



Research article

Lactic fermentation of cereals aqueous mixture of oat and rice flours with and without glucose addition



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ABSTRACT

Studies of the ability of probiotics to ferment cereal flours are necessary to obtain products with enhanced nutritional value. In this study, *Lactobacillus paracasei* CBA-L74 was used to ferment cereal aqueous mixtures containing both oat (7.5% w/v) and rice flours (7.5% w/v), with and without glucose, to understand whether glucose addition could have any effect on growth and metabolism. Viability, pH, metabolites production during fermentation (24 h, 37 °C) and substrates reduction were analysed. The strain showed good growth in the cereal aqueous mixture both with and without glucose addition, but suspensions prepared with glucose showed the best results. A bacterial concentration of 7 log CFU mL⁻¹, a pH value of 4.70 and lactic acid production of 1250 mg L⁻¹ were achieved when fermentation was performed without glucose addition, while in the presence of glucose, a t₂₄ bacterial growth of 8 log CFU mL⁻¹ was reached, with a pH value of 3.11 and lactic acid production of 6050 mg L⁻¹.

Practical applications

The aim of the present study was the fermentation of suspensions prepared by mixing equal parts of rice and oat flours with water to obtain a semi-finished product to use as a functional ingredient for the production of new dairy- or cereal-based products. Fermentation of single flours suspension was previously studied. By mixing two different flours, we wished to obtain semi-finished products with nutritional potentialities greater than those products obtained by fermenting single flours suspensions. In this sense, the role of glucose was also investigated.

1. Introduction

It is now a certainty that some foods or food components can have beneficial effects on health. In fact, nutritional science not only allows us to understand the correct intake of nutritional components to avoid deficiencies but is also increasingly interested in identifying new food components that can optimize the state of health and reduce the risk of disease. Some foods, such as fruits, milk and vegetables, naturally contain components that are useful for improving health. New products have

been developed to enhance or add in them these beneficial components (Hasler, 2002). All these foods can be defined as functional, as indicated in the literature (Roberfroid, 2002; Diplock et al., 1999; Huggett and Schlitter, 1996). Examples of functional foods are probiotics, live micro-organisms that, when supplied in adequate amounts, confer a health benefit to the host (Joint FAO/WHO, 2001). A microbial concentration considered sufficient for a beneficial effect on the host, according to the International Dairy Federation (International Dairy Federation, 1997), is equal to 6 and 7 Log₁₀ CFU mL⁻¹ based on a 100 mL daily dose. In most cases the mechanism by which these functional foods act is not known. However, most of them exert a beneficial effect by acting on the immune response (Isolauri, 2001; Biancone et al., 2002). In most cases, this is due to stimulation of natural immunity (Newburg et al., 2005; Galdeano and Perdigón, 2006). Most of the probiotics on the market are dairy based, although cereals are becoming a viable alternative, as they bypass problems such as lactose intolerance or impact on cholesterolaemia (Prado et al., 2008). Several studies have demonstrated the feasibility of lactic fermentation on cereals and the probiotic effect that fermented cereals can exert (Sarno et al., 2014; Gallo et al., 2018, 2019a; Salameh et al., 2019; Labruna et al., 2019). The advantages offered by cereals

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therefore represent a boost for the design of new cereals-based foods and ingredients. Several are the key parameters to be considered for the production of these types of foods, starting from the processing of cereal grains, their formulation, the ability to be fermented, the organoleptic properties of the fermented product and so on. The choice of the substrate and its composition, as well as the selection of the strain, are key aspects to be considered in order to monitor the final metabolic products (Lönner and Preve-Åkesson, 1988; De Vuyst, 2000). In several works, cereal-based foods were fermented with indigenous strains (Blandino et al., 2003). In most cases, in these studies, a single kind of cereal was fermented as substrate, and a single kind of culture was used (Angelov et al., 2006; Charalampopoulos et al., 2003; Helland et al., 2004; Kedia et al., 2008; Muyaja et al., 2003). Mixed cultures were used by Kedia et al. (2007) to ferment matrices composed of single cereals, but to the best of our knowledge, no work has studied how cereal composition and inocula can affect the fermentation process. Different cereals have a complex composition that can consistently modify, when mixed, the carbon and nitrogen source profile of the substrate and the content of the prebiotic substances originally contained in the raw materials affecting microorganism growth and metabolism. The feasibility of the fermentation of rice and oat flours, with and without added glucose, have already been investigated in previous studies (Gallo et al., 2019b; c), which showed differences between the two types of substrate. On the basis of those results, we investigated the possible effects that mixing the two flours could have, assuming that the different nutritional compositions of the two flours could influence microbial metabolism during fermentation. Therefore, in this work, we aimed to understand how the mixing of two different flours with different nutritional compositions can influence the fermentation, growth and metabolism of *Lactobacillus paracasei* CBA L-74 in aqueous suspension of a mixture of rice and oat flours, also with the addition of a small amount of glucose.

2. Materials and methods

2.1. Cereal fermentation substrates

The substrate was prepared by mixing 150 g of a mixed cereal flour with 850 mL distilled water. The mix was formed from 75 g of rice and 75 g of oat flours. The cereal flours were air treated at 120 °C for 90 min in an electric oven, while distilled water (or water +2% w/v of glucose) was autoclaved at 121 °C for 20 min. These ingredients were then mixed aseptically in a laminar flow hood in a sterilized fermenter. Subsequently, the suspension, under continuous mixing, was tyndallized, applying two consecutive cycles of heating (70 °C) and cooling (37 °C) to reduce and control the microbial load.

2.2. Microorganisms and inoculation

The strain used as starter culture was *Lactobacillus paracasei* CBA L74, (Heinz Italia SpA), International Depository Accession Number LMG P-24778.

The strain is a gram-positive homofermentative, facultative anaerobic bacterium. It was stored in freeze-dried form at -20 °C and revitalized in a 0.9% sodium chloride solution 10 min before each fermentation. The inoculum was made after bringing the fermentation substrate (mixed cereal media) to a temperature of 37 °C to guarantee the best conditions for microorganism growth.

2.3. Batch fermentation

All fermentations were carried out in a batch reactor under no pH control conditions and at 37 °C. The entire fermentation process lasted 24 h. Sampling times were 0, 4, 18, 20, 22, and 24 h. Samples, aseptically collected, were subjected to pH, microbial count, substrate and metabolite analysis.

2.4. Analytical methods

pH was monitored during fermentation using a Mettler Toledo device (equipped with an autoclavable In Pro 3100 probe) for continuous measurements of pH and temperature during the fermentation process. Growth evaluation was conducted immediately after sampling. After serial dilutions, samples were seeded on Petri plates, prepared with MRS agar (Oxoid) specific for *Lactobacillus* growth and incubated for 48 h at 37 °C under anaerobic conditions, guaranteed by the use of special anaerobic kits (Anaerogen Compact, Oxoid). High-performance liquid chromatography (HPLC) (Agilent Technologies 1100) was used to separate, identify, and quantify the lactic acid, which was the only expected product of fermentation; HPLC was fitted with a UV variable wavelength detector set at 218 nm. The column used was a 5- μ m silica C18 (Agilent Zorbax C18). The mobile phase was 0.1 M $\text{NH}_4\text{H}_2\text{PO}_4$ with a flow rate of 0.8 ml min⁻¹. To verify the presence of any contamination, the production of secondary metabolites, such as butyric, propionic and acetic acids, was analysed by gas chromatography (Agilent Technologies 6890). A capillary Poraplot Q column (25 m \times 0.32 mm) was used. The flow rate was 200 ml min⁻¹. The mobile phase was helium gas. The internal standard was caproic acid at 5 g L⁻¹. Determination of starch and sugars (sucrose, fructose and glucose) contents during the fermentations was obtained by Megazyme enzymatic kits (Total Starch Assay Kit (AA/AMG) and Sucrose/D-Fructose/D-Glucose Assay Kit (K-SUFRG), respectively).

2.6. Statistical analysis

Each test was performed in triplicate. Statistical analysis and graphics were obtained from GraphPad Prism (San Diego, CA). Means and standard deviations for the experiment were calculated; their significance was evaluated by Student's t-test accepting as significant only those results showing values of $p < .05$.

3. Results

The growth results, pH values and organic acid production obtained during the cereal mix fermentations performed with and without glucose addition are all shown in Figure 1. The initial *Lactobacillus* concentration was approximately 5×10^5 CFU mL⁻¹ for both formulations, as shown in Figure 1A. From 18 h until the end of the process, statistically significant differences were noted at each sampling time (t_{18} , t_{20} , t_{22} , t_{24}). After 24 h of fermentation, a final concentration of $3.3 \times 10^7 \pm 7.2 \times 10^6$ CFU mL⁻¹ was reached for the fermentation performed without glucose addition, while a final concentration of $6.2 \times 10^8 \pm 7.1 \times 10^7$ CFU mL⁻¹ was measured in the case with glucose addition ($p < .001$). The final pH values, measured after 24 h of fermentation, were 4.7 ± 0.21 when no glucose was added and 3.11 ± 0.13 with glucose addition, starting from the same values, as shown in Figure 1B. Also in this case, from 18 h until the end of the process, statistically significant differences were noted at each sampling time (t_{18} , t_{20} , t_{22} , t_{24}). The trend of lactic acid production was congruent with microbial growth. The concentration of lactic acid began to increase after 4 h, reaching higher values at the end of fermentation of 1250 ± 107.92 mg L⁻¹ and 6050 ± 103.41 mg L⁻¹ in the cases of no addition and addition of glucose, respectively, as shown in Figure 1C. From t_{18} to t_{24} , statistically significant differences ($p < .001$) were noted.

Additionally, the contents of acetic, propionic and butyric acids were measured. Concentrations of an order of magnitude lower than lactic acid concentrations for both conditions (data not shown) were detected, demonstrating no viability of contaminants in the fermenting medium. Starch and sugars concentrations were evaluated for the two different process conditions, as shown in Figure 2. Starch concentrations measured at t_0 , t_{20} and t_{24} for both conditions are shown in Figure 2A. Starting from an initial starch concentration of 106 g L⁻¹ for both cases, a reduction in the starch content was notable at 24 h only when no glucose was added (t_{24} 103.33 ± 0.58 g L⁻¹); the difference in starch concentration at t_{24}

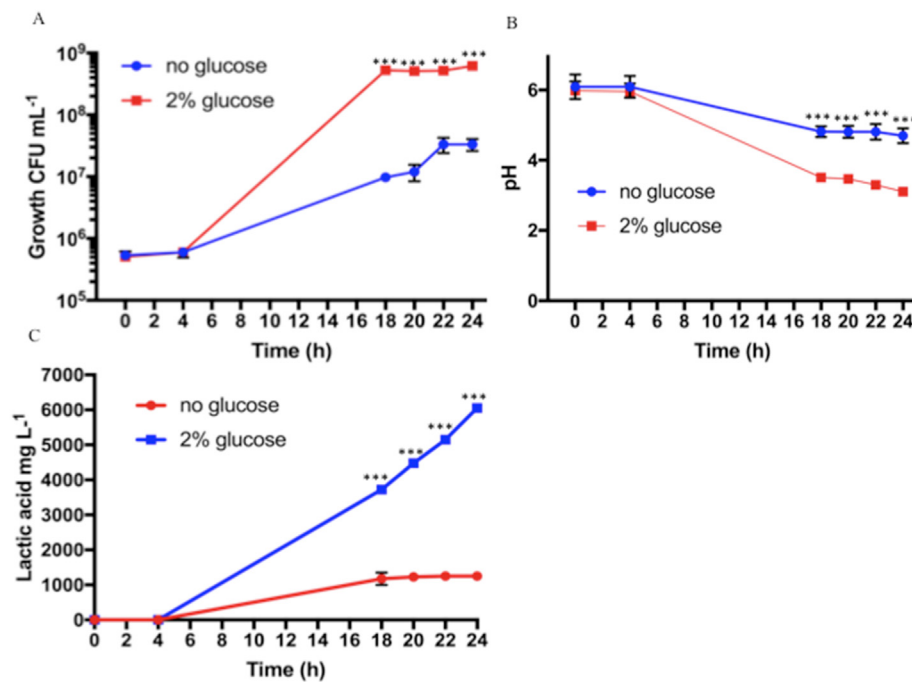


Figure 1. Analytical results obtained for the fermentation of mixed rice-oat suspension with and without glucose addition at different sampling times (t_0 , t_4 , t_{18} , t_{20} , t_{22} , t_{24}). A) Bacterial growth (CFU mL⁻¹) observed for the rice-oat fermentation performed without glucose addition and by adding 2% of glucose. B) pH values observed for the fermentations performed without glucose addition and by adding 2% of glucose; C) Lactic acid production in mg L⁻¹. Bars represent standard deviation of three independent experiments. Student t-test *** $p < .001$.

was statistically significant ($p < .05$). Sucrose, fructose and glucose concentrations are shown in Figure 2B for both the fermentations performed with and those performed without glucose addition. The initial sugars concentrations of 2.713 ± 0.155 g L⁻¹ sucrose, 0.183 ± 0.025 g L⁻¹ glucose and 0.040 ± 0.017 g L⁻¹ fructose were detected in the suspension fermented without glucose addition. At the end of the fermentation process, final values of 0.803 ± 0.210 , 0.083 ± 0.015 and 0.013 ± 0.002 g L⁻¹ of sucrose, glucose and fructose, respectively, were reached.

Regarding fermentations with glucose addition, starting with initial sugars concentrations of 2.707 ± 0.158 , 24.267 ± 2.281 and 0.039 ± 0.013 g L⁻¹ of sucrose, glucose and fructose, respectively, final values of 1.303 ± 0.199 , 19.067 ± 1.097 and 0.004 ± 0.002 g L⁻¹ were reached. All the results presented so far are summarized in Table 1. To make the trend of the concentrations of the different sugars more evident, the results were also represented in two distinct graphs: Figure 2C for the sugars concentrations measured when no glucose was added and

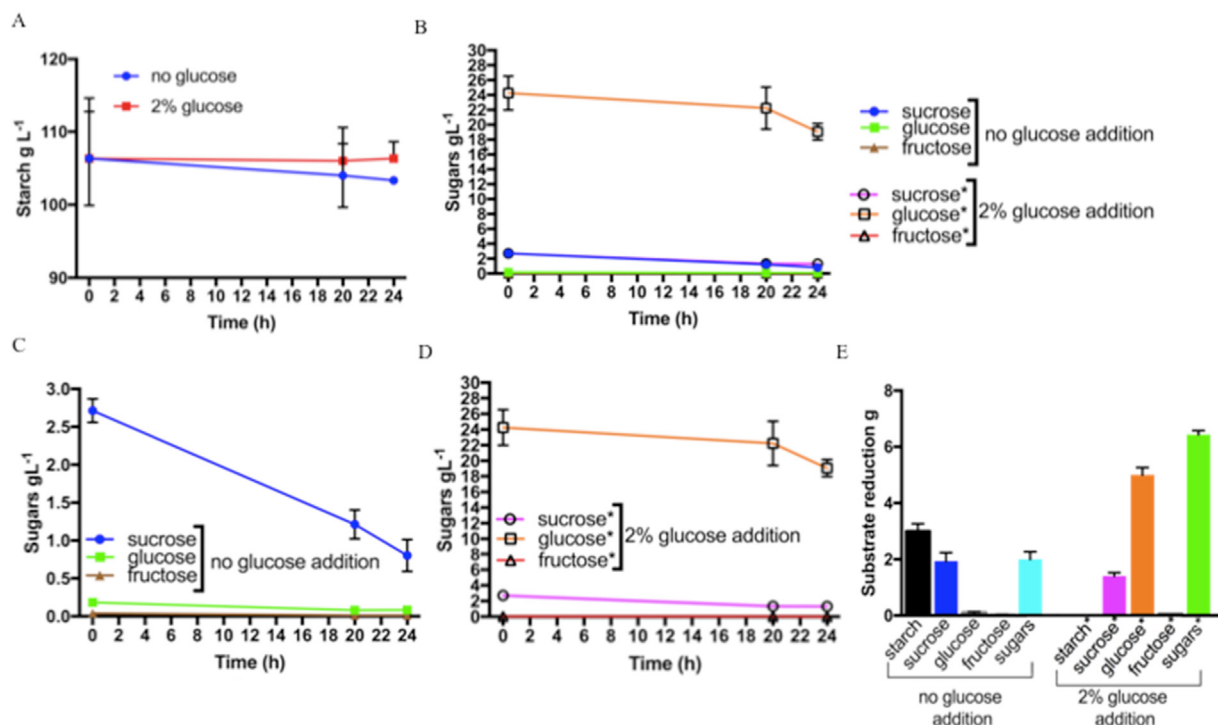


Figure 2. Substrates reduction observed during the mixed rice-oat fermentations with and without glucose. A) Starch concentrations (g L⁻¹) measured at t_0 , t_{20} , t_{24} . B) Sugars concentrations (g) measured at t_0 , t_{20} , t_{24} , for both conditions tested (with and without glucose addition) C) Sucrose, glucose and fructose concentrations (g L⁻¹) measured at t_0 , t_{20} , t_{24} , when no glucose was added. D) Sucrose, glucose and fructose concentrations (g L⁻¹) measured at t_0 , t_{20} , t_{24} , when 2% of glucose was added. E) Sucrose, glucose, fructose (g) and sugars (to be understood as the sum of the observed reductions for sucrose, glucose and fructose) reduction measured for both the conditions tested (with and without glucose addition). Bars represent standard deviation of three independent experiments.

Figure 2D for the sugars concentration detected when 2% glucose was added. The reduction of all the substrates observed is shown in Figure 2E and presented in Table 2. In particular, a total substrate reduction of 5.037 g was measured when no glucose was added and of 6.639 g when glucose was added. However, when no glucose was added, a starch reduction was observed, but it was not possible to affirm that all the starch was truly consumed, considering that the possible intermediates of starch hydrolysis were not evaluated. However, it is possible to say with certainty that a consumption of simple sugars of 2.037 g was observed when no glucose was added.

Finally, the biomass and the product yields were calculated according to equation 1 [$Y_{X/S} = \Delta X / \Delta S = (X - X_0) / (S - S_0)$] and equation 2 [$Y_{P/S} = \Delta P / \Delta S = (P - P_0) / (S - S_0)$], respectively, where X_0 (g L⁻¹) is the concentration of biomass at time 0, X (g L⁻¹) is the concentration of biomass at the end of the process, S_0 (g L⁻¹) is the concentration of substrate at time 0, S (g L⁻¹) is the concentration of biomass at the end of the process, P_0 (g L⁻¹) is the concentration of lactic acid found at time 0, and P (g L⁻¹), is the concentration of lactic acid measured at the end of the process. The biomass concentration in g L⁻¹ was determined by measuring cell growth (CFU mL⁻¹) in revitalising animal-free broth after a 24-h incubation by the spread plate method and determining the grams of biomass corresponding to a colony forming unit. The revitalising broth was centrifuged, the supernatant was discharged, and the pellet weight was measured. In this way, for the biomass concentration, a conversion factor of 10⁻⁹ was found to convert CFU mL⁻¹ in g L⁻¹ of biomass. Biomass yields of 0.006 and 0.093 were found when no glucose and 2% glucose were added, respectively. In the same way, product yields of 0.248 and 0.911 were found when no glucose and 2% glucose were added, respectively. Biomass and product yields were also calculated considering as ΔS (concentration of substrate consumed) only the sugars. In this way, the same yields were obtained for the fermentation performed with 2% glucose addition, while when no glucose was added, the biomass yield and product yield were 0.016 and 0.614, respectively. All these results are summarized in Table 3.

4. Discussion

Cereal fermentation, particularly rice and oat flours fermentation, has already been investigated (Gallo et al., 2018; Gallo et al., 2019b; c). In these fermentation processes, rice and oat flours were individually

studied as fermentation substrates. Rice flour showed the best results in terms of bacterial growth and lactic acid production with no glucose addition, and no difference was noted with the presence of glucose, while oat flour showed improved bacterial growth and lactic acid production during fermentation with glucose addition. On these bases, to check whether each flour, with its own characteristics, could affect the fermentation performance and the functionality of the other flour, we designed fermentation substrates mixing rice and oat flours. By mixing equal parts of these two flours, with no addition of glucose, an apparent reduction in microbial growth, and consequently in the production of lactic acid, could be observed compared to what was reported by Gallo et al. (2019b) (with 3.3×10^7 CFU mL⁻¹ and 1250 mg L⁻¹ being the microbial growth and the lactic acid production obtained at 24 h in the present study, with no glucose addition, whereas 9.5×10^8 CFU mL⁻¹ and 2×10^8 CFU mL⁻¹ were the rice and oat concentrations reached by Gallo et al. (2019b) with lactic acid production of 3100 mg L⁻¹ for rice and 2000 mg L⁻¹ for oat flour). However, taking into account the initial bacterial charge of both studies (10⁵ CFU mL⁻¹ in the present study; 10⁶ CFU mL⁻¹ in Gallo's study), a final increase of approximately 2 log was reached in both cases. Therefore, it is possible to state that without glucose addition, no substantial difference was noted concerning microbial growth. The lactic acid produced in the present study was lower than that obtained for the rice flour in particular but also for the oat flour, and this could be linked to the lower bacterial concentration reached in the present study.

Mixed oat-rice flours fermentations were also carried out by adding 2% w/v glucose because in the past study, this glucose concentration was evaluated as the lowest concentration able to guarantee a significant increase in bacterial growth (Gallo et al., 2019c). Comparing the results obtained by fermentation with the addition of glucose in the present study with those reported previously (Gallo et al., 2019c), a final increase of 3 log was observable in both studies, but the production of lactic acid was significantly higher by mixing the flours, rather than the lactic acid produced by single flours (with lactic acid concentrations being 6050 mg L⁻¹ for mixed rice-oat suspensions with glucose addition in the present study, 3200 mg L⁻¹ for rice flour with glucose addition and 3300 mg L⁻¹ for oat flour with glucose addition). Thanks to this study, it is therefore possible to confirm and strengthen the results obtained with those previously reported (Gallo et al., 2019b; c) on the role of glucose in increasing microbial growth and lactic acid production, for oat flour

Table 1. Analytical results obtained by fermenting rice-oat mixed suspension without and with glucose addition.

Sampling Time	W/O GLUCOSE		2% GLUCOSE	
	0	24	0	24
Growth CFU mL ⁻¹	$5.3 \times 10^5 \pm 5.0 \times 10^4$	$3.3 \times 10^7 \pm 7.2 \times 10^6$	$5.1 \times 10^5 \pm 1.1 \times 10^5$	$6.2 \times 10^8 \pm 7.1 \times 10^7$
pH	6.10 ± 0.35	4.7 ± 0.21	5.98 ± 0.26	3.11 ± 0.13
Lactic Acid mg L ⁻¹	0	1250 ± 107.92	0	6050 ± 103.41
Starch g L ⁻¹	106.33 ± 6.43	103.33 ± 0.58	106.33 ± 8.33	106.33 ± 2.31
Sucrose g L ⁻¹	2.713 ± 0.155	0.803 ± 0.210	2.707 ± 0.158	1.303 ± 0.199
Glucose g L ⁻¹	0.183 ± 0.025	0.083 ± 0.015	24.267 ± 2.281	19.067 ± 1.097
Fructose g L ⁻¹	0.040 ± 0.017	0.013 ± 0.002	0.039 ± 0.013	0.004 ± 0.002

Table 2. Substrates reduction (g L⁻¹) measured at the end of rice-oat mixed suspension fermentation performed without and with glucose addition.

Substrates reduction	W/O GLUCOSE	2% GLUCOSE
	(g L ⁻¹)	(g L ⁻¹)
Starch	3.00	0
Sucrose	1.91	1.404
Glucose	0.10	5.2
Fructose	0.027	0.035
Total reduction	5.037	6.639
Sugars reduction	2.037	6.639

Table 3. Biomass and product yields (g g^{-1}) calculated for both conditions tested (with and without glucose addition), according to the equations $Y_{X/S}$ and $Y_{P/S}$, respectively, where S has been estimated both as an overall substrate reduction (starch + sugars) and as sugars only.

g g^{-1}	W/O GLUCOSE		2% GLUCOSE	
	S = starch + sugars	S = sugars	S = starch + sugars	S = sugars
Biomass Yield	0.006	0.016	0.093	0.093
Product Yield	0.248	0.614	0.911	0.911

alone and for the mixture of rice-oat flours but not for rice flour alone. Furthermore, it is possible to state that flours mixing in the presence of glucose can give the best results in terms of lactic acid production. This could be due to a positive influence of the oat flour (for which improved results were noted in past studies by adding glucose) on the rice flour (for which no improving results were noted) in the presence of glucose. Therefore, a synergistic effect of mixing and glucose addition can be supposed. This could be due to differences between rice and oat flours in terms of chemical composition: oat flour is richer in protein (approximately 13% for oat versus 7% for rice) and vitamin contents than rice flour (INRAN, 2000). Several studies have shown how nitrogen and vitamin supplementation could improve lactic acid production during lactic fermentation (John et al., 2007). Furthermore, oat flour is a rich source of β -glucans, which act as prebiotic components (Jayachandran et al., 2018). Another aspect to be considered is substrate reduction during the two types of fermentations. We prefer to talk of “substrate reduction” instead of “substrate consumption” considering that only the starch concentration was evaluated, and no data on intermediates of starch hydrolysis were obtained. Substrate reductions of 5.037 g and of 6.639 g were registered when no glucose and when 2% glucose was added, respectively, considering the starch reduction as well, while considering only the sugars reductions, 2.037 g was the reduction measured when no glucose was added, and the same reduction of 6.639 g was found when glucose was added. Thus, in the absence of the addition of glucose, starch and sucrose were the substrates of choice; with the addition of glucose, the metabolism moved towards simple sugars, primarily glucose. The addition of glucose allows better use of the substrate both for the production of biomass and for the production of lactic acid, as shown by the yields, and this is probably due to the simpler and faster accessibility of the substrate (glucose instead of starch or sucrose). Although no complete data are available on starch consumption, it is possible to affirm that the addition of glucose determines better yields, taking into account that these yields are, however, higher than those obtained without adding glucose, whether the starch is included (and therefore the yields are overestimated) and not in the evaluations. In any case, it is necessary to note that only 5 g of glucose was consumed, which may lead to speculation that the addition of 2% glucose is too high. It can be assumed that the minimum percentage to be added to obtain the same results could be 0.5% w/v or slightly higher. However, if the aim of fermentation is lactic acid production, the results obtained by Gallo et al. (Gallo et al., 2019b; c) on the effect of increasing glucose concentrations (i.e., as the glucose added increased, the lactic acid increased) should be considered. Therefore, a future perspective could be to test different glucose concentrations on mixed rice-oat suspensions to understand whether, using the same substrate, lactic acid increases as glucose increases and whether the addition of glucose concentrations lower than 2% (i.e., 0.5%) can give the same results in terms of bacterial growth and lactic acid production.

5. Conclusions

This work allowed us to confirm the feasibility of cereal fermentation and to better understand the effect of mixing flours. The flours mixing, without glucose addition, did not improve bacterial growth and lactic acid production, while if glucose was added, growth equivalent to that of the single flours, accompanied by doubled lactic acid production, was noted. Therefore, from this study, it is possible to confirm the importance

of glucose on bacterial growth and on the production of lactic acid, but a glucose concentration of less than 2% w/v is likely sufficient. Additionally, starch reduction was monitored: starch, together with sucrose, was used as the substrate only when no glucose was added. Starch can therefore be considered a second-choice substrate to be used in the absence of glucose. This can also be confirmed by examining the biomass and product yields.

The role of mixing also emerged from this experimentation, since a synergistic effect of both glucose addition and mixing was noted. In fact, by mixing the flours, it is possible to compensate for the possible deficits of each one, obtaining a product with greater nutritional value and greater industrial potential; therefore, further studies on other flours or more generally on the mixing of different substrates would be important in this sense.

Declarations

Author contribution statement

Marianna Gallo: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

Francesca Passannanti: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Rosa C. Cante, Federica Nigro, Paola Schiattarella, Salvatore Zappulla: Performed the experiments.

Andrea Budelli: Contributed reagents, materials, analysis tools or data.

Roberto Nigro: Conceived and designed the experiments; Analyzed and interpreted the data.

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Competing interest statement

The authors declare the following conflict of interests: Dr Andrea Budelli; [currently employed by Heinz BV, Netherlands. He provided the raw materials (rice, oat and wheat flours) and *Lactobacillus paracasei* CBA L74 and participated in the design of the study. He did not have any additional role in the data collection and analysis, decision to publish, or preparation of the manuscript.]

Additional information

No additional information is available for this paper.

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