^{\circ}\text{C}$) and duration (24, 60, 96, 156, and
80 216 h). Germination process was carried out in triplicate for each experimental condition and sprouts were
81 freeze-dried (Virtis Company, INC Gardiner, NY, USA). Oat seeds and freeze-dried sprouts were milled,
82 passed through a mesh sieve (0.5 mm pore size) and stored under vacuum at $-20\text{ }^{\circ}\text{C}$.

83 *2.3. Total protein content.*

84 Nitrogen content was analyzed by the Dumas method, using a nitrogen analyzer (LECO Corp., St. Joseph,
85 MI). A factor of 5.83 was used for protein content conversion. The results were expressed as g/100 g dry
86 matter (d.m.)

87 *2.4. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE).*

88 Protein profiles of oat flours were determined by SDS-PAGE in NuPAGE[®] Novex 4-12% Bis-Tris Gels, using
89 NuPAGE[®] MES-SDS as running buffer and Novex[®] Sharp Prestained Protein Standard (Invitrogen, Madrid,
90 Spain) as molecular weight marker.

91 *2.5. Beta-glucan content.*

92 The β -D-glucan content was quantified by 1,3:1,4 mixed-linkage β -glucan kit (Megazyme, Wicklow, Ireland),
93 following the manufacturer instructions. This method is accepted by the American Association of Cereal
94 Chemists (AACC method 32-23.01) and the Association of Official Analytical Collaboration (AOAC
95 International method 995.16). The results were expressed as mg β -glucan/100 g d.m.

96 *2.6. Free phenolic content.*

97 Free phenolic compounds (FPC) were extracted and quantified using Folin–Ciocalteu’s phenol reagent as
98 described previously (Cáceres et al., 2014). The results were expressed as mg of gallic acid equivalents
99 (GAE)/100 g d.m.

100 *2.7. Antioxidant capacity.*

101 Antioxidant capacity was determined by the oxygen radical absorbance capacity (ORAC) method following
102 the procedure previously reported (García-Mora et al., 2014). The results were expressed as mg of Trolox
103 equivalents (TE)/100 g d.m.

104 *2.8. α -amylase activity.*

105 The determination of α -amylase activity was carried out by the α -amylase assay procedure (Amylase SD
106 method, Megazyme, Wicklow, Ireland), following the manufacturer instructions. This method was based on
107 AOAC method 2002.01 and AACC method 22-02.01. The Results were expressed as amylase units (U)/g d.m.
108 One unit of activity was defined as the enzyme amount that releases 1 μ mol of p-nitrophenol/min from 4,6-O-
109 ethylidene- α -4-nitrophenyl-maltoheptaoside (EtPNG7).

110 *2.9. Protease activity.*

111 Enzyme extraction was carried out in 50 mM sodium acetate buffer (pH 5.5), containing 0.02 mM L-cysteine.
112 The flour samples were homogenized (0.5 g flour/ 4 mL of buffer) in a T25 Ultra-Turrax (Ika, Königswinter,
113 Germany) at 4 °C for 1 min. The blended extract was centrifuged (10,000 rpm, 15 min, 4 °C) and the Pierce
114 Fluorescent Protease Assay Kit (Thermofisher Scientific Inc.) was used for evaluating protease activity in the
115 supernatant, with some modifications. The sample extracts were incubated for 10 min at 37 °C in darkness,
116 and the pH was adjusted above 6 using 2 % (w/v) of Tris buffer (pH 9). Then, fluorescence was measured in
117 at λ excitation of 485 nm and λ emission of 538 nm. Trypsin was used as standard (0-500 ng/mL). The results

were expressed units (U)/g d.m. One unit was defined as the amount of enzyme able to hydrolyse one μmol of N- α -tosyl-L-arginine methyl ester (TAME) per minute.

2.10. Lipase activity.

An amount of 0.5 g of oat flour sample defatted with hexane was mixed with 0.2 M Tris-HCl, pH 8.5 (3 mL). The mixture was homogenized in a T25 Ultra-Turrax (Ika, Königswinter, Germany) at 4 °C for 4 min and centrifuged (10,000 rpm, 15 min, 4 °C). Oat extracts (40 μL) were incubated with 100 mM Tris-HCl containing 5 mM CaCl_2 , pH 7 (140 μL) and dimethylsulfoxide (DMSO, 20 μL) into 96-well microplates at 37 °C for 15 min. As a sample control, buffer (175 μL) was incubated with DMSO (20 μL). Subsequently, 10 mM *p*-nitrophenol butyrate (pNPB, 5 μL) was added in every plate except sample control. The absorbance was measured at 405 nm and data were collected every 5 min for a total of 15 min. *p*-nitrophenol was used as standard. Results were expressed as lipase units/g d.m. One unit was defined as the amount of lipase necessary to release one mmol of *p*-nitrophenol/min at 37°C from pNPB.

2.11. Experimental design and data modelling.

Response Surface Methodology (RSM) was used to study the influence of germination temperature and time (independent variables) on contents of protein, β -glucan, free phenolic compounds, antioxidant capacity, and enzymatic activities (response variables) in sprouted flours obtained from the two studied oat varieties. A face-centred composite design, with three factor levels (-1, 0, +1), two axial points and three replications of the central point was used.

The correlation between germination temperature and time and the response variables were expressed by a second-order polynomial equation (Eq. 1), as shown below:

$$Y = \beta_0 + \sum_{i=1}^2 \beta_i X_i + \sum_{i=1}^2 \beta_{ii} X_i^2 + \sum_{i=1}^2 \beta_{ij} X_j \quad (\text{Eq. 1})$$

where Y represents the predicted response values and β_0 , β_i , β_{ii} , β_{ij} are the regression coefficients for intercept, linear, quadratic and interaction terms, respectively. X_i and X_j represent both independent variables (temperature and time). The significance of regression coefficients was determined by ANOVA.

The coefficient of determination (R^2) and adjusted coefficient of determination ($\text{Adj-}R^2$) were used to determine the degree of fitness of the regression models to experimental data. The developed quadratic

models were statistically validated by multivariate ANOVA, Fisher's *F*-test and the *p* value (chosen at 95% confidence interval level), as well as by lack-of-fit. Three-dimensional response surface plots were generated to graphically visualize the impact of germination conditions on response variables. The Derringer's Desirability function (D) was used to determine the optimal germination conditions that maximize the content of protein, β -glucan, free phenolic compounds, protease and antioxidant activities, minimizing, at the same time, the activities of α -amylase and lipase.

2.12. Statistical analysis.

Data were expressed as the mean $\pm$ standard deviation of three replicates. One-way analysis of variance (ANOVA) and Duncan's test (Statgraphics Plus software version 5.1, Statistical Graphics Corporation, Rockville, MD, USA) were used to determine differences between mean experimental values. The confidence level was chosen at 95% ($\alpha = 0.05$). The Statistica v7.0 software (Statsoft, CA, USA) was employed for the statistical analysis of RSM models.

3. Results and Discussion

3.1. Effect of germination conditions on protein content and profile in oat flour.

Control flour obtained from var. Barra (Table 1) exhibited a lower protein content than that from var. Meeri (Table 2). Hull constitutes between 23 and 35% of oat kernel and it is rich in cellulose and poor in proteins (Burrows, 2011), fact that could explain the lower protein levels in flours obtained from the hulled variety. The protein content sharply increased during in both varieties, reaching sprouted flours protein levels ranging 9.47-12.69 g/100 g d.m. (var. Barra, Table 1) and 11.03-11.92 g/100 d.m. (var Meeri, Table 2). The magnitude of the changes produced in the protein content clearly depended on the germination conditions. Germination at 16 °C for 216 h and 20 °C for 96 h produced the highest protein accumulation in varieties Barra (Table 1) and Meeri (Table 2), respectively. The losses of dry weight at high temperatures and times, mainly in the form of carbohydrates through respiration, could be responsible for the apparent rise of protein levels. The synthesis of new proteins during germination might also contribute to the enhanced protein levels in sprouted oat flours.

As it is illustrated in Fig. 1, the flours from both varieties presented similar protein profile, showing bands ranging from 14 to 110 kDa. However, Meeri variety flours (Fig. 1b) presented lower intensity and number of protein bands, probably due to the lack of hull. Globulins 3S (48-52 kDa), 7S (50-70 kDa), 12S α -

171 polypeptide (32-35 kDa), 12S β -polypeptide (22-24 kDa) and avenins (17 and 37 kDa) were clearly visible
172 (Fig. 1a,b, Lane 1).

173 Germination induced a partial hydrolysis of some oat proteins that was reflected by the loss of
174 intensity of several bands (Fig. 1), phenomenon that was more pronounced in var. Meeri (Fig. 1b). Longer
175 germination periods (96-216 h) combined with higher temperatures (16-20 °C) caused a more pronounced
176 disappearance of large protein bands (>40 kDa) (Figure 1a, lanes 5, 6, and 8; Figure 1b, lanes 3, 5, 6 and 8).
177 Germination for longer times (156-216) at 16-18 °C also reduced the intensity of bands <20 kDa, effect that
178 was more evident for var. Barra (Fig. 1a, lanes 5 and 6). Proteolysis during oat germination might enhance the
179 health promoting properties of the derived sprouted flours since a recent study has demonstrated that
180 germination favors the release of bioactive peptides from soybean sprouts in the gastrointestinal tract
181 (González-Montoya, Hernández-Ledesma, Mora-Escobedo, & Martínez-Villaluenga, 2018).

182 3.2. *Effect of germination conditions on β -glucan content in oat flour.*

183 Oat flours from varieties Barra and Meeri presented a β -glucan content of 2.03 and 3.45 mg/100 g d.m.
184 (Tables 1 and 2, respectively), which are in the range of values reported in other oat cultivars (Chappell et al.,
185 2017). Levels of this bioactive compound in oat are strongly influenced by genetic diversity and
186 environmental factors during growing and harvesting periods (Stewart & McDougall, 2014), findings that
187 explain the differences between both oat varieties. Moreover, the presence of hull in Barra variety can
188 partially explain its lower β -glucan content, since hull is mainly composed by insoluble fiber. Similarly,
189 Wilhelmson et al. (2001) observed higher levels of this compound in a naked oat variety than in a hulled one.

190 Germination for short periods (24-96 h) combined with medium temperatures (12-16 °C) retained β -
191 glucan levels in oat, but longer germination times (156-216 h) caused reductions of 47-64 % of this compound
192 (Tables 1 and 2). The high retention of β -glucan in sprouted oat flours observed in the preset study is of
193 particular interest considering that much higher β -glucan losses (up to 67%) were observed in oat and barley
194 germinated at 15-16 °C for 96 h (Betts et al., 2017; Wilhelmson et al., 2001), being this compound almost
195 totally degraded beyond this period of time (Willhemson et al., 2001). Grain endogenous β -glucanases
196 increase their activity during germination (Betts et al., 2017), causing the hydrolysis of β -glucan. β -glucan

197 degradation is slow in the early stages of germination, until a sufficient β -glucanase activity has developed,
198 being faster after 96 h of germination, as it has been showed by our results.

199 It has been established that daily consumption of ≥ 3 g of oat β -glucan reduces blood total and LDL-
200 cholesterol concentrations (Ferguson et al., 2020), and 4 g of β -glucan per 30 g of available carbohydrates
201 consumed in a meal decreases postprandial glycemic response (EFSA, 2011). Thus, the retention of β -glucan
202 in sprouted oat flours is desirable for enhancing their health benefits.

203 3.4. *Effect of germination conditions on the content of free phenolic compounds (FPC) in oat flour.*

204 FPC contents in control flours were very similar (Tables 1 and 2), values much higher than those observed in
205 other oat varieties (Li et al., 2017). Germination process caused a significant ($p \leq 0.05$) enhancement of FPC
206 content in sprouted flours, which was between 1.6-fold and 2.8-fold higher in than control samples when
207 germination took place at high temperatures (16-18 °C) for longer times (156-216 h) (Tables 1 and 2). Higher
208 phenolic contents were observed in Meeri sprouted flours than in var. Barra, suggesting that the absence of
209 hull favored the accumulation of phenolics. The release of bound phenolic forms due to the activation of
210 hydrolytic enzymes that degrade cell wall during germination (Tian et al., 2010) is partially responsible of the
211 enhancement of FPC levels in germinated oat. Moreover, *de novo* synthesis of phenolic compounds during
212 sprouting by activation of shikimate and phenylpropanoid pathways, as it has been observed in wheat (Zilic et
213 al., 2014), might contribute also to the highest FPC levels in oat sprouted flours.

214 3.5. *Effect of germination conditions on the antioxidant activity in oat flour.* Control flours exhibited
215 antioxidant capacity values in the range of 537-575 mg TE/100 g d.m. (Tables 1 and 2), and not important
216 differences were observed between both cultivars, suggesting that the presence of hull did not exert a notable
217 influence on the oat antioxidant potential. Low concentration of antioxidant compounds in the hull (Emmons
218 and Peterson, 1999) may explain these results.

219 Germination caused a noticeable enhancement ($p \leq 0.05$) of antioxidant capacity in oat, which was
220 between 1.4-4.5-fold higher than in non-germinated grain. The extent of the increase depended on the oat
221 variety and germination conditions. ORAC values were higher for Meeri sprouted flours, likely due to the
222 greater contents of FPC, but also β -glucan, compound that also exert significant antioxidant capacity (Kofugi,

223 Aoki, & Murata, 2012), in these sprouted flours (Table 2). High temperatures (16-18) and longer germination
224 times (156-216 h) positively influence the antioxidant capacity of sprouted oat flours. A gradual increase of
225 antioxidant activity during oat germination at 25 °C for 24-72 h were also observed by Kaur & Gill (2020).
226 However, to the best of our knowledge, this is the first study investigating the influence of germination
227 temperature on the antioxidant potential of oat flour. Recently, Shahverdi et al. (2019) have reported higher
228 antioxidant enzyme activities at high temperatures in stevia seedlings, phenomenon that could explain the
229 larger antioxidant capacity in oat sprouted flours obtained at higher temperatures.

230 3.6. *Effect of germination conditions on the α -amylase activity in oat flour.*

231 Control flours exhibited low α -amylase activity (0.16 and 0.65 U/g d.m., respectively) (Tables 1 and 2), in
232 agreement with previous literature data (Krapf, Kandzia, Brühn, Walther, & Flöter, 2019; Mäkinen, Zannini,
233 & Arendt, 2013). Germination process triggered a significant increase ($p \leq 0.05$) in α -amylase activity of oat
234 flours, reaching levels in the range of 0.92-30 U/g d.m., depending on germination conditions and the oat
235 variety (Tables 1 and 2). Our results clearly evidenced that germination conditions exerted different effects in
236 α -amylase activity in both varieties, suggesting a strong influence of genotype and presence/absence of hull.
237 For hulled Barra variety, the highest activity ($p \leq 0.05$) was observed at 14 °C for 156 h and the lowest at 14-16
238 °C for 24-60 h (Table 3). In var. Meeri flours, temperatures of 16-18 °C and times of 156-216 h affected
239 positively α -amylase activity. The maximal α -amylase activity in sprouted oat flours was observed at
240 temperatures lower
241 than those recently reported in a Canadian oat variety by Krapf et al. (2019), indicating again the activity of
242 this enzyme during germination depended on the cultivar studied.

243 Alpha-amylase plays a key role during germination since it catalyzes the hydrolysis of α -1,4
244 glucosidic linkages of starch, yielding maltose and glucose necessary for seedling development. The activity
245 of this enzyme increased considerably during oat sprouting (Krapf et al., 2019) but it is also *de novo*
246 synthesized during this process, as it has been reported in wheat (Olaerts, De Bondt, & Courtin, 2017). High
247 glucose content in sprouted flour can increase its glycemic index (GI). Foods with low GI are beneficial due to
248 low postprandial glucose response compared to foods with a high GI, reducing the risk of type 2 diabetes and

obesity (Kumar, et al., 2020). Hence, the careful selection of germination conditions is crucial to modulate α -amylase activity in oat for obtaining healthier sprouted flours with lower GI.

3.7. Effect of germination conditions on the protease activity in oat flours.

Both control flours presented similar protease activity (0.3 U/g d.m.) (Tables 1 and 2), which was increased drastically during germination, reaching values between 5-fold and 40-higher than in control flours. Longer germination times (156-216 h) applied at 16-18 °C conducted to the highest proteolytic activity in both varieties (Tables 1 and 2), results in agreement with largest protein degradation observed by SDS-PAGE (Figure 1). Mäkinen et al. (2013) also observed a 3-fold enhancement of protease activity in oat sprouts obtained at 15 °C for 5 days. However, this is the first study aimed at evaluating the combined influence of germination temperature and time on protease activity in sprouted oat flours. Storage proteins mobilization during germination is initiated by *de novo* synthesized proteases, providing small peptides and amino acids that are used by the embryo to form new structural proteins (Mäkinen et al., 2013). Higher proteolytic activity in the hulled variety sprouted flour (Table 3) was observed in the present study, results in accordance with those reported by Hosseini, Kadivar & Shahedi (2010). The presence/absence of hull determined different moisture retention during oat germination, phenomenon that could contribute to the differences observed in protease activity between both varieties.

3.8. Effect of germination conditions on the lipase activity in oat flours.

Lipase activity in oat grains was high (3.3-3.6 U/g d.m.) (Tables 1 and 2), as recently reported (Liu et al., 2019). In accordance with previous literature data (Mäkinen et al., 2013), lipase activity experienced a significant increase ($p \leq 0.05$) during germination of variety Barra (Table 3), reaching the highest activity at longer germination times (96-216 h). Surprisingly, the statistical analysis indicated that lipase activity decreased in sprouted var. Meeri flour during germination, phenomenon also observed in an early study performed in dehulled oat grains by Peterson (1999). Our results are interesting since they suggest that there must be an important lipase activity in oat hull. Lipase hydrolyse triglycerides to free fatty acids, that are prone to oxidation and cause rancidity of cereal flours (Liu et al., 2019). According to our results, the use of dehulled oat grains is desirable to obtain sprouted oat flours with increased stability and longer shelf life.

3.9. Multi-response modeling of germination temperature and time and effects in sprouted oat flours

276 In the present study, RSM has been applied to oat germination process with the aim to better understand the
277 effect of germination conditions on nutritional and bioactive potential of sprouted oat flours as well as on the
278 activity of key enzymes related with flour quality. In this sense, response variables (contents of protein, β -
279 glucan and FPC, antioxidant capacity and enzymatic activities) were modelled as a function on germination
280 temperature and time. The second polynomial regression equations obtained are shown in Table 3. Non-
281 significant regression coefficients ($p>0.05$) were discarded from the equations. All models showed a non-
282 significant ($p>0.05$) lack-of-fit and p -values below 0.05 determined by ANOVA (results not shown),
283 indicating their reliability to model the experimental data. Large F -values obtained for models (Table 3) also
284 indicated their statistical significance. The degree of fitness of the models to the experimental data obtained
285 were evaluated by the coefficient of determination (R^2) and adjusted coefficient of determination (R^2 -adj). All
286 models exhibited values for both coefficients higher than 0.75, indicating their suitability to explain the
287 variability of experimental data. The only exception were the models obtained for protein content (in both
288 varieties) and that for lipase activity (in variety Meeri) that showed a significant lack-of-fit, and low F , R^2 and
289 R^2 -adj values, which implies poor agreement between experimental and predicted values. Therefore, these
290 models were not considered for the multi-response optimization using the desirability function.

291 As it is evident from the Table 3, all the models obtained fitted to a second-order polynomial
292 equations, indicating that germination temperature and time exhibited significant linear and quadratic effects
293 on most response variables, being the interaction between both factors also significant. The regression
294 equations obtained for each response variable were very different for both oat varieties, suggesting that the
295 effect of germination conditions strongly depended on the grain genetic diversity and the presence/absence of
296 hull, as it was observed in the experimental data (Tables 1 and 2).

297 To facilitate the visualization of the influence of germination conditions on the parameters studied in
298 oat flours, three-dimensional response surface graphs were depicted (Figures 2 and 3). The figures clearly
299 evidenced the highest reduction of β -glucan content in both oat varieties as the germination time and
300 temperature increased (Figure 2a and 3a). Contrarily, high temperatures and larger germination times caused
301 the highest FPC (Figure 2b and 3b) and antioxidant activity (Figures 2c and 3c) accumulation in both oat
302 varieties. The 3D-plot obtained for α -amylase was different in both varieties, showing a pronounced quadratic

influence of germination temperature and time in var. Barra (Figure 2d), while for var. Meeri, time had mainly a linear effect and temperature exerted both linear and quadratic effects (Figure 3d). Similarly, effects of germination conditions on protease activity depended on the oat variety considered. Short germination times combined with high temperatures or longer times combined with low temperatures positively affected protease activity in var. Barra, while higher temperatures and longer periods increased this activity in var. Meeri. The presence/absence of hull might determine the different effects of germination conditions on α -amylase and protease activities in both varieties. Lipase activity in var. Barra increased as germination temperature and time increased (Figure 2f). The three-dimensional plot was not generated for var. Meeri, since the model exhibited low reliability (Table 3),

3.10 Identification of optimal germination conditions. Desirability function is the most currently used multicriteria methodology in the optimization of processes with more than one response. It is based on the simultaneous consideration of all responses and on the concept that a model is acceptable if any response variable fulfil a desirable limit. In the present study, a desirability (D) function approach was used to identify the optimal germination conditions that maximize the contents of β -glucan, free phenolic compounds, protease activity and antioxidant capacity and minimize the activity of undesirable enzymes (α -amylase and lipase activities). Only significant regression models were considered for desirability function calculation. Based on this criterion, the global desirability value obtained for variety Barra was $D=0.5880$ that corresponded to germination temperature of 16 °C and time of 216 h (Figure 4a,b left panel), while for variety Meeri the value was $D=0.5603$ that corresponded to 18 °C and time of 120 h (Figure 4a, right panel).

3.11. Validation of predictive models. Regression models adequacy was further validated by comparison of the experimental and predicted values obtained under the optimal germination conditions determined by desirability function. For validation of var. Meeri models, 16 °C and 96 h were selected since these conditions are within the range of maximum desirability region (Figure 4b, right panel). The experimental values were in close agreement with the predicted ones, and no significant differences ($p \leq 0.05$) between them were found (Table 4), confirming the suitability of the regression equations for modeling the influence of germination conditions on the studied response variables in sprouted oat flours obtained from both varieties.

4. Conclusions

This study demonstrates that germination conditions are key factors affecting the nutritional and bioactive potential, as well as the enzymatic activities in sprouted oat flours. The influence of germination temperature and time in oat depended on the presence/absence of hull. Germination enhanced dramatically the content of phenolics, antioxidant capacity, protease and α -amylase activities in both oat varieties, especially at higher temperatures and longer times. Short germination times (24-96 h) retained β -glucan levels in sprouted flours, but longer germination times caused strong reductions. Lipase activity was enhanced during germination in variety Barra but it was reduced in var. Meeri. The multivariate optimization performed by RSM allowed to identify the optimal germination conditions to obtain high quality sprouted oat flours (16 °C-216 h and 18 °C-156 h for Barra and Meeri varieties, respectively). Dehulled oat var. Meeri grains are an excellent option to produce sprouted gluten-free flours due to their high protein, β -glucan and phenolic compounds contents as well as low lipase activity. The obtained sprouted oat flours can fulfil the consumer demands for healthier plant-based foods and might be a source of innovation for food industry. Techno-functional properties of sprouted oat flours and consumer acceptability of foods formulated with them will be further studied.

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430 **Figure captions**

431 **Figure 1.** Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under denaturing
432 conditions of oat grains from variety Barra (a) and Meeri (b) and their sprouts obtained at different
433 germination conditions. Lane 1: non-germinated grain; lane 2: 14 °C for 60 h; lane 3: 14 °C for 156 h; lane 4:
434 18 °C for 60 h; lane 5: 18 °C for 156 h; lane 6: 16 °C for 216 h; lane 7: 12 °C for 96 h; lane 8: 20 °C for 96 h;
435 lane 9:16 °C for 96 h. MW: molecular weight marker.

436 **Figure 2.** Three dimensional surface plots for the combined effects of germination temperature and time on:
437 (a) β -glucan content, (b) free phenolic compounds content, (c) antioxidant activity, (d) α -amylase activity, (e)
438 protease activity and (e) lipase activity in sprouts obtained from oat var. Barra.

439 **Figure 3.** Three dimensional surface plots for the combined effects of germination temperature and time on:
440 (a) β -glucan content, (b) free phenolic compounds content, (c) antioxidant activity, (d) α -amylase activity, and
441 (e) protease activity in sprouts obtained from oat var. Meeri.

442 **Figure 4.** (a) Global desirability function profile and (b) bidimensional contour plot for desirability as
443 function of germination time and temperature in sprouted flours obtained from oat var. Barra (left panel) and
444 var. Meeri (right panel)

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446

Table 1. Contents of protein (g/100 g d.m.), β -glucan (g/100 g d.m.), FPC (mg GAE/100 g d.m.), antioxidant capacity (mg TE/100 g d.m) and activity (U/g d.m.) of enzymes α -amylase, protease and lipase in control and sprouted flours obtained from oat variety Barra at different combinations of germination temperatures and times.

Temperature (°C)	Time (h)	Protein	β -glucan	FPC	Antioxidant capacity	α -amylase	Protease	Lipase
Control flour		8.42 $\pm$ 0.10 ^a	2.03 $\pm$ 0.07 ^{de}	227.26 $\pm$ 20.27 ^a	574.58 $\pm$ 52.58 ^c	0.16 $\pm$ 0.07 ^a	0.28 $\pm$ 0.02 ^a	3.26 $\pm$ 0.28 ^a
14	60	11.00 $\pm$ 0.27 ^d	2.78 $\pm$ 0.23 ^g	232.08 $\pm$ 7.41 ^a	508.01 $\pm$ 43.32 ^b	0.92 $\pm$ 0.03 ^b	6.16 $\pm$ 0.32 ^b	3.80 $\pm$ 0.02 ^b
14	156	11.99 $\pm$ 0.15 ^f	1.66 $\pm$ 0.07 ^c	298.65 $\pm$ 24.83 ^c	817.13 $\pm$ 75.46 ^e	30.14 $\pm$ 1.05 ^h	9.12 $\pm$ 0.72 ^c	4.62 $\pm$ 0.22 ^c
18	60	9.61 $\pm$ 0.15 ^b	1.98 $\pm$ 0.06 ^d	243.15 $\pm$ 15.41 ^a	567.01 $\pm$ 52.87 ^c	6.75 $\pm$ 0.14 ^e	9.36 $\pm$ 0.19 ^c	4.50 $\pm$ 0.40 ^c
18	156	10.17 $\pm$ 0.25 ^c	0.72 $\pm$ 0.10 ^a	365.01 $\pm$ 38.33 ^d	1494.56 $\pm$ 110.28 ^f	3.35 $\pm$ 0.08 ^d	9.77 $\pm$ 0.61 ^c	6.30 $\pm$ 0.50 ^e
16	24	11.10 $\pm$ 0.07 ^{de}	2.54 $\pm$ 0.09 ^f	235.85 $\pm$ 19.32 ^a	425.60 $\pm$ 40.05 ^a	0.26 $\pm$ 0.01 ^a	6.17 $\pm$ 0.51 ^b	3.92 $\pm$ 0.11 ^b
16	216	12.69 $\pm$ 0.03 ^g	1.08 $\pm$ 0.14 ^b	372.02 $\pm$ 20.28 ^d	1563.94 $\pm$ 93.24 ^g	1.76 $\pm$ 0.02 ^c	10.88 $\pm$ 0.67 ^d	6.44 $\pm$ 0.26 ^e
12	96	9.47 $\pm$ 0.05 ^b	2.52 $\pm$ 0.27 ^f	235.31 $\pm$ 19.84 ^a	465.33 $\pm$ 45.20 ^{ab}	1.33 $\pm$ 0.05 ^{bc}	9.33 $\pm$ 0.49 ^c	5.48 $\pm$ 0.11 ^d
20	96	11.29 $\pm$ 0.20 ^{de}	2.21 $\pm$ 0.19 ^e	284.67 $\pm$ 26.66 ^{bc}	686.54 $\pm$ 57.08 ^d	14.92 $\pm$ 0.31 ^f	9.32 $\pm$ 0.67 ^c	7.34 $\pm$ 0.20 ^f
16	96	11.32 $\pm$ 0.31 ^e	1.77 $\pm$ 0.15 ^c	273.83 $\pm$ 22.25 ^b	563.90 $\pm$ 56.28 ^c	20.97 $\pm$ 1.41 ^g	5.71 $\pm$ 0.53 ^b	5.39 $\pm$ 0.40 ^d

Data are the mean $\pm$ standard deviation of three replicates. Different lowercase letters within a column indicate statistical differences among germination conditions (p $\leq$ 0.05, Duncan test). FPC: free phenolic compounds.

Table 2. Contents of protein (g/100 g d.m.), β -glucan (g/100 g d.m.), FPC (mg GAE/100 g d.m.), antioxidant capacity (mg TE/100 g d.m) and activity (U/g d.m.) of enzymes α -amylase, protease and lipase in control and sprouted flours obtained from oat variety Meeri at different combinations of germination temperatures and times.

Temperature (°C)	Time (h)	Protein	β -glucan	FPC	Antioxidant capacity	α -amylase	Protease	Lipase
Control flour		10.06 $\pm$ 0.04 ^a	3.45 $\pm$ 0.14 ^f	221.87 $\pm$ 18.32 ^a	537.79 $\pm$ 59.14 ^a	0.65 $\pm$ 0.02 ^a	0.27 $\pm$ 0.03 ^a	3.55 $\pm$ 0.13 ^e
14	60	11.53 $\pm$ 0.09 ^{de}	3.92 $\pm$ 0.09 ^g	353.03 $\pm$ 22.46 ^c	1431.15 $\pm$ 100.96 ^c	1.57 $\pm$ 0.15 ^b	1.36 $\pm$ 0.03 ^b	2.68 $\pm$ 0.16 ^{cd}
14	156	11.40 $\pm$ 0.09 ^{cd}	2.03 $\pm$ 0.06 ^c	517.09 $\pm$ 31.07 ^e	1957.76 $\pm$ 101.89 ^e	10.81 $\pm$ 0.58 ^f	1.34 $\pm$ 0.14 ^b	2.41 $\pm$ 0.08 ^c
18	60	11.24 $\pm$ 0.06 ^c	3.07 $\pm$ 0.19 ^e	379.94 $\pm$ 27.79 ^d	1698.36 $\pm$ 30.66 ^d	1.47 $\pm$ 0.07 ^b	1.71 $\pm$ 0.22 ^c	1.84 $\pm$ 0.18 ^a
18	156	11.72 $\pm$ 0.05 ^{ef}	1.52 $\pm$ 0.09 ^a	589.41 $\pm$ 33.94 ^f	2525.07 $\pm$ 122.77 ^g	19.00 $\pm$ 0.51 ^g	5.07 $\pm$ 0.01 ^f	2.50 $\pm$ 0.08 ^{cd}
16	24	11.03 $\pm$ 0.07 ^b	3.61 $\pm$ 0.16 ^f	246.32 $\pm$ 9.94 ^b	527.45 $\pm$ 31.40 ^a	0.81 $\pm$ 0.02 ^a	1.42 $\pm$ 0.14 ^{bc}	2.01 $\pm$ 0.14 ^{ab}
16	216	11.74 $\pm$ 0.06 ^{ef}	1.67 $\pm$ 0.09 ^{ab}	588.21 $\pm$ 16.15 ^f	2383.74 $\pm$ 150.48 ^f	25.12 $\pm$ 1.12 ^h	4.21 $\pm$ 0.06 ^e	2.72 $\pm$ 0.19 ^{cd}
12	96	11.26 $\pm$ 0.15 ^c	3.67 $\pm$ 0.23 ^f	402.98 $\pm$ 31.59 ^d	909.99 $\pm$ 46.10 ^b	2.75 $\pm$ 0.15 ^c	1.49 $\pm$ 0.19 ^{bc}	1.90 $\pm$ 0.19 ^a
20	96	11.92 $\pm$ 0.06 ^g	1.77 $\pm$ 0.14 ^{bc}	529.37 $\pm$ 23.26 ^e	2408.99 $\pm$ 124.19 ^f	5.39 $\pm$ 0.19 ^e	4.15 $\pm$ 0.28 ^e	2.76 $\pm$ 0.17 ^d
16	96	11.62 $\pm$ 0.26 ^{de}	2.73 $\pm$ 0.22 ^d	523.87 $\pm$ 12.12 ^e	1719.59 $\pm$ 64.59 ^d	4.07 $\pm$ 0.51 ^d	2.27 $\pm$ 0.30 ^d	2.16 $\pm$ 0.36 ^b

Data are the mean $\pm$ standard deviation of three replicates. Different lowercase letters within a column indicate statistical differences among germination conditions (p $\leq$ 0.05, Duncan test). FPC: free phenolic compounds.

Table 3. Regression models and fitting parameters for response variables as a function of germination temperature and time in sprouted oat flours obtained from varieties Barra and Meeri.

Variables	Variety Barra				Variety Meeri			
	Predictive model	R ²	R ² -adj	F-value	Predictive model	R ²	R ² -adj	F-value
Protein	$Y = -8.93 + 2.15T + 0.05t - 0.06T^2 + 0.00005t^2 - 0.03Tt$	0.46	0.82	0.48	$Y = 12.48 - 0.00003t^2 + 0.0017t$	0.71	0.66	2.92
β-glucan	$Y = 13.30 + 1.27 + 0.04T^2 + 0.00004t^2 - 0.0017t$	0.84	0.82	8.18	$Y = 9.73 + 0.36T - 0.04t + 0.00003t^2 + 0.00109t$	0.93	0.92	20.72
FPC	$Y = 46.77 + 26.21T - 1.45t - 1.00T^2 + 0.13Tt$	0.79	0.78	25.92	$Y = -510.33 + 76.71T - 2.29t + 3.36T^2 - 0.014t^2 + 0.11Tt$	0.94	0.93	26.97
ORAC	$Y = 1825.94 - 27.04t - 2.25T^2 + 0.03t^2 + 1.68Tt$	0.92	0.91	7.32	$Y = -1582.85 + 202.47T - 4.54T^2 - 0.05t^2 + 0.90Tt$	0.88	0.87	6.87
α-amylase	$Y = -370.71 + 34.26T + 2.17t - 0.75T^2 - 0.002t^2 - 0.10Tt$	0.85	0.84	8.02	$Y = 23.06 - 1.08T - 0.02T^2 + 27.26t^2 + 22.00Tt$	0.98	0.98	38.3
Protease	$Y = 50.86 - 6.23T + 0.22t + 0.07T^2 + 0.0002t^2 - 0.006Tt$	0.79	0.77	4.12	$Y = 17.62 - 1.60T + 0.13T^2 + 0.009Tt$	0.96	0.96	37.62
Lipase	$Y = 25.46 - 2.69T - 0.03t + 0.08T^2 + 0.003Tt$	0.79	0.77	10.88	$Y = 9.99 - 0.47T + 0.01T + 0.002Tt$	0.28	0.21	0.40

FPC: free phenolic compounds; ORAC: oxygen radical absorbance capacity

Table 4. Comparison of predictive and experimental values for response variables in sprouted flours obtained from oat varieties Barra and Meeri at optimal germination conditions

Response variables	Var. Barra		Var. Meeri	
	Predicted	Experimental	Predicted	Experimental
β -glucan (g/100 g d.m.)	0.94	1.08 $\pm$ 0.14	2.69	2.73 $\pm$ 0.22
FPC (mg GAE/100 g d.m.)	372.81	372.02 $\pm$ 20.28	482.02	523.87 $\pm$ 12.12
ORAC (mg TE/100 g d.m.)	1670.39	1563.94 $\pm$ 93.24	1838.84	1719.59 $\pm$ 64.59
Protease activity (U/g d.m.)	11.05	10.88 $\pm$ 0.67	2.16	2.27 $\pm$ 0.30
α -amylase activity (U/g d.m.)	2.50	1.76 $\pm$ 0.02	4.94	4.10 $\pm$ 0.52
Lipase activity (U/g d.m.)	6.19	6.44 $\pm$ 0.26	-	-

FPC: free phenolic compounds; ORAC: oxygen radical absorbance capacity

Fig. 1.

Fig. 2.

Fig. 3.

Fig. 4.

Varity Barra

Varity Meeri

a)

b)

Natalia Aparicio: Conceptualization, Methodology, Formal analysis, Investigation, Writing-Original Draft; **Cristina Martínez-Villaluenga:** Conceptualization, Methodology, Validation, Investigation, Resources, Writing-Review & Editing; Visualization, Supervision; **Juana Frias:** Methodology, Validation, Investigation, Resources, Writing-Review & Editing, Visualization; **Elena Peñas:** Conceptualization, Methodology, Validation, Formal analysis, Investigation, Resources, Writing-Original Draft, Review & Editing, Visualization, Supervision, Project administration, Funding acquisition;

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