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(Article begins on next page)

1 **A new practical approach for the biological treatment of a mixture of cheese whey and white wastewaters**
2 **using Kefir grains**

3

4 Lobna Elleuch¹, Olfa Ben Salem-Berrabah^{2,3}, Yasmin Cherni¹, Besma Sghaier-Hammami⁴, Mariam Kasmi¹, Cristian
5 Botta⁵, Ikram Ouerghi¹, Irene Franciosa⁵, Luca Coccolin⁵, Ismail Trabelsi¹, Abdelwaheb Chatti^{1,6}

6

7 ¹Laboratory of Treatment and Valorization of Water Rejects, Water Researches and Technologies Center, Borj-
8 Cedria Technopark, University of Carthage, 8020 Soliman, Tunisia

9 ² Laboratory of Environmental Science and Technologies, Higher Institute of Sciences and Technology of
10 Environment, University of Carthage, 2050 Borj-Cedria, Tunisia

11 ³ Department of Process Engineering, General Directorate of Technological Studies, Higher Institute of
12 Technological Studies of Zaghouan, Mogren, 1121 Zaghouan, Tunisia

13 ⁴Laboratoire des plantes extrêmophiles, Centre de Biotechnologie de Borj-Cédria, BP 901, 2050 Hammam-Lif,
14 Tunisia

15 ⁵Department of Agriculture, Forest and Food Sciences, University of Torino, Turin, Italy

16 ⁶Laboratory of Biochemistry and Molecular Biology, Faculty of Science of Bizerte, University of Carthage, 7021
17 Jarzouna, Tunisia

18

19 **Keywords:** Cheese wastes, Microbial consortium, Box-Behnken design, Reusability, 16S rDNA sequence analysis,
20 Barley

21

22 **Abstract**

23 Kefir grains are a microbial consortium of different genera of bacteria and yeasts. In this study, the performance
24 of Tunisian Kefir grains during the biological treatment of a mixture of Gouda cheese whey and white
25 wastewaters (GCW) in ratio 1:1 with very high organic matter concentration is investigated. The biological

26 process was evaluated and optimized through the response surface methodology. Under the optimum
27 conditions, Kefir grains concentration of 1.02%, temperature at 36.68 °C, and incubation time of 5.14 days, the
28 removal efficiencies of COD, PO₄³⁻, and NO₃⁻ were 87, 37.48, and 39.5%, respectively.

29 Interestingly, the reusability tests of the grains proved not only their high resistance to harsh environmental
30 conditions but also their great potential for more practical applications. Particularly, different strains were
31 isolated from the grains and identified as *Kluyveromyces marxianus*, *Lactococcus lactis*, *Lactobacillus kefir*, and
32 *Bacillus spp.* using 16S rDNA sequence analysis and rep-PCR fingerprinting. At the biological level, the raw GCW
33 (RGCW) has a negative impact on the *Hordeum vulgare* both on seed germination, and on the growth parameters
34 of seedlings. Interestingly, after Kefir grains treatment, the treated GCW (TGCW) allow a seedlings growth and
35 germination rate similar to those soaked in water.

36

37 **Introduction**

38 In recent years, dairy and cheese industries have been known among the fastest growing agrofood companies
39 worldwide. These industries produce a significant volume of different liquid effluents especially wastewaters
40 (Martínez-Suller et al. 2010) and valuable byproducts mainly whey (Panesar et al. 2007). The produced volume
41 and the chemical composition of these effluents are significantly variable and depend largely on the different
42 stages used during the making process and the final products (Pattnaik et al. 2007; Carvalho et al. 2013).
43 Wastewaters, with low organic loads ranging from 2.5 to 3 L per L of processed milk on average (Singh et al.
44 2014), mainly contain milk losses and washing water from equipment sections, bottles, and tanks (Carvalho et
45 al. 2013). Globally, the volume of cheese whey (CW), with an average value around 0.9 L generated from 1 L of
46 processed milk (Nicolás et al. 2019), accounts for about one-third of the total effluents of cheese factory
47 (Chatzipaschali and Stamatis 2012). Generally, it comprises 85–95% of the milk volume and retains about 55% of
48 the milk constituents (Ryan and Walsh 2016). Besides, it is characterized by relatively high concentrations of
49 biodegradable organic matter (Chatzipaschali and Stamatis 2012).

50 From a valorization point of view, approximately 50% of 190 million tons of whey produced worldwide every

51 year is processed for effective purposes in the medical, pharmaceutical, agroindustrial (Baldasso et al. 2011; Ryan
52 and Walsh 2016), and agricultural fields (Prazeres et al. 2012, 2016). Recently, research on the production of
53 kefir, a natural probiotic beverage, from CW fermentation with Kefir grains has shown exponential interest in its
54 potential effective benefits to human health (Rosa et al. 2017). These grains are irregularly shaped hard granules
55 with a yellowish-white color which resemble miniature cauliflower blossoms (Leite et al. 2012; Rosa et al. 2017).
56 They are a symbiotic association of bacteria especially *Lactobacillus*, *Leuconostoc*, *Lactococcus*, and *Acetobacter*,
57 and yeasts mainly *Kluyveromyces*, *Saccharomyces*, *Candida*, and *Torulaspora*. These bacteria and yeasts are
58 naturally immobilized in a matrix of proteins and heteropolysaccharide “kefiran” and their different combi-
59 nations at the species level generally characterize each local product (Zanirati et al. 2015). According to the
60 literature, the use of Kefir grains in agro-food industries is soaring given the fact that the grains can be
61 successfully produced on a large scale in a low-cost culture. They also exhibit excellent resistance to physical and
62 chemical stresses (Magalhães et al. 2010; Londero et al. 2015; Plessas et al. 2017). Yet, in the environmental
63 field, there are no reports on the biological treatment of dairy wastewater using Kefir grains. In addition, our
64 previous research (Elleuch et al. 2020) was, to our knowledge, the first study to report on the effective and low-
65 cost biological pretreatment of wastewater (landfill leachate) using Kefir grains with its high organic matter
66 content and toxicity. Under the optimum conditions, the overall removal rates of TOC, COD, NH_4^+-N , and PO_4^{3-}
67 were 93, 83.33, 70, and 88.25%, at an initial COD concentration of 24,000 mg/L, respectively. Besides, the grains
68 exhibit excellent reusability and resistance to harsh environmental conditions (Elleuch et al. 2020).

69 In Tunisia, the dairy and milk processing sector includes 45 companies, and the cheese subsector is composed of
70 25 enterprises. The dairy production approximately reached 1.2 billion liters in 2014 with a daily processing
71 capacity of about 3.8 million liters and an average of 0.5 million liters are processed daily for cheese making (APII
72 2014). Consequently, different dairy effluents with a high organic matter content are produced and their disposal
73 without treatment and valorization represents a serious environmental problem causing considerable
74 economical losses (Kasmi 2016). Over the past decades, many studies have focused on the treatment of dairy
75 wastewaters using different biological and physicochemical methods; only a few studies, however, have dealt

76 specifically with the CW treatment without biotechnological valorization strategies.

77 Recently, it has become worthwhile research to turn to the strategic environmental challenge for effective
78 treatment and advanced valorization technologies of different wastes generated from the entire dairy chain into
79 economic incentives. In this context and from an economic point of view, recent studies have highlighted the
80 potential reuse of dairy wastes for nutritive components production (Kasmi et al. 2017a), isolation and selection
81 of lactic acid bacteria for their antimicrobial activities against different pathogenic bacteria causing nosocomial
82 infections (Ghodhbane et al. 2016), and low-cost lactic acid bacteria growth media production (Kasmi et al. 2018).

83 On the other hand, Tsolcha et al. (2018) described the efficiency of a *Leptolyngbya*-based microbial consortium
84 for biological treatment of second CW effluent and biodiesel production. Furthermore, Paçal et al. (2019)
85 reported the effective treatment of CW wastewater and biogas production using anaerobic dynamic membrane
86 bioreactor. In addition, anaerobic digestion based on the biological reduction of organic compounds to biogas is
87 proposed as ecofriendly technology for industrial dairy wastewater (Mainardis et al. 2019; Charalambous et al.
88 2020;Treu et al. 2019). In Tunisia, the reuse of treated wastewater as an alternative
89 water source in agriculture has been growing rapidly since 2013 (Sdiri et al. 2018). Interestingly, Toumi et al.
90 (2015) reported that treated dairy wastewaters have the potential to be reused as biofertilizers. Furthermore,
91 recent results confirmed that the treated dairy wastewaters, following the required Tunisian legislation, have
92 effectively improved the growth parameters of wheat (Sioud et al. 2016) and the biomass production of olive
93 plants of the variety "Chemlali" (Sdiri et al. 2018). In this research, the performance of the Tunisian Kefir
94 grains process of a mixture of Gouda cheese whey and white wastewaters (GCW) was investigated using Box-
95 Behnken design (BBD). In addition, different strains of yeasts and bacteria were isolated from the grains and
96 identified using 16S rDNA sequence analysis and rep-PCR fingerprinting. Furthermore, the impact of treated GCW
97 (TGCW) with different dilution on morphophysiological parameters: germination rate, fresh weight, shoot and
98 root lengths, and chlorophyll contents of the seedlings of *Hordeum vulgare*, in comparison to raw GCW (RGCW)
99 and control (water) was evaluated.

100

101 **Materials and methods**

102

103 **Samples characterization**

104 In this study, the selected wastes, CW, and white wastewaters (WW) were collected from a regional cheese-
105 making factory located in the industrial zone of Ben Arous, Tunisia, and stored at – 20 °C to avoid their
106 acidification and chemical composition modification. CW obtained from the manufacturing of Gouda cheese and
107 WW, mainly rich in milk and water, is generated after the procedure of equipment washing. After preliminary
108 experiments, a mixture of these two Gouda cheese wastes (GCW) in ratio 1:1 was selected to be treated by a
109 biological process with Kefir grains. The physicochemical characterization of the raw CW, WW, and GCW is
110 presented in Table 1. The COD and pH values of GCW were 46.080 g/L and 4.36, respectively. In general, the
111 physicochemical properties of CW were characterized by high variability, and a COD range of 49.87–78.73 g/l
112 was reported in the study of Mainardis et al. (2019) while a COD value of 77.5 g/l was described in the work of
113 Treu et al. (2019).

114

115 **Kefir grains**

116 In this study, the grains were prepared on CW according to the method of Magalhães et al. 2010 with slight
117 modifications. The grains 10% (w/v) were inoculated into fresh CW at 25 °C for 24 h without stirring. The
118 experiment was repeated three times, and the activated grains were used for further analyses. The reusability
119 of the grains was tested as described by Elleuch et al. (2020).

120

121 **Isolation and identification of microorganisms from Tunisian Kefir grains**

122 Free Kefir cells were isolated from activated grains as follows: 10 g of the grains sample were suspended in 27.6 mL
123 of sterile Ringer solution (Sigma-Aldrich, Saint Luis, MO, USA) and homogenized using a Stomacher 400. Serial
124 dilutions were used for microbial enumeration and isolation on different media. The following microbial species
125 were enumerated: lactobacilli on Man Rogosa Sharpe (MRS; Lab M®, Heywood, Lancashire, UK) supplemented

126 with 0.025 g/mL of Delvocid (Sigma) and cultivated at 30 °C for 48 h and yeasts on W.L nutrient agar (Lab M®)
127 nutrient agar supplemented with 0.05 g/mL of tetracycline (Sigma) and incubated at 25 °C for 48 h. Results were
128 expressed as the decimal logarithm of colony-forming units (CFU) per gram of Kefir grains (\pm standard deviations).
129 The isolated bacteria and yeasts were further purified, grown in MRS and YPD (Lab M®) broth, respectively,
130 and stored at – 20 °C with 20% glycerol.

131

132 **Rep-PCR**

133 The genomic DNAs of the different isolated bacteria and yeasts were extracted as described by Cocolin et al.
134 2001. Then, rep-PCR fingerprinting was carried out using the primer (GTG)₅ (5' - GTGGTGGTGGTGGTG-3')
135 according to Dal Bello et al. 2010. The obtained products were visualized under ultraviolet light, and the resulting
136 profiles were determined by a digital image capturing, using a CCD UVI pro Platinum 1.1 (Eppendorf). The
137 BioNumerics 4.6 software package was used to analyze the rep-PCR fingerprints. Group differences in the
138 microbial community structure of Kefir grains were performed using unweighted pair group method with
139 arithmetic mean (UPGMA), and the Pearson's correlation coefficient was used to assess the similarity between
140 profiles.

141

142 **16S rDNA gene sequencing of Kefir grains isolates**

143 Representative microbial isolates of subcluster at 70% of similarity were identified by sequencing the partial
144 rRNA amplicon. The 16S rDNA was amplified from the bacterial strains with the primers FD1 (5' -
145 AGAGTTTGATCCTG GCTCAG-3') and RD1 (5' -AAGGAGGTGATCCAGCC-3') as described by Weisburg et al.
146 (1991). For the yeasts, the Internal transcribed spacer (ITS) region (ITS1-5.8S-ITS2) was amplified with the two
147 primers ITS1 (5' -TCC GTA GGT GAA CCT TGC GG-3') and ITS4 (5' -TCC TCC GCT TAT TGA TAT GC-3')
148 according to Korabečná et al. (2003).

149

150 **Biological process**

151 The optimization of Kefir grains treatment parameters for GCW was carried out by using BBD based on response
152 sur- face methodology with the statistical software design expert Version 10.0.6 (Stat- Ease Inc., MN, USA).
153 Fifteen experiments were conducted with three independent factors: tempeature (X1), incubation time (X2), and
154 Kefir grains concentration (X3) at three different levels and under different conditions to evaluate their
155 interactions and the importance of their effectiveness on the removal of COD (Y1), PO₄³⁻ (Y2), and NO₃⁻ (Y3),
156 selected as responses (Table 2). The biological process was carried out at a small-scale system under non aseptic
157 conditions, and the experiments were performed with 50 mL of GCW inoculated with Kefir grains in a 250-mL
158 Erlenmeyer flask and incubated without any pH adjustment and agitation. The evaluated response Y was
159 calculated using the following equation:

$$Y = a_0 + \sum_i a_i X_i + \sum_{ij} a_{ij} X_i X_j + \sum_{ii} a_{ii} X_i^2$$

160

161 where Y is defined as the evaluated response for the removal efficiency of pollutants, a₀, a_i (i = 1,2,3) a_{ii} (i =
162 1,2,3), and a_{ij} (i = 1,2,3; j = 1,2,3) are the model coefficients and X_i and X_j the coded independent variables.
163 NemrodW software (LPRAI version 2000) was used to analyze the variance (ANOVA) results and perform form
164 the response surface curves.

165

166 **Analytical methods**

167 Chemical analyses of the raw and treated cheese effluents were performed using standard methods described
168 by Rodier et al. 2009. PO₄³⁻ and NO₃⁻ were determined using an ion chromatography Metrohm 761.
169 Conductivity/pH meter con- sort C860 was used to determine the pH and the conductivity of the samples. The
170 turbidity was determined by using a turbidimeter (WTWTurb 555). The different analyses were per- formed in
171 triplicate.

172

173 **Evaluation of treated GCW on seedlings growth of barley**

174 The seeds of barley (*Hordeum vulgare*) were sterilized with HgCl₂ solution (0.1%) and thoroughly washed with
175 sterile distilled water. Five uniform seeds were placed in 90-mm Petri dishes lined with a filter paper moistened
176 with 15 mL of one of the different dilutions (25, 50, and 100X) of RGCW and TGCW. Three replicates were carried
177 out for the different samples, including the control with distilled water (H₂O), and the Petri dishes were
178 incubated in a dark incubator at 20 ± 2 °C for 3 days then in a photoperiod (16h light/8h dark) for 1 week. The
179 germination rate (GR) was calculated according to Komilis et al. 2005, and the different growth parameters, leaf
180 and root lengths and fresh weight, were determined after 10 days of germination of the seeds with regular
181 observation at an interval of every 24 h.

182

183 **Determination of chlorophyll content**

184 Fresh leaves (0.1 g) were homogenized with 10 mL 80% chilled acetone in a prechilled mortar and pestle.
185 Concentrations of chlorophylls a (Chl a), b (Chl b), and total chlorophyll were calculated according to Arnon 1949.

186

187 **Results and discussion Microbiological**

188

189 **Microbiological analysis of Tunisian Kefir grains**

190 Different genera and species of yeasts and bacteria have been isolated and identified from Kefir grains collected
191 from different locations (Garofalo et al. 2015; Dertli and Çon 2017; Gut et al. 2019). Interestingly, it has been
192 proved that their complex microbial composition is extremely variable and depends mainly on geographical
193 regions and culture conditions (Marsh et al. 2013; Zanirati et al. 2015; Arslan 2015). In this section, Tunisian Kefir
194 grains were analyzed microbiologically to identify the predominant microbial populations. The MRS counts were
195 5.04 ± 0.57 Log CFU/g while yeasts were present in the grains at 6.25 ± 0.05 Log CFU/g. A total of 81 isolates (54
196 bacteria and 27 yeasts) were subjected to rep-PCR fingerprinting technique in order to group them at
197 genus/species level. Later on, 11 bacteria and 4 yeasts were chosen as representatives of each subcluster obtain-
198 ed (70% of similarity) and identified as the bacteria *Lactococcus lactis*, *Lactobacillus kefir*, and *Bacillus* spp. (Fig.

199 1a)and the yeast *Kluyveromyces marxianus* (Fig. 1b). These findings are in accordance with previous observations
200 of Garofalo et al. (2015). It is worth noticing, in this vein, that several studies have highlighted the successful use
201 of these genera of bacteria (Kasmi et al. 2017a, b; Ghasemi et al. 2017; Al-Wasify et al. 2017) and the the yeast
202 *Kluyveromyces marxianus* (Yadav et al. 2014) for the biological treatment of dairy effluents.

203

204 **Kefir grains process**

205 **Optimization of Kefir grains process using BBD**

206 The preliminary experiments showed the significant effects of the culture conditions especially temperature (X1),
207 incubation time (X2), and Kefir grains concentration (X3) of Kefir grains on the removal of COD, PO_4^{3-} , and NO_3^- .
208 On the basis of these findings, BBD was specifically selected to evaluate the interactions between these factors
209 and the importance of their effectiveness on the removal of COD (Y1), PO_4^{3-} (Y2), and NO_3^- (Y3). Table 3 shows the
210 values of the independent factors and the predicted and experimental values of the responses.

211

212 **Analysis of the experimental data**

213 The optimization of Kefir grains process by BBD includes the study of the response of the designed combinations
214 of factors, the estimation of the different coefficients, the response prediction of the fitted model and the testing
215 of the significance, and adequacy of the model. Firstly, the ANOVA tests were conducted for the three studied
216 responses: COD (Table 4), PO_4^{3-} (Table 5), and NO_3^- (Table 6) removal rates. In statistics, coefficients with P value
217 less than 5% show that model terms are significant whereas, the coefficients with P value more than 5% are
218 considered as insignificant.

219 For COD removal rate (Y1), the obtained results indicated that the three studied factors are insignificant (Table4)
220 and the response was calculated using the following equation:Y1 ¼ 68:52 Concerning the PO_4^{3-} removal
221 efficiency (Y2), it is evident from Table 5 that only the incubation time fac- tor (X2) is positively significant (P value
222 < 5%), and the different interactions have no significant effect.

223 Consequently, the mathematical model is obtained as follows:

224 $Y_2 = 32.6 + 4.08 X_2$

225 In contrast, as shown in Table 6, the temperature is more significant than the grains concentration and the
226 incubation time on the removal of NO_3^- . In addition, only the interaction effect between temperature and Kefir
227 grains concentration is significant. Thus, the response Y_3 was calculated as follows:

228 $Y_3 = 32.29 + 5.23 X_1 - 4.26 X_2 - 2.2 X_3 + 5.92 X_{11} + 3.60 X_{33} - 5.46 X_{13}$

229 As presented in Table 7, the significance and adequacy of the model are confirmed by the variance analysis and
230 Fisher's F test values.

231

232 **Effect of variables on COD, PO_4^{3-} , and NO_3^- removal rates**

233 The concurrent effect of temperature, incubation time, and Kefir grains concentration on COD, PO_4^{3-} , and NO_3^-
234 removal efficiencies during Kefir grains process was evaluated (Fig. 2). According to the response surface plots,
235 the maximum removals of COD and NO_3^- were obtained at the low level of Kefir grains concentration. In
236 addition, the increase in incubation time improved the biological treatment, whereas, temperature proved to be
237 an irrelevant factor.

238

239 **Optimization using desirability functions**

240 The reuse of treated industrial wastewaters with a relatively low concentration on COD and no excessive amount
241 of nutrients especially, nitrogen (N) and phosphorus (P) used as growth factors for plants is a common practice
242 of irrigation in many parts of the world. In this study, RGCW was treated with Kefir grains in order to enhance
243 their quality for further application in agriculture. Hence, the main goal of the biological process with Kefir grains
244 is to maximize the removal rate of COD and reduce the rates of PO_4^{3-} and NO_3^- to the desired concentrations
245 with recalculating the values of responsible factors by using the desirability function approach. According to the
246 BBD results, the maximum predicted values of COD, PO_4^{3-} and NO_3^- are found to be 86.78, 35.95, and 38.76%,
247 respectively, during the biological process with 1.02% Kefir grains at 36.68 °C during 5.14 days without agitation
248 and any pH adjustment. Under these conditions, an additional experiment was performed, and the obtained

249 results showed that the removal efficiencies of COD, PO_4^{3-} , and NO_3^- were 87 ± 0.5 , 37.48 ± 0.74 , $39.5\% \pm 0.39$,
250 respectively. It is clear that Kefir grains can reduce the organic compounds of GCW characterized by a high COD
251 concentration (25,920 mg/L). This is in line with other studies proving that the different organic compounds
252 from CW can be significantly reduced during the biological process with the pure culture of *Bacillus* sp., coculture
253 of *Bacillus* sp. with *Cupriavidus* sp. (Reddy et al. 2019) and mixed culture of the two yeasts *Kluyveromyces*
254 *marxianus* and *Candida krusei* (Yadav et al. 2014). In general, CW contains soluble proteins, lipids, vitamins,
255 mineral salts, and mostly lactose responsible for high BOD and COD content (Saini et al. 2017). Lactose is a
256 disaccharide fermented only by microorganisms expressing both the membrane transporter, lactose permease,
257 and the hydrolytic enzyme β -galactosidase (Grba et al. 2002). *Lactococcus lactis* strains are homofermentative
258 bacteria ferment lactose into pyruvic acid, which is, then, reduced to lactic acid by the reducing power previously
259 produced in the form of NADH. While, *Lactobacillus kefir* strains classified as heterofermentative lactobacilli
260 produce acetate, carbon dioxide, ethanol, and/or acetic acid in addition to lactic acid as the end product of
261 fermentation-phosphoketolase pathway (Bintsis 2018). Additionally, various metabolites are released from
262 proteins and lipid fractions present in cheese whey through many enzymatic reactions (Burgain et al. 2014). On
263 the other hand, *Kluyveromyces marxianus* is lactose-fermenting yeast with useful physiological features such as
264 high growth rate and thermotolerance. The coregulated genes LAC4 and LAC12 encoding β -galactosidase and
265 lactose permease, respectively, are responsible for lactose uptake and hydrolysis to glucose and galactose (Saini
266 et al. 2017). So far, several studies have reported various biological processes of synthetic dairy wastewater
267 under aseptic conditions while a few studies have focused on the biological treatment of real wastewater under
268 non-aseptic conditions. In this work, the biological treatment of a mixture of two real dairy effluents with very
269 high organic matter concentration was studied under non-aseptic conditions. Tzolcha et al. (2018) have studied
270 the removal of organic and inorganic compounds from dairy wastewater at dilution ratio (8:100) by a mixed
271 microbial consortium. The effluent contains initial pollutants concentrations lower than those presented in this
272 research and after biological treatment under non-aseptic conditions, the removal rates of COD, NO_3^- , and PO_4^{3-}
273 were 93.5, 54.5, and 83.2%, respectively.

274

275 **Reusability tests of Kefir grains during GCW treatment**

276 Currently, the level of reusability of immobilized microorganisms is an important issue for practical
277 environmental applications. Therefore, the reusability tests of Kefir grains during GCW treatment were studied
278 for two cycles and at the end of the second cycle, the removal rate of COD, PO_4^{3-} , and NO_3^- were 82.6, 30.8, and
279 32.3%, respectively (Table 8). Overall, Kefir grains process can be regarded as an economical and ecofriendly
280 process with no secondary pollution effects since the produced biomass can be reused and allows efficient
281 removal of pollutants. Its advantages include feasibility, practicability, reliability, simplicity, and absence of un-
282 pleasant odors. Compared with free or immobilized strains, Kefir grains
283 display many features that make it particularly suitable for industrial dairy applications under non-aseptic
284 conditions. Indeed, Kefir grains are a natural consortium containing a unique, complex, and stable microbial
285 community with the predominance of lactic acid bacteria, acetic bacteria, yeasts, and fungi (Laureys and De Vuyst
286 2014; Garofalo et al. 2015). All these microorganisms exist in a state of symbiotic equilibrium in a natural
287 exopolysaccharide and a protein matrix (Gao and Zhang, 2019). Interestingly, Kefir grains are a natural mixed
288 starter culture commercially used in food industries for the production of cheese, bread (Plessas et al. 2017), and
289 various probiotic beverages with interesting healthy properties (Gao and Zhang 2019). In addition, they can be
290 stored for long periods (Fiorda et al. 2017). Besides, the freeze-dried culture retains a high survival rate and
291 shows good metabolic activity and fermentation efficiency which is important for their industrial applications
292 (Prado et al. 2015). Furthermore, their recovery is very easy, and CW has been frequently used in large scale as
293 a low-cost substrate for growth and biomass production under non-aseptic conditions (Magalhães et al. 2010;
294 Plessas et al. 2017). Regarding wastewater treatment, the use of Kefir grains has been studied for the first time
295 in biological pre-treatment of landfill leachate with its high organic matter content and toxicity, and the grains
296 exhibit excellent reusability and resistance to harsh environmental conditions (Elleuch et al. 2020). Yet, during
297 the biological process with free microbial cells dispersed throughout the mixed culture medium and raw effluent,
298 it is practically very difficult to harvest them for other cycles of reuse (San et al. 2014). Additionally, the culture

299 of free or immobilized strains generally requires commercial media under specific and sterile conditions which
300 increases the process costs and therefore limits their application and reusability in large- scale and industrial-
301 scale systems. Generally, the direct use of the biological process presents some disadvantages such as a large
302 amount of sludge generation, slower treatment time, and unintended inhibition with an output of noisome smell
303 (Gogate et al., 2020). In this study, the performance of Kefir grains for the cheese wastewater treatment was
304 investigated at a small-scale system; therefore, it is difficult to discuss the disadvantages of the process.

305 306 **Impact of GCW on the morpho-physiological parameters of *Hordeum vulgare***

307 The impact of RGCW and TGCW with their different dilutions (25, 50, and 100X) on barley was studied. The
308 different parameters of the seedlings growth, germination rate (GR), fresh weight (FW), and shoot and root
309 lengths, were evaluated in comparison to control (Fig. 3). It is noted that the different effects of RGCW and TGCW
310 on *Hordeum vulgare* seedlings growth traits may mainly depend on GCW quality and dilution. RGCW completely
311 inhibits seed germination (data not shown). These results confirm the findings from previous studies which
312 focused on the evaluation of the effects of cheese and dairy effluents on the germination and growth of crops
313 (Prazeres et al. 2014; Toumi et al. 2015; Sioud et al. 2016; Abou-Dahab et al. 2019). According to these
314 researchers, the negative effect of RGCW may be attributed to the toxicity caused by the different amounts of
315 organic and inorganic compounds present in the effluent. Figure 3 shows that the Kefir grains process improved
316 the quality of GCW and all the studied parameters were significantly better with TGCW than those obtained with
317 RGCW (Fig. 4). The results of TGCW at 50 and 100X are similar.

318 Subsequently, the effect of TGCW 50X on the content of leaf photosynthetic pigments was studied. Regarding
319 the total chlorophyll content, similar results were obtained between the different seedlings either soaked with
320 RGCW or TGCW (Fig. 5a).

321 Similarly, previous research showed that the interaction between olive mill wastewater-compost and foliar
322 application with ZnSO₄ increased the growth parameters of *H. vulgare*, while no significant differences in total
323 chlorophyll content were observed (Abdel-Ati and Eisa 2015). Regarding the chlorophyll b, RGCW and TGCW

324 have a negative impact compared to control plants (Fig. 5c). Concerning chlorophyll a, barley seedlings registered
325 different results between RGCW and TGCW (Fig. 5b).
326 Interestingly, the highest chlorophyll a content was obtained with TGCW. Similarly, Sdiri et al. (2018) reported a
327 sig- nificant difference between the results of the effect of dairy wastewater on chlorophylls contents of olive
328 leaves and indicated that treated wastewater improved significantly chlorophyll a content. Generally, treated
329 wastewater is a valuable source of water and nutrients which are the major factors enhancing chlorophyll
330 contents (Ashrafi et al. 2016). According to Croce and van Amerongen (2014), the differ- ence in chlorophyll a
331 and chlorophyll b contents could be related to the fact that chlorophyll a acts the first in the light- harvesting
332 complexes and contrarily, chlorophyll b, cannot act as the primary donor within the reaction centers. This study
333 is a first attempt to investigate the performance of Kefir grains for cheese wastewater treatment at a small- scale
334 system under non-aseptic conditions. Removal rates of COD, PO_4^{3-} and NO_3^- reached 87, 37.48, and 39.5%,
335 respectively under the optimum treatment conditions. Therefore, this study can be a starting point for further
336 re- search to be performed gradually at lab-scale system, pilot- scale system, and full-scale industrial in
337 wastewater treatment plants to establish the best operating parameters in terms of pollutants removal from
338 cheese wastewaters. Interestingly, the treated effluent has a positive effect on barley seedlings growth
339 parameters and chlorophyll a content, and further tests should be executed in order to use treated effluent as
340 liquid fertilizer by mixing it with soil after appropriate dilution.

341

342 **Conclusion**

343 In the present study, a new practical approach for biological treatment with Kefir grains of a mixture of GCW was
344 successfully developed. The BBD was applied to deter- mine the effect of three different biological process
345 variables: temperature, incubation time, and Kefir grains con- centration on the removal efficiencies of COD,
346 PO_4^{3-} and NO_3^- . Experimental results revealed that the incubation time factor is positively significant on the
347 percentage of PO_4^{3-} removal. However, the temperature is more significant than Kefir grains concentration and
348 incubation time on the removal of NO_3^- , and only the interaction effect between the two variables temperature

349 and Kefir grains concentration is significant. Under the optimum conditions of the grains concentration of 1.02%,
350 temperature at 36.68 °C and incubation time of 5.14 days, about 87% of COD, 37.48% of PO₄³⁻, and 39.5% of NO₃
351 – were removed after the biological process. The reusability tests of the grains showed that COD removal rate is
352 more than 80% up to two cycles, suggesting that the biological process with Kefir grains could be a promising
353 approach for industrial GCW treatment. After 10 days, a positive effect on barley seedlings growth parameters
354 and chlorophyll a content of was observed with TGCW 50X in comparison with RGCW and control. Future
355 research should focus on testing the effect of TGCW on barley seedlings growing in pots on growth, productivity
356 and antioxidant enzymes activities during prolonged periods (1, 2, and 3months).

357

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361

362 **References**

- 363 Abdel-Ati AA, Eisa SS (2015) Response of barley grown under saline condition to some fertilization treatments.
364 Ann Agric Sci 60:413– 421
- 365 Abou-Dahab TAM, Ewis STA, El-Kady AFY (2019) Towards sustain- able landscape: feasibility of using different
366 cheese whey types in the fertigation of Schinus molle L. seedlings. J Clean Prod 235: 1051–1060
- 367 Al-Wasify RS, Ali MN, Hamed SR (2017) Biodegradation of dairy wastewater using bacterial and fungal local
368 isolates. Water Sci Technol 76:3094–3100
- 369 APII (2014) Les Industries Agroalimentaires en Tunisie: Industrie des Boissons. Agency for the Promotion of
370 Industry and Innovation, Tunisia
- 371 Arnon DI (1949) Copper enzymes isolated chloroplasts, polyphenoloxidase in Beta vulgaris. Plant Physiol 24:1–
372 15
- 373 Arslan S (2015) A review: chemical, microbiological and nutritional characteristics ofkefir. CyTA-J Food 13:340–

374 345

375 Ashrafi N, Nikbakht A, Gheysari M (2016) Effect of recycled water applied by surface and subsurface irrigation on
376 the growth, photo- synthetic indices and nutrient content of young olive trees in Central Iran. *J Water Reuse Desal*
377 *7*:246–252

378 Baldasso C, Barros TC, Tessaro IC (2011) Concentration and purification of whey pro-teins by ultrafiltration.
379 *Desalination* 278:381–386

380 Bintsis T (2018) Lactic acid bacteria as starter cultures: an update in their metabolism and genetics. *AIMS*
381 *Microbiol* 4:665–684

382 Burgain J, Scher J, Francius G, Borges F, Corgneau M, Revol-Junelles AM, Cailliez-Grimal C, Gaiani C (2014) Lactic
383 acid bacteria in dairy food: surface characterization and interactions with food ma- trix components. *Adv Colloid*
384 *InterfSci* 213:21–35

385 Carvalho F, Prazeres AR, Rivas J (2013) Cheese whey wastewater: char- acterization and treatment. *Sci Total*
386 *Environ* 445-446:385–396

387 Charalambous P, Shin J, Shin SG, Vyrides I (2020) Anaerobic digestion of industrial dairy wastewater and cheese
388 whey: performance of internal circulation bioreactor and laboratory batch test at pH 5-6. *Renew Energy* 147:1–
389 10

390 Chatzipaschali AA, Stamatis AG (2012) Biotechnological utilization with a focus on anaerobic treatment of cheese
391 whey: current status and prospects. *Energies* 5:3492–3525

392 Cocolin L, Manzano M, Cantoni C, Comi G (2001) Denaturing gradient gel electrophoresis analysis of the 16S rRNA
393 gene V1 region to monitor dynamic changes in the bacterial population during fermentation of Italian sausages.
394 *Appl Environ Microbiol* 67:5113–5121

395 Croce R, van Amerongen H (2014) Natural strategies for photosynthetic light harvesting. *lightharvesting*.
396 *NatChem Biol*10:492–501

397 Dal Bello B, Rantsiou K, Bellio A, Zeppa G, Ambrosoli R, Civera T, Cocolin L (2010) Microbial ecology of artisanal
398 products from north west of Italy and antimicrobial activity of the autochthonous popu- lations. *LWT-Food Sci*

399 Technol 43:1151–1159

400 Dertli E, Çon AH (2017) Microbial diversity of traditional kefir grains and their role on kefir Aroma. *LTW-Food Sci*

401 *Technol* 85:151–157

402 Elleuch L, Messaoud M, Djebali K, Attafi M, Cherni Y, Kasmi M, Trabelsi I, Chatti A (2020) A new insight into highly

403 contaminated landfill leachate treatment using Kefir grains pre-treatment combined with Ag-doped TiO₂

404 photocatalytic process. *J Hazard Mater* 382:121119

405 Fiorda FA, de Melo Pereira GV, Thomaz-Soccol V, Rakshit SK, Pagnoncelli MGB, Vandenberghe LP d S, Soccol CR

406 (2017) Microbiological, biochemical, and functional aspects of sugary kefir fermentation - a review. *Food*

407 *Microbiol* 66:86–95

408 Gao W, Zhang L (2019) Comparative analysis of the microbial community composition between Tibetan kefir

409 grains and milks. *Food Res Int* 116:137–144

410 Garofalo C, Osimani A, Milanović V, Aquilanti L, De Filippis F, Stellato G, Mauro SD, Turchetti B, Buzzini P, Ercolini

411 D, Clementi F (2015) Bacteria and yeast microbiota in milk kefir grains from different Italian regions. *Food*

412 *Microbiol* 49:123–133

413 Ghasemi M, Ahmad A, Jafary T, Azad AK, Kakooei S, Wan Daud WR, Sedighi M (2017) Assessment of immobilized

414 cell reactor and microbial fuel cell for simultaneous cheese whey treatment and lactic acid/electricity

415 production. *Int J Hydrogen Energy* 42:9107–9115

416 Ghodhbane H, Alessandria V, Snoussi M, Elleuch L, Trabelsi I, Abdely C, Sabatier JM, Cocolin L, Regaya I (2016)

417 Genetic characterization of lactic acid bacteria isolated from tunisian milk waste and their antimicrobial activity

418 against some bacteria implicated in nosocomial infections. *Infect Disord Drug Targets* 16:1–10

419 Gogate PR, Thanekar PD, Oke AP (2020) Strategies to improve biological oxidation of real wastewater using

420 cavitation based pre-treatment approaches. *Ultrason Sonochem* 64:105016

421 Grba S, Stehlik-Tomas V, Stanzer D, Vahèiæ N, Škrilin A (2002) Selection of yeast strain *Kluyveromyces marxianus*

422 for alcohol and biomass production on whey. *Chem Biochem Eng Q* 16:13–16

423 Gut AM, Vasiljevic T, Yeager T, Donkor ON (2019) Characterization of yeasts isolated from traditional kefir grains

424 for potential probiotic properties. *J Funct Foods* 58:56–66

425 Kasmi M (2016) Biological processes as promoting way for both treat- ment and valorization of dairy industry
426 effluents. *Waste Biomass Valorization* 9:195–209

427 Kasmi M, Hamdi M, Trabelsi I (2017a) Processed milk waste recycling via thermal pretreatment and lactic acid
428 bacteria fermentation. *Environ Sci Pollut Res* 24:13604–13613

429 Kasmi M, Djebali K, Hamdi M, Trabelsi I (2017b) Physical-chemical treatment process optimization for high
430 polluting dairy effluents prior fermentation. *Int J Environ Sci Technol* 15:779–790

431 Kasmi M, Elleuch L, Dahmeni A, Hamdi M, Trabelsi I, Snoussi M (2018) Novel approach for the use of dairy industry
432 wastes for bacterial growth media production. *J Environ Manag* 212:176–185

433 Komilis DP, Karatzas E, Halvadakis CP (2005) The effect of olive mill wastewater on seed germination after various
434 pretreatment tech- niques. *J Environ Manage* 74:339–348

435 Korabečná M, Liška V, Fajfrlik K (2003) Primers ITS1, ITS2 and ITS4 detect the intraspecies variability in the internal
436 transcribed spacers and 5.8 S rRNA gene region in clinical isolates of fungi. *Folia Microbiol* 48:233–238

437 Laureys D, De Vuyst L (2014) Microbial species diversity, community dynamics, and metabolite kinetics of water
438 kefir fermentation. *Appl Environ Microbiol* 80:2564–2572

439 Leite AMO, Mayo B, Rachid CTCC, Peixoto RS, Silva JT, Paschoalin VMF, Delgado S (2012) Assessment of the
440 microbial diversity of Brazilian kefir grains by PCR-DGGE and pyrosequencing analysis. *Food Microbiol* 31:215–
441 221

442 Londero A, Iraporda C, Garrote G, Abraham A (2015) Cheese whey fermented with kefir microorganisms:
443 Antagonism against *Salmonella* and immunomodulatory capacity. *Int J Dairy Technol* 68:118–126

444 Magalhães KT, De Melo Pereira GV, Nicolau A, Dragone G, Domingues L, Teixeira JA, de Almeida Silva JB, Schwan
445 RF (2010) Production of fermented cheese whey-based beverage using kefir grains as start- er culture: evaluation
446 of morphological and microbial variations. *Bioresour Technol* 101:8843–8850

447 Mainardis M, Flaibani S, Trigatti M, Goi D (2019) Techno-economic feasibility of anaerobic digestion of cheese
448 whey in small Italian dairies and effect of ultrasound pre-treatment on methane yield. *J Environ Manag* 246:557–

449 563

450 Marsh AJ, O’Sullivan O, Hill C, Ross RP, Cotter PD (2013) Sequencing- based analysis of the bacterial and fungal
451 composition of kefir grains and milks from multiple sources. *PLoS One* 8:e69371

452 Martínez-Suller L, Provolo G, Carton OT, Brennan D, Kirwan L, Richards KG (2010) The composition of dirty water
453 on dairy farms in Ireland, Irish. *J Agric Food Res* 49:67–80

454 Nicolás P, Ferreira ML, Lassalle V (2019) A review of magnetic separation of whey proteins and potential
455 application to whey proteins recovery, isolation and utilization. *J Food Eng* 246:7–15

456 Paçal M, Semerci N, Çallı B (2019) Treatment of synthetic wastewater and cheese whey by the anaerobic dynamic
457 membrane bioreactor. *Environ Sci Pollut Res* 26:32942–32956

458 Panesar P, Kennedy J, Gandhi D, Bunko K (2007) Bioutilisation of whey for lactic acid production. *Food Chem*
459 105:1–14

460 Pattnaik R, Yost RS, Porter G, Masunaga T, Attanandana T (2007) Improving multi-soil-layer (MSL) system
461 remediation of dairy effluent. *Ecol Eng* 32:1–10

462 Plessas S, Nouska C, Mantzourani I, Kourkoutas Y, Alexopoulos A, Bezirtzoglou E (2017) Microbiological
463 exploration of different types of kefir grains. *Fermentation* 3:1–10

464 Prado MR, Blandón LM, Vandenberghe LPS, Rodrigues C, Castro GR, Thomaz-Soccol V, Soccol CR (2015) Milk
465 kefir: composition, microbial cultures, biological activities, and related products. *Front Microbiol* 6:1177

466 Prazeres AR, Carvalho F, Rivas J (2012) Cheese whey management: a review. *J Environ Manag* 110:48–68

467 Prazeres AR, Carvalho F, Rivas J, Patanita M, Dôres J (2014) Reuse of pretreated cheese whey wastewater for
468 industrial tomato production (*Lycopersicon esculentum* mill.). *Agric Water Manag* 140:87–95

469 Prazeres AR, Rivas J, Almeida MA, Patanita M, Dôres J, Carvalho F (2016) Agricultural reuse of cheese whey
470 wastewater treated by NaOH precipitation for tomato production under several saline conditions and sludge
471 management. *Agric Water Manag* 167:62–74

472 Reddy MV, Mawatari Y, Onodera R, Nakamura Y, Yajima Y, CYC (2019) Bacterial conversion of waste into
473 polyhydroxybutyrate (PHB): a new approach of bio-circular economy for treating waste and energy generation.

474 Bioresour Technol Rep 7:100246

475 Rodier J, Legube B, Merlet N, Brunet R (2009) Résiduaires eaux. In: Rodier J (ed) L'analyse de l'eau: eaux
476 naturelles, eaux résiduaires, eaudemer. Dunod, Paris, pp987–991

477 Rosa DD, Dias MMS, Grześkowiak ŁM, Reis SA, Conceição LL, Peluzio M d CG (2017) Milk kefir: nutritional,
478 microbiological and health benefits. *Nut Res Rev* 30:82–96

479 Ryan MP, Walsh G (2016) The biotechnological potential of whey. *Rev Environ Sci Biotechnol* 15:479–498

480 Saini P, Beniwal A, Kokkiligadda A, Vij S (2017) Evolutionary adaptation of *Kluyveromyces marxianus* strain for
481 efficient conversion of whey lactose to bioethanol. *Process Biochem* 62:69–79

482 San NO, Celebioglu A, Tümtaş Y, Uyar T, Tekinay T (2014) Reusable bacteria immobilized electrospun nanofibrous
483 webs for decolorization of methylene blue dye in wastewater treatment. *RSC Adv* 4: 32249–32255

484 Sdiri W, Chehab H, Reyns T, Van Loco J, Mechri B, Boujnah D, Bua GD, Ben Mansour H, Di Bella G (2018) Incidence
485 of dairy wastewater on morphological and physiological compartment of Chemlali and Chetoui olive. *Water*
486 *Resour Indust* 20:29–36

487 Singh NB, Singh R, Imam MM (2014) Waste water management in dairy industry: pollution abatement and
488 preventive attitudes. *Int J Environ Sci Technol* 3:672–683

489 Sioud O, Beltifa A, Ayeb N, Mansour HB (2016) Characterization of industrial dairy wastewater and contribution
490 to reuse in cereals culture: study of phytotoxic effect. *Austin J Environ Toxicol* 2:1013

491 Toumi J, Miladi B, Farhat A, Nouria S, Hamdi M, Gtari M, Bouallagui H (2015) Microbial ecology overview during
492 anaerobic codigestion of dairy wastewater and cattle manure and use in agriculture of obtained bio-fertilisers.
493 *Bioresour Technol* 198:141–149

494 Treu L, Tsapekos P, Peprah M, Campanaro S, Giacomini A, Corich V, Kougias PG, Angelidaki I (2019) Microbial
495 profiling during anaerobic digestion of cheese whey in reactors operated at different conditions. *Bioresour*
496 *Technol* 275:375–385

497 Tsolcha ON, Tekerlekopoulou AG, Akrotos CS, Antonopoulou G, Aggelis G, Genitsaris S, Moustaka-Gouni M,
498 Vayenas DV (2018) A *Leptolyngbya*-based microbial consortium for agro-industrial wastewaters treatment and

499 biodiesel production. *Environ Sci Pollut Res* 25:17957–17966

500 Weisburg WG, Barns SM, Pelletier DA, Lane DJ (1991) 16S ribosomal DNA amplification for phylogenetic study. *J*

501 *Bacteriol* 173:697–703

502 Yadav JSS, Bezawada J, Ajila CM, Yan S, Tyagi RD, Surampalli RY (2014) Mixed culture of *Kluyveromyces marxianus*

503 and *Candida krusei* for single-cell protein production and organic load removal from whey. *Bioresour Technol*

504 164:119–127

505 Zanirati DF, Abatemarco M, Sandes SH d C, Nicoli JR, Nunes AC, Neumann E (2015) Selection of lactic acid bacteria

506 from Brazilian kefir grains for potential use as starter or probiotic cultures. *Anaerobe* 32:70–76

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509 **Legend of figures:**

510

511 **Fig. 1** Dendrograms of bacteria (a) and yeast (b) from Kefir grains obtained by the cluster analysis of rep-PCR
512 (GTG)5 fingerprints. The dendrogram is based on the Pearson coefficient of similarity with the unweighted pair
513 group method with arithmetic averages clustering algorithm (UPGMA)

514

515 **Fig. 2** Response surface graphs showing the effect of the interaction between temperature, T (X1), incubation
516 time, t (X2) and Kefir grains concentration, C (%) (X3) on COD (Y1), PO_4^{3-} (Y2), and NO_3^- (Y3) removal rates; (a)
517 Interaction X1X2, (b) interaction X1X3, and (c) interaction X2X3

518

519 **Fig. 3** Effect of RGCW and TGCW at different dilutions (25, 50, and 100X), on barley germination (a), fresh weight
520 (b), root (c), and shoot length (d)

521

522 **Fig. 4** Effect of RGCW and TGCW at different dilutions (50 and 100X) on barley growth

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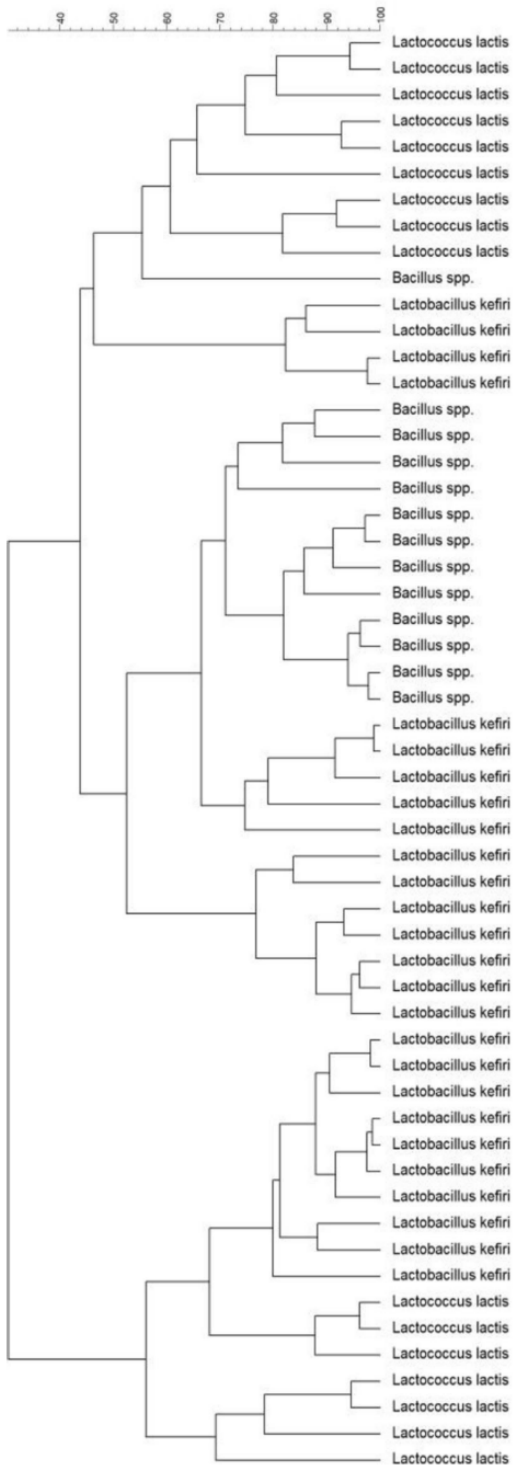
524 **Fig. 5** Total chlorophyll (a), chlorophyll a (b), and chlorophyll b (c) contents of barley seedlings soaked with H₂O
525 (control), RGCW 50X, and TGCW 50X

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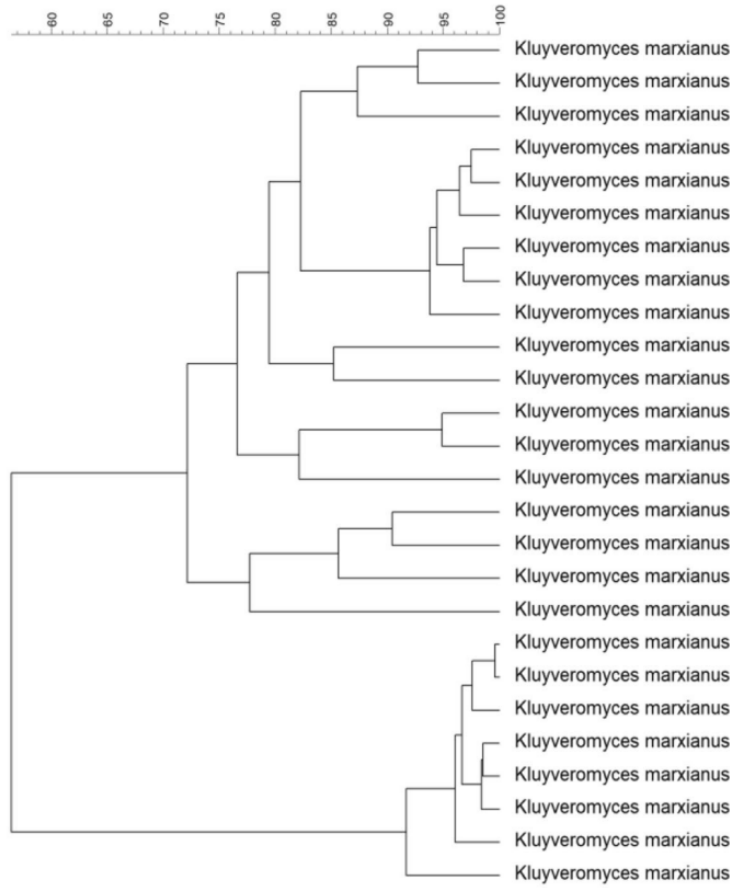
Pearson correlation [0.0%-100.0%]
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Pearson correlation [0.0%-100.0%]
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Figure 1

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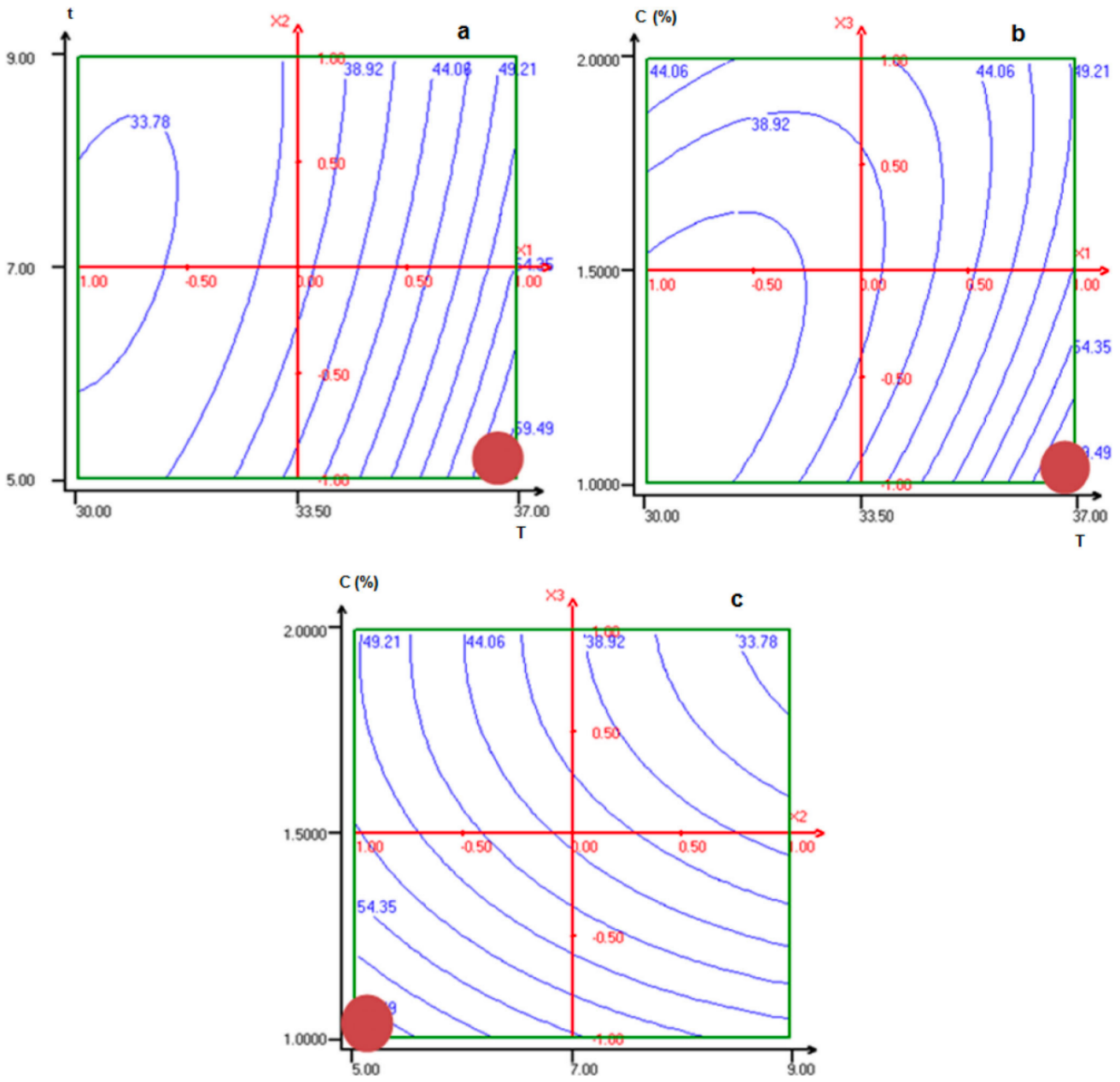


Figure 2

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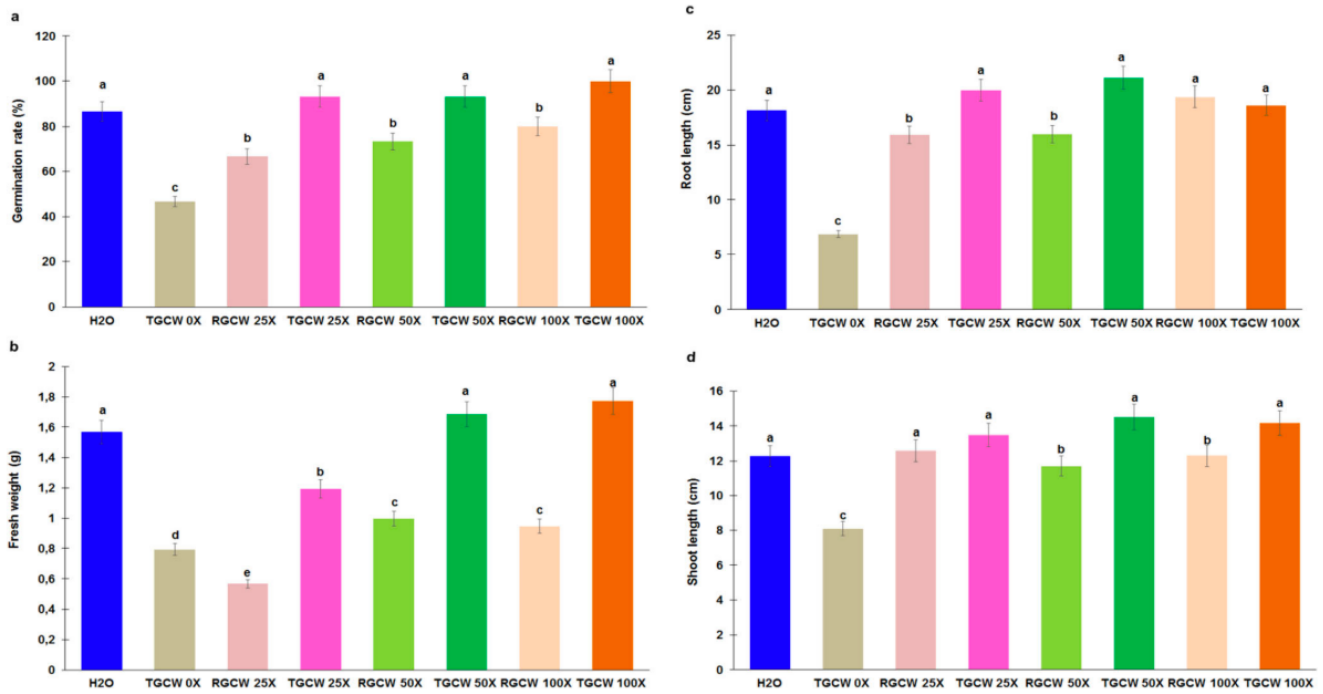
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Figure 3

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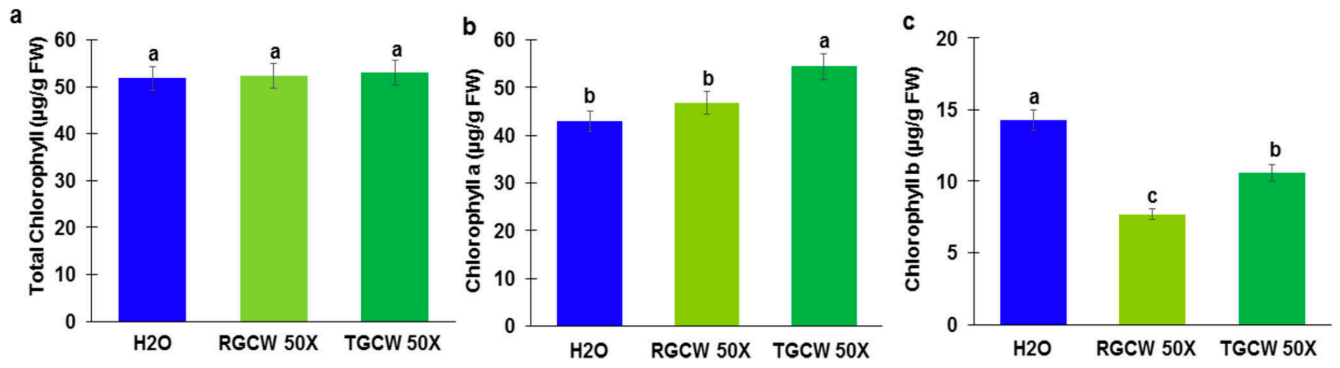
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Figure 4

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Figure 5

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561 **Tables**

562

563 **Table 1** Characteristics of raw cheese whey (CW), white wastewaters (WW) and their mixture (GCW) in ratio 1:1

Parameters	Unit	CW	WW	GCW
pH	–	4.36 ± 0.3	6.09 ± 0.25	4.48 ± 0.5
COD	mg L ⁻¹	46,080 ± 24	700 ± 15	25,920 ± 20
BOD ₅	mg L ⁻¹	19,200 ± 19	–	9800 ± 10
PO ₄ ³⁻	mg L ⁻¹	2.94 ± 0.08	1.88 ± 0.03	2.115 ± 0.05
NO ₃ ⁻	mg L ⁻¹	3920.95 ± 33	3725.78 ± 25	4309.28 ± 28
Conductivity	mS cm ⁻¹	12.47 ± 0.1	0.76 ± 0.05	5.35 ± 0.045
Turbidity	NTU	2144.70 ± 19	2.10 ± 0.047	930 ± 14

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566 **Table 2** Factors and levels of experiment

Levels	Factors		
	X ₁ Temperature (°C)	X ₂ Incubation time (day)	X ₃ Kefir grains concentration (%)
- 1	30	5	1
0	33.5	7	1.5
+ 1	37	9	2

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576 **Table 3** Experimental design matrix and responses (COD, PO₄³⁻ and NO₃⁻ – removal rates) during Kefir grains
 577 treatment using different concentrations of Kefir grains (1; 1.5 and 2%), at 30; 33.5 and 37 °C during 5; 7 and 9
 578 days

Experiment	Independent variables			Responses (Y, removal rates %)					
	X ₁ (°C)	X ₂ (day)	X ₃ (%)	COD		PO ₄ ³⁻		NO ₃ ⁻	
	Temperature	Incubation time	Kefir grains concentration	O*	P*	O	P	O	P
1	30	5	1.5	50	59.09	31.46	31.64	38.39	35.92
2	37	5	1.5	66.66	67.42	38.16	39.90	53.28	52.10
3	30	9	1.5	72.22	71.46	50.63	48.89	31.94	33.121
4	37	9	1.5	61.11	52.02	39.13	38.95	35.40	37.87
5	30	7	1	66.67	67.43	42.01	40.57	36.82	33.32
6	37	7	1	61.11	70.20	41.52	38.52	59.49	54.70
7	30	7	2	72.22	63.13	34.33	37.33	35.06	39.85
8	37	7	2	50	49.24	36.25	37.69	35.89	39.39
9	33.5	5	1	88.88	79.03	35.77	37.03	36.40	42.37
10	33.5	9	1	66.67	66.67	41.52	44.70	34.57	36.89
11	33.5	5	2	55.55	55.55	37.69	34.51	43.33	41.01
12	33.5	9	2	55.05	64.90	44.40	43.14	35.43	29.46
13	33.5	7	1.5	72.22	68.52	30.02	32.60	33.90	32.29
14	33.5	7	1.5	66.67	68.52	38.23	32.60	31.77	32.29
15	33.5	7	1.5	66.67	68.52	29.54	32.6	31.21	32.29

579 O* observed, P* Predicted

580

581 **Table 4** Statistical analysis of BBD and significance of the independent variables and their interactions for COD
 582 removal (%)

Coefficient	Value	SD	t student	Signification (%)
a ₀	68.52	6.04	11.34	***
a ₁	-2.78	3.70	-0.75	49.10
a ₂	-0.76	3.70	-0.20	84.0
a ₃	-6.31	3.70	-1.71	14.70
a ₁₁	-5.03	5.44	-0.92	40.10
a ₂₂	-0.99	5.44	-0.18	85.60
a ₃₃	-0.99	5.44	-0.18	85.70
a ₁₂	-6.94	5.23	-1.33	24.10
a ₁₃	-4.17	5.23	-0.80	46.60
a ₂₃	5.43	5.23	1.04	34.90

583 ***P < 0.1%; SD standard deviation

584

585 **Table 5** Statistical analysis of BBD and significance of the independent variables and their interactions for
 586 PO₄³⁻removal (%)

Coefficient	Value	SD	<i>t</i> student	Signification (%)
<i>b</i> ₀	32.60	2.57	12.67	***
<i>b</i> ₁	- 0.42	1.58	- 0.27	79.40
<i>b</i> ₂	4.08	1.58	2.59	*
<i>b</i> ₃	- 1.02	1.58	- 0.65	55.10
<i>b</i> ₁₁	2.97	2.32	1.28	25.7
<i>b</i> ₂₂	4.28	2.32	1.85	12.3
<i>b</i> ₃₃	2.95	2.32	1.28	25.7
<i>b</i> ₁₂	- 4.55	2.23	- 2.04	9.5
<i>b</i> ₁₃	0.60	2.23	0.27	79.2
<i>b</i> ₂₃	0.242	2.23	0.11	91.5

587 *****P* < 0.1%, **P* < 5%, *SD* standard deviation

588

589 **Table 6** Statistical analysis of BBD and significance of the independent variables and their interactions for NO₃
 590 - removal (%)

Coefficient	Value	SD	t- student	Signification (%)
<i>c</i> ₀	32.29	0.82	39.41	***
<i>c</i> ₁	5.23	0.50	10.42	**
<i>c</i> ₂	- 4.26	0.50	- 8.48	*
<i>c</i> ₃	- 2.20	0.50	- 4.38	*
<i>c</i> ₁₁	5.92	0.74	8.02	*
<i>c</i> ₂₂	1.54	0.74	2.08	17.3
<i>c</i> ₃₃	3.60	0.74	4.88	*
<i>c</i> ₁₂	- 2.86	0.71	- 4.03	5.4
<i>c</i> ₁₃	- 5.46	0.71	- 7.69	*
<i>c</i> ₂₃	- 1.52	0.71	- 2.14	16.7

591 *****P* < 0.1%, ***P* < 1%, **P* < 5%, *SD* standard deviation

592

593 **Table 7** Analysis of variance results for COD, PO₄³⁻, and NO₃⁻ removal (%)

Response (Y, %)	Source of variance	SS	DF	MS	Ratio	Signification (%)
COD removal	Regression	861.20	9	95.69	0.87	59.5
	Residual	547.19	5	109.44		
	Lack of fit	526.66	3	175.55		
	Pure error	20.53	2	10.27		
	Total	1408.39	14			
PO ₄ ³⁻ removal	Regression	342.96	9	38.11	1.92	24.40
	Residual	99.32	5	19.86		
	Lack of fit	51.61	3	17.20		
	Pure error	47.72	2	23.86		
	Total	442.29	14			
NO ₃ ⁻ removal	Regression	732.58	9	81.40	40.41	0.039***
	Residual	171.35	5	34.27		
	Lack of fit	167.35	3	55.78		
	Pure error	4.029	2	2.01		
	Total	903.94	14			

594 SS: Sum of Squares, DF: Degree of Freedom, MS: Mean Square, *** P < 0.1 %, * P > 5%

595

596 **Table 8** Reusability test results of Kefir grains after two cycles for COD, PO₄³⁻, and NO₃⁻ removal from GCW using

597 1.02% Kefir grains at 36.68 °C during 5.14 days

	Kefir grains treatment		
	Pollutants removal rates (%)		
	COD	PO ₄ ³⁻	NO ₃ ⁻
Native	87 ± 0.5	37.48 ± 0.74	39.5 ± 0.39
Cycle 1	80 ± 0.33	65.3 ± 0.45	80.5 ± 0.28
Cycle 2	82.6 ± 0.20	30.8 ± 0.35	32.3 ± 0.27

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