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In situ riboflavin fortification of different kefir-like cereal-based beverages using selected Andean LAB strains

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ABSTRACT

Cereal-based functional beverages represent social, economic, and environmental sustainable opportunities to cope with emerging trends in food consumption and global nutrition. Here we report, for the first time, the polyphasic characterization of three cereal-based kefir-like riboflavin-enriched beverages, obtained from oat, maize and barley flours, and their comparison with classical milk-based kefir. The four matrices were successfully fermented with commercial starters: i) milk-kefir and ii) water-kefir, proving the potential of cereal ingredients in the formulation of dairy-like fermented beverages with milk-kefir starter behavior better in these matrices. In the light of their potentiality, seven riboflavin-producing Andean Lactic Acid Bacteria (LAB) were tested for tolerance to food stresses commonly encountered during food fermentation. Moreover, the LAB strains investigated were screened for spontaneous riboflavin overproducing derivatives. *Lactobacillus plantarum* M5MA1-B2 with outstanding response to stress, was selected to improve riboflavin content in an *in situ* fortification approach. The combination of *L. plantarum* M5MA1-B2 riboflavin overproducing strain with milk kefir starter in oat, lead to cover, for one serving of 100 g, 11.4% of Recommended Dietary Allowance (RDA). Besides, addition of *L. plantarum* M5MA1-B2 improved performance of water kefir in oat and maize matrices. Proton Transfer Reaction Time-of-Flight Mass Spectrometry (PTR-ToF-MS) analysis provided the on-line Volatile Organic Compounds profiles supporting the best combination of starter, LAB and cereal matrix for novel functional foods development.

1. Introduction

Development of new functional beverages is a growing sector of the food industry due to an increase demand for foods that confer health benefits and reduce the risk of human diseases. Fermented milks are the most popular functional beverages in Western Europe and North America (Marsh et al., 2014). However, non-dairy functional beverages are particularly attractive in order to avoid dairy allergens and intolerances, to reduce cholesterol-intake and to support some alternative dietary lifestyles (Kandylis et al., 2016; Prado et al., 2008). Furthermore, food matrices that are different from milk can provide valuable combinations of antioxidants, dietary fibers, minerals and vitamins, increasing the health attributes of the final products. Cereal-based fermented products well fit in this emerging field, since they are, among other, a cheap source of natural prebiotic molecules (e.g. beta-glucans) (Pérez-Ramos et al., 2017; Russo et al., 2016; Luana et al., 2014).

Despite yogurt is the best known fermented milk product in the world, kefir represents another important fermented milk that became very popular during the 20th century for its potential contribution to improve the health of Caucasian communities (De Oliveira Leite et al., 2013). Kefir is an acidic, viscous, somewhat effervescent, slightly alcoholic milk beverage. At the basis of fermentative process there is a symbiotic combination of bacteria (several species of lactic acid bacteria and *Acetobacter* spp.) and yeasts, bound within a polysaccharide matrix, known as kefir 'grains' (Bourrie et al., 2016; Garofalo et al., 2015; Garrote et al., 2001). Water kefir is similar in concept to milk

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kefir since a symbiosis of bacteria and yeasts contained within grains of dextran ferment sweetened water, to which figs and lemon are added to improve flavour and nutritional content (Laureys and De Vuyst, 2014). Although the composition of water kefir can vary, it is known to contain mainly lactic acid bacteria (LAB), and *Bifidobacterium* spp. (Marsh et al., 2014; Gulitz et al., 2011). In recent years, some works challenged the formulation of kefir-like products manufactured using non-dairy raw materials, such as soy milk (Liu and Lin, 2000; Abdolmaleki et al., 2015), cocoa pulp juice (Puerari et al., 2012), apple juice (Sabokbar et al., 2015), walnut milk (Cui et al., 2013) and Mediterranean fruit juices (Randazzo et al., 2016), or comparing soy kefir product with the traditional beverage (Abdolmaleki et al., 2015). Related to cereals, reports on the use of kefir culture to ferment cereal matrices are still scarce. Indeed, apart from ethnic traditional cereal-based fermented products such as *Boza, Kvass* and *Kile* (Baschali et al., 2017; Tamang et al., 2016), kefir culture has been recently used with oat and wheat fibers to optimize the fermented soy products formulation (Baú et al., 2013), or for the production of Thai pigmented rice milk kefirs (Deeseenthum et al., 2018).

Kefir-based functional beverages can also benefit from the metabolic activities of other microorganisms. Fermentations and microbial bioconversions of raw materials are crucial for the production of functional metabolites (De Vos, 2005) and food grade LAB with remarkable technological or functional traits, could be exploited for the formulation of new fermented foods in order to improve their nutritional value. The biosynthetic capacity and metabolic versatility of LAB, are some of the principal features that facilitate the application of LAB in foods for producing, releasing and/or increasing specific beneficial compounds. Among these, vitamin production by LAB has recently gained the attention of the scientific community. For instance, several LAB were successfully employed to improve B-group vitamins content of fermented food (Juarez del Valle et al., 2014; Capozzi et al., 2012; Burgess et al., 2009) and, recently, LAB able to overproduce vitamin B_2 (also known as riboflavin), were selected and used as starter to increase riboflavin content of cereals based fermented food (Russo et al., 2014). Humans are unable to synthesize riboflavin, a vitamin that plays an important role in energy metabolism of the cell and it is involved in different redox reactions (Abbas and Sibirny, 2011). Therefore, the selection of LAB able to synthesize riboflavin and their application with an *in situ* fermentation may be considered a food grade approach in order to improve the healthy value of a given fermented food (LeBlanc et al., 2011). According to the European Food Information Council (2006), a balanced diet normally supplies the riboflavin Recommended Daily Allowance (RDA) of 1.4 mg/day for an adult. Recent studies have reported a decreasing intake of B_2 vitamin in some population groups (Mensink et al., 2013) leading to deficiencies risk in elderly people (Fabian et al., 2012). Thus, it may be advisable for specific groups of individuals a supplementation of daily riboflavin intake. Nevertheless, the industrial production of new fermented foods such as riboflavin enriched food, have to deal with the adaptability of LAB during food production and storage. Indeed, several food stress including low pH, freezing, temperature, osmotic pressure, occurrence of ethanol, may significantly affect LAB viability and/or their technological performances (Laureys et al., 2017; Capozzi et al., 2016; Ferrando et al., 2015).

With this work, we aimed to propose and characterize, marketable cereal-based kefir-like products using three different matrices fermented with both, classic milk kefir starter cultures and water kefir starter cultures. Moreover, we were able to select, among LAB isolated from indigenous fermented Andean foods/beverages (Jiménez et al., 2018; Elizaquível et al., 2015), some strains harboring a riboflavin over-producing phenotype. The selected strains, in combination with commercial kefir starter cultures, may be useful to improve the nutritional value of the final products.

2. Material and methods

2.1. Microorganisms and growth conditions

LAB used in the present study were five *Lactobacillus plantarum* strains (M5MA1, M9MG6, M9MM1, M9MM4 and M9Y2) and two *Leuconostoc mesenteroides* strains (M9MG2b and T1M3), previously characterized and screened for some biotechnological traits (Jiménez et al., 2018). They were isolated from "*chicha*", an alcoholic maize-based fermented beverage from North-western Argentina (Elizaquível et al., 2015), and from "*tocosh*", naturally fermented potatoes from Peruvian Andes (Jiménez et al., 2018). LAB strains were routinely grown on MRS broth (Oxoid, Basingstoke, UK) at 30 °C for 24–48 h or stored at −20 °C in a 10% (w/v) dilution of the same broth medium supplemented with 20% (w/v) sterile glycerol prior usage.

2.2. Evaluation of technological stress tolerance of the LAB strains

Overnight cultures of each LAB strains were used to inoculate MRS medium in order to test their tolerance to four different stresses. For this, growth was monitored by measuring the optical density (OD) at 600 nm under the following stress conditions: temperatures (10 °C, 20 °C, 37 °C and 42 °C), salt (2 and 4% of NaCl, w/v), ethanol (8 and 10%, v/v) and low pH (3 and 4). The OD_{600nm} measurements for all stress conditions were performed in three independent biological replicates. In order to estimate the maximum OD_{600nm} reached, bacterial growth was monitored until stationary phase and statistically significant adjustment were performed according to the reparametrized Gompertz equation proposed by Zwietering et al. (1990) which also takes into account the maximum specific speed (μ_{max}) of the strains (Alonso-del-Real et al., 2017).

2.3. Isolation of roseoflavin-resistant derivative strains

In order to obtain spontaneous riboflavin overproducing derivatives, the LAB strains were grown on a chemically defined medium (CDM) vitamin B_2-free (Russo et al., 2014), and exposed to increasing concentrations of roseoflavin (10 mg/L, 50 mg/L, 100 mg/L, and 200 mg/L) according to Burgess et al. (2006). Cultures treated with 200 mg/L of roseoflavin were spread onto MRS agar plates to obtain isolated colonies. A total of 35 randomly chosen colonies (five for each treated strain) were inoculated again in CDM broth without B_2 vitamin. Conversion of the medium to yellow was evaluated for the selection of potential riboflavin overproducing derivatives (Russo et al., 2014). For each strain, the derivative showing the most intense yellow color was selected and cryopreserved for further assays.

2.4. Extraction and quantification of riboflavin

Riboflavin was quantified in whole-cell extracts of cultures at stationary phase, as well as in kefir product samples studied in the present work, as previously reported (Russo et al., 2014). Briefly, 5 g of samples were mixed with 25 mL 0.1 M HCl and hydrolysed by autoclaving at 121 °C for 30 min. Then, the pH was adjusted to 4.5 with 4 M sodium acetate and the samples were submitted to enzymatic treatment by adding a 5-mL solution containing α-amylase (420 U), papain (12 U), acid phosphatase (22 U), and 0.1% of glutathione (all purchased from Sigma Aldrich, St Louis, MO, USA). The samples were diluted to a final volume of 50 mL with 0.01 M HCl and quantified by HPLC according to Jakobsen (2008).

2.5. Tolerance of selected strains to kefir-related stress conditions

Three promising LAB strains and their corresponding roseoflavin-resistant natural derivatives were tested for their ability to grow under stress conditions simulating the growth during kefir and kefir-like

fermentation process. Following the same procedure reported in section 2.2, the growth of the selected strains was monitored for 24 h at 25 °C in MRS at pH 4 and supplemented with ethanol 2% (v/v). Assays were performed in triplicate.

2.6. Elaboration of cereal based kefir-like products

Cereal-based fermentable products were obtained from oat, maize and barley flours, according to the method previously reported by Coda et al. (2012) with some modifications. Briefly, three mixtures with each flour (12% w/v) and distilled water (88% v/v) were obtained in sterile plastic containers. To achieve gelatinization, the mixtures were heated at 95 °C in a water bath for 10 min with manual shaking every 2 min. After that, the mixtures were aliquoted (75 mL) in sterile plastic containers to perform the fermentations. Fermentations of the three cereal products were carried out using two commercial kefir starters "*kefir fai da te*" (BioNova snc, Villanova sull'Arda, Italy): i) water kefir preparation and ii) milk kefir preparation as previously described by Randazzo et al. (2016) for fruit juices. Furthermore, milk was included in the study as a control of standard kefir production. According to manufacturer specifications, two 25X suspensions of freeze-dried microbial mixtures (6.25% w/v) were prepared to rehydrate milk and water kefir starters during 1 h at room temperature. Inoculation was performed at a final concentration of approximately 6 log CFU/mL. Fermentations were carried out at 25 °C for 48 h in two independent biological replicates for each combination of matrices (4) and kefir starters (2). Besides, four batches (barley, maize, oat and milk) were included without any inoculum, as controls. In parallel, another fermentation series was carried out combining the matrices (4) and kefir starters (2) that, in addition, were inoculated with a riboflavin overproducing strain at a final known concentration of approximately 8 log CFU/mL, according to the procedure previously described (Russo et al., 2016).

2.7. Physico-chemical and microbiological analysis

Content in lactic and acetic acids were analysed in all batches at the beginning and after 48 h of fermentation process. Viscosity and microbial analysis were determined only for fermented samples. Lactic and acetic acid concentrations were determined using a commercial kit (Biogamma, Setteville di Guidonia, Italy) following the manufacturer's instructions. Determinations were performed in triplicate. Rheological measurements were analysed with a rotational Brookfield LV, DV-II-Pro viscometer (Brookfield, Harlow, England). Approximately 50 mL of each sample were placed in the concentric cylindrical cup. For the analysis, the spindle n° 3 was used, applying a speed equal to 40 g for 30 s. The viscosity was expressed in centipoise (cP). LAB and yeast viability during fermentation was determined by plate count analysis on MRS agar (Oxoid) and Yeast Extract-Peptone-Dextrose agar (YPD, Oxoid) added with 10 mg/L of cycloheximide and chloramphenicol, respectively, after incubation at 30 °C for 72 h.

Sensory evaluation was carried out by comparing the flavor of cereal-based matrices (oat, maize and barley flours) fermented with water kefir or milk kefir starter, with the standard milk kefir product used as control.

2.8. Volatile organic compounds (VOC) determination by Proton Transfer Reaction Time-of-Flight Mass Spectrometry (PTR-ToF-MS)

All fermentation processes were also monitored in order to detect and quantify VOC production over time using a multifunctional autosampler provided with a robotic arm (Gerstel, Mülheim an der Ruhr, Germany). For each experimental mode (20 in all: the four food matrices, two kefir starter additions, with or without riboflavin *L. plantarum* B_2-overproducing strain and non-inoculated matrices), five replicates were prepared accounting for one hundred vials employed. The resulting kefir products were monitored for 48 h. The whole experiment was performed in two separate days, randomizing the position of replicates in the analyser. Headspace measurements of the fermented products were performed with a commercial PTR-ToF-MS 8000 apparatus from Ionicon Analytik GmbH (Innsbruck, Austria), as was already described by Capozzi et al. (2017) and Benozzi et al. (2015).

2.9. Statistical analysis

Significant differences between stress tolerance of LAB strains as well as physicochemical and microbiological characterization of the milk kefir and cereal kefir-like products were analysed by using the one-way ANOVA module of the Statistica 7.0 software.

For the stress tests, LAB strains growth was evaluated with the maximum estimated OD_{600nm} values which were introduced as dependent variables, and mean values were grouped using the Tukey HSD test ($\alpha = 0.05$). Regarding kefir and kefir-like products characterization, the dependent variables were: the variation between initial time and after 48 h-fermentation of lactic and acetic acids, the viscosity after 48 h and the Log of microbial counts on MRS and YPD at the end of fermentations. In this case, the obtained means were grouped using the Tukey HSD test ($\alpha = 0.1$).

3. Results

3.1. Performance of LAB strains under food-related stress conditions

LAB strains used in this study were previously selected among isolates from traditional Andean fermented foods by exhibiting interesting biotechnological properties, such as the ability to produce group-B vitamins, and, in particular, high amounts of riboflavin (Jiménez et al., 2018). With the aim of a potential industrial application, the behavior of the strains was investigated using different food related stressors. As reported in Fig. 1, all the strains were able to grow at the different temperatures studied with the best growth rates recorded at 20 °C, followed by 37 °C. In particular, *L. plantarum* strain M5MA1 reached the maximum estimated OD_{600nm} values at all the temperature investigated with some marked differences at 37 °C and 20 °C. Growth of *Lc. mesenteroides* T1M3 and M9MG2b showed no statistical differences when compared with *L. plantarum* M9Y2 at 37 °C, or with *L. plantarum* M9MM4 at 20 °C. Overall, the growth temperature range observed, suggested a strain rather than a species dependent feature. As expected, temperature of 10 °C drastically affected growth of all the strains analysed (Fig. 1a).

Regarding salt stress, all strains were able to grow at 2% and at 4% of NaCl albeit to a lesser extent. *L. plantarum* M5MA1 and M9MG6 reached the maximum OD_{600nm} at 2% NaCl while *Lc. mesenteroides* strains showed the lowest OD_{600nm} at both salt concentrations tested (Fig. 1b). Growth in the presence of 8% ethanol was between 2 and 4 times higher than at 10% ethanol for *L. plantarum* strains, being M5MA1 and M9MM1 strains those reached the highest OD_{600nm} either at 8% or 10% ethanol. However, *Lc. mesenteroides* strains hardly grew at 10% ethanol (Fig. 1c).

Regarding low pH stress, a significantly different behavior was observed for *L. plantarum* M5MA1. Indeed, only this strain was able to reach the highest OD_{600nm} at pH 3 and 4, while a lower growth was observed for the remnant *L. plantarum* strains. In contrast, *Lc. mesenteroides* M9MG2b and T1M3 showed no or very slight growth at pH 3 and pH 4 (Fig. 1d). Considering all the stress conditions analysed, *L. plantarum* M5MA1 showed the best tolerance to low pH, temperature, salt and ethanol conditions. Moreover, it showed the major μ_{max} at pH 3 and 4, as well as at 20 °C, 37 °C and 42 °C.

3.2. Selection of spontaneous riboflavin overproducing strain-derivatives

In order to obtain spontaneous riboflavin overproducers, the wild type strains were exposed to the selective pressure of roseoflavin,

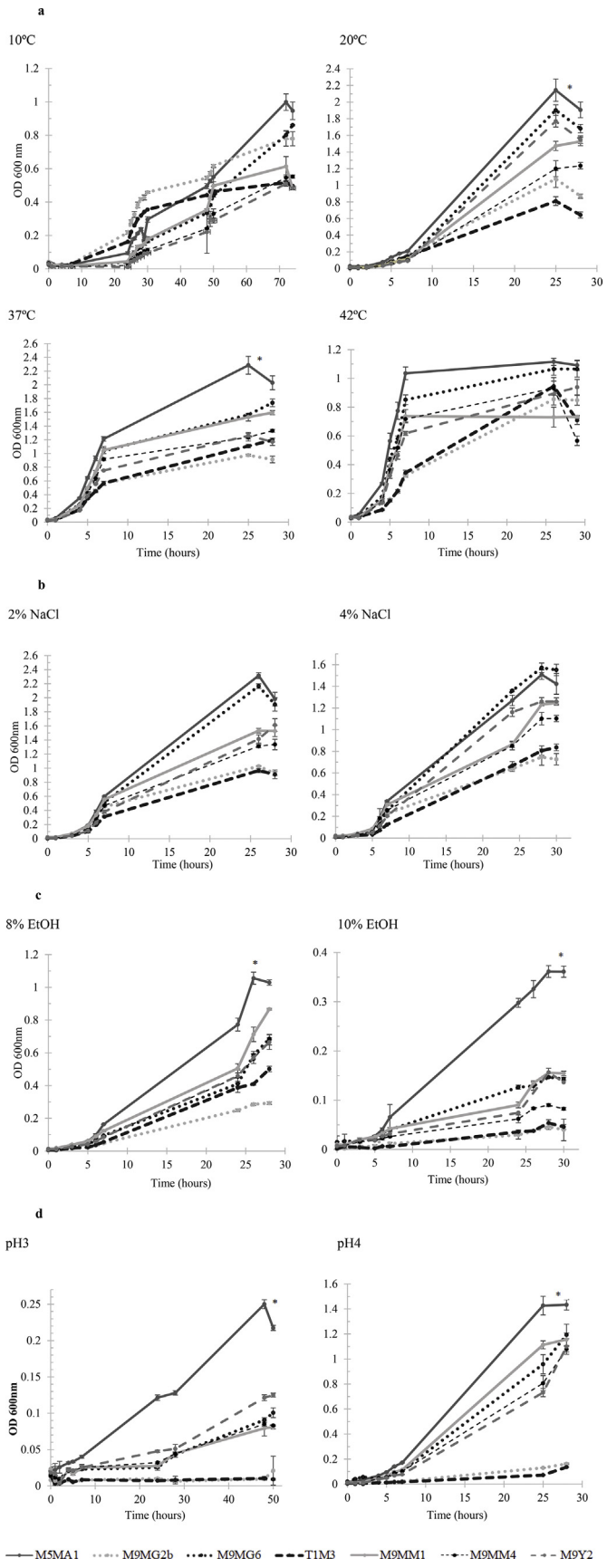

Fig. 1. Growth of L. *plantarum* and *Lc. mesenteroides* strains at different stress conditions: a) low and high temperatures, b) different concentrations of NaCl (w/v), c) presence of ethanol (EtOH) (v/v) and d) acidic pH. The increase in OD_{600nm} is shown as a function time (hours) and monitored until the achievement of the stationary phase. Data shown are means ± standard errors of the results from triplicates. Maximum OD_{600nm} value for each strain was obtained through an adjustment to Gompertz (Zwietering et al., 1990). An ANOVA analysis was carried out and values significantly higher according to the Tukey HSD test (α = 0.05) are followed by an asterisk.

according to the previously reported procedure by Burgess et al. (2006). The riboflavin overproduction after roseoflavin exposition was qualitatively detected by the conversion of the medium from white to yellow (Russo et al., 2014). The best overproducing derivatives, isolate for each strain, were selected based on the most intense yellow color. Furthermore, the corresponding level of vitamin B_2 in the whole-cell extracts was analytically quantified. In particular, the riboflavin content was in the range between 2.5 and 2.8 mg/L for all the *L. plantarum* overproducing strain derivatives. In contrast, a lower ability to synthesize vitamin B_2 was observed in *Lc. mesenteroides* derivatives (approximately 0.5 mg/L for both strains) (Fig. S1).

3.3. Exposure of selected strains to kefir-related stress conditions

Due to their similar ability to overproduce riboflavin, and according to higher tolerance to the stress assayed, only *L. plantarum* strains M5MA1, M9MG6 and M9MM1 isolated from *chicha*, and their roseoflavin-resistant natural derivatives were selected as candidates for the elaboration of milk kefir and cereal kefir-like products. Thus, to evaluate the suitability of the selected *L. plantarum* and their riboflavin overproducer derivatives, bacterial growth was monitored in MRS simulating kefir conditions, namely 2% ethanol (v/v), pH 4 and 25 °C. All strains were able to grow under these conditions and no significant differences were observed between wild type strains and the corresponding riboflavin overproducing natural derivative (Fig. S2). Therefore, according to the highest stress tolerance previously reported, *L. plantarum* riboflavin overproducing strain M5MA1-B2 was selected and inoculated in combination with commercial starters in cereal kefir-like fermentations.

3.4. Physico-chemical and microbiological characterization of milk kefir and cereal kefir-like

The production of lactic and acetic acids in the three cereals kefir-like and milk kefir products was determined as the difference in their concentrations at the end and at the beginning of the fermentation (Table 1). Moreover, in order to assess the rheological quality of the kefir-like products, viscosity was determined after the fermentation process (Table 1).

Regarding differences between the two kinds of starters, the highest content in lactic acid was recorded in those products fermented with milk starter. In contrast, the inoculations with commercial milk or water kefir starter unaffected the production of acetic acid, which variability appears to be mainly dependent from the matrix. Interestingly, all batches fermented with kefir milk starter showed a remarkably increase of the viscosity respect to the unfermented corresponding matrix. In particular, the employment of milk kefir starter in milk matrix results in a fourteen-fold higher viscosity than the corresponding control. Although to a lesser extent, similar results were found in cereal based products that, when fermented by milk kefir cultures, showed an increase in their viscosity of more than 1.5-folds.

Co-inoculation with *L. plantarum* M5MA1-B2 improved the concentration of lactic acid in cereal kefir-like, when compared with the corresponding matrix fermented by the commercial starters. In contrast, a reduction of lactic acid was detected when milk was co-fermented with M5MA1-B2 strain. No significant effects on the content in

Table 1
Quantification of lactic acid, acetic acid, and viscosity of milk kefir and cereal kefir-like beverages performed with water kefir starter (a), milk kefir starter (b), or co-inoculated (β) with riboflavin overproducer *L. plantarum* strain M5MA1-B2.

Matrix	Batch	Lactic acid (g/L)[a]	Acetic acid (g/L)[a]	Viscosity (cP)[b]
Milk	a	7.96 ± 0.70^{ad}	0.73 ± 0.07^{a**}	80.0 ± 0.00^{ad}
	b	8.77 ± 0.99^{a}	1.01 ± 0.15^{b}	560 ± 56.57^{b}
	aβ	5.47 ± 0.17^{b}	1.21 ± 0.09^{b}	440 ± 0.00^{b}
	bβ	4.29 ± 0.55^{b}	1.09 ± 0.15^{b}	520 ± 0.00^{b}
	C[c]	–	–	40.0 ± 0.00^{a}
Oat	a	−0.01 ± 0.01^{c}	−0.72 ± 0.05^{d}	520 ± 25.00
	b	0.50 ± 0.31^{c}	−0.73 ± 0.06^{d}	1300 ± 44.00
	aβ	1.10 ± 0.13^{cd}	−1.11 ± 0.00^{e}	1180 ± 39.00
	bβ	2.69 ± 0.75^{e}	−0.97 ± 0.00^{de}	1460 ± 26.00
	C	–	–	680 ± 12.00
Barley	a	0.19 ± 0.04^{f}	−1.12 ± 0.28^{g}	480 ± 13.0^{c}
	b	2.20 ± 0.38^{gh}	1.24 ± 0.16^{h}	860 ± 0.00^{cd}
	aβ	1.60 ± 0.38^{fgh}	−0.63 ± 0.00^{gi}	1080 ± 0.00^{d}
	bβ	3.04 ± 0.86^{h}	1.04 ± 0.00^{hi}	960 ± 0.00^{cd}
	C	–	–	600 ± 0.00^{cd}
Maize	a	0.48 ± 0.06^{i}	−0.83 ± 0.09^{j}	820 ± 19.0^{e}
	b	2.79 ± 0.26^{j}	−0.19 ± 0.01^{k}	1880 ± 0.00^{f}
	aβ	0.66 ± 0.42^{i}	0.32 ± 0.00^{l}	1400 ± 0.00^{ef}
	bβ	3.44 ± 0.26^{j}	0.21 ± 0.00^{kl}	1520 ± 0.00^{ef}
	C	–	–	1400 ± 0.00^{ef}

*An ANOVA analysis was carried out and values in the same column with different superscripts are significantly different according to the Tukey HSD test ($\alpha = 0.1$).

[a] Lactic and acetic acid productions were expressed as difference in concentrations at the end of the fermentation and the uninoculated matrix.

[b] Viscosity in centipoise (cP) was determined after 48 h fermentation.

[c] C, uninoculated matrix.

Table 2
Determination by plate counting of viable LAB and yeasts in milk kefir and cereal-based kefir-like, inoculated with water kefir starter (a), milk kefir starter (b), or co-inoculated (β) with riboflavin overproducer *L. plantarum* strain M5MA1-B2, after 48 h fermentation at 25 °C. C, uninoculated matrix.

		MRS	YPD + chloramphenicol
		Med Log ± Desv Log	Med Log ± Desv Log
Milk	a	7.90 ± 0.16	7.66 ± 0.10
	b	7.54 ± 0.07	3.16 ± 0.13
	aβ	8.38 ± 0.25	6.88 ± 0.07
	bβ	8.05 ± 0.08	2.97 ± 0.02
	C	3.75 ± 0.02	2.23 ± 0.08
Oat	a	7.28 ± 0.25	7.23 ± 0.21
	b	6.91 ± 0.04	3.15 ± 0.22
	aβ	8.48 ± 0.12	6.91 ± 0.04
	bβ	8.25 ± 0.15	3.70 ± 0.15
	C	3.98 ± 0.02	4.06 ± 0.23
Barley	a	8.37 ± 0.32	8.11 ± 0.22
	b	8.30 ± 0.26	3.16 ± 0.26
	aβ	8.91 ± 0.09	7.64 ± 0.08
	bβ	8.91 ± 0.06	1.26 ± 0.04
	C	4.02 ± 0.02	1.34 ± 0.06
Maize	a	8.31 ± 0.13	7.87 ± 0.09
	b	7.67 ± 0.13	3.27 ± 0.17
	aβ	8.62 ± 0.10	8.81 ± 0.11
	bβ	8.71 ± 0.05	2.71 ± 0.16
	C	4.13 ± 0.06	2.83 ± 0.08

acetic acid were observed by fermentation with the riboflavin overproducer strain. Interestingly, *L. plantarum* M5MA1-B2 strongly improved the texture of the matrices co-inoculated with water kefir starter being approximately 5-folds higher in milk, and approximately 2-folds higher in cereal-based kefir-like. These findings point out the potential application of M5MA1-B2 strain as bio-thickening agent. Regarding sensory characterization, oat and barley based products fermented with the milk kefir starter, showed a nice buttermilk flavor, while the maize based product had a distasteful aroma.

Microbial viable counts (Table 2) recorded similar values independently from the food matrix analysed. In particular, viable LAB counts in milk kefir or cereal based kefir-like co-inoculated with *L. plantarum* M5MA5-B2, were slightly higher than the corresponding kefir obtained by fermentation with commercial starters. Interestingly, yeasts counts in samples inoculated with milk kefir starter was about 3 log CFU/mL and similar to the corresponding uninoculated matrix, suggesting a different microbial composition, at least in yeast content, between milk and water kefir starter formulations.

3.5. Production of riboflavin in kefir products

Riboflavin content in the final fermented products and in the corresponding non-fermented matrices are reported in Fig. 2. Significant differences were detected in the vitamin B_2 content of the unfermented matrices since milk contains almost 1 mg/L, barley around 0.2 mg/L, while maize and oat presented undetectable values of riboflavin. Interestingly, in the case of all milk kefir samples, including even those inoculated with the overproducing *L. plantarum* M5MA1-B2, a reduction of about 250 mg/L of riboflavin after 48 h of fermentation was observed.

Cereal kefir-like fermented with water and milk starter cultures, presented levels of riboflavin similar to the non-inoculated controls. However, when cereal samples were co-inoculated with the overproducing *L. plantarum* M5MA1-B2, an increase in this vitamin was generally detected. In particular, the riboflavin content of maize kefir-like increased from undetectable levels up to about 0.5 mg/L in samples co-inoculated with M5MA1-B2. Similar vitamin B_2 content was found in samples fermented with water or milk starter in barley kefir-like, although in these samples the riboflavin increased only about 1.5 – fold when compared to the control. Oat kefir-like showed a higher riboflavin production when fermented with water starter (about 0.6 mg/L) co-inoculated with M5MA1-B2. Interestingly, the vitamin B_2 content increased until 1.5 mg/L (about 2.5-fold higher) when the same matrix was fermented by *L. plantarum* M5MA1-B2 and the commercial kefir milk starter formulation.

3.6. VOC profiling of kefir products

PTR-ToF-MS was used to determine the volatile compound profile of milk kefir and cereal kefir-like products experimental modes during the monitored 24 h' fermentation process. Fourteen VOC were produced in important concentrations and were identified as contributors to aroma and flavour. They were tentatively identified as: 5 ketones (2-butanone, 2-pentanone, acetone, diacetyl and metyl vinil ketone), 2 aldehydes (acetaldehyde and hexanal), 2 esters (acetoin and ethyl butyrate), 2 alcohols (isobutanol and ethanol), 2 carboxylic acids (acetic acid and hexyl acetate) and 1 sulphur compound (dimethyl sulphide). In this article, the totality of acquired data was studied by principal component analysis (PCA) (Fig. 3) in order to perform a preliminary investigation on the impact, in the different studied matrices, of VOCs diversity associate with i) the different starter cultures (i.e. starter cultures for milk kefir and for water kefir) and ii) the riboflavin overproducing bacteria inoculated. The first two principal components explain 96% of variability among samples in the dataset, with the first one capturing most of the variance (94%) (Fig. 3). A general evolution in the time of kefir/kefir-like associated volatilomes has been observed, with a particular trend depicted in the case of barley. On the other hand, concerning the influence of *L. plantarum* M5MA1-B2 in fermentation, our evidences indicate: i) a slight impact on barley- and milk-based beverages, and ii) a pronounced impact on maize- and oat-based beverages. In particular, the latter led to a reduction of VOC variability among experimental modes and in the time, because of bacterium inoculation, suggesting a possible impact on the sensory proprieties of the

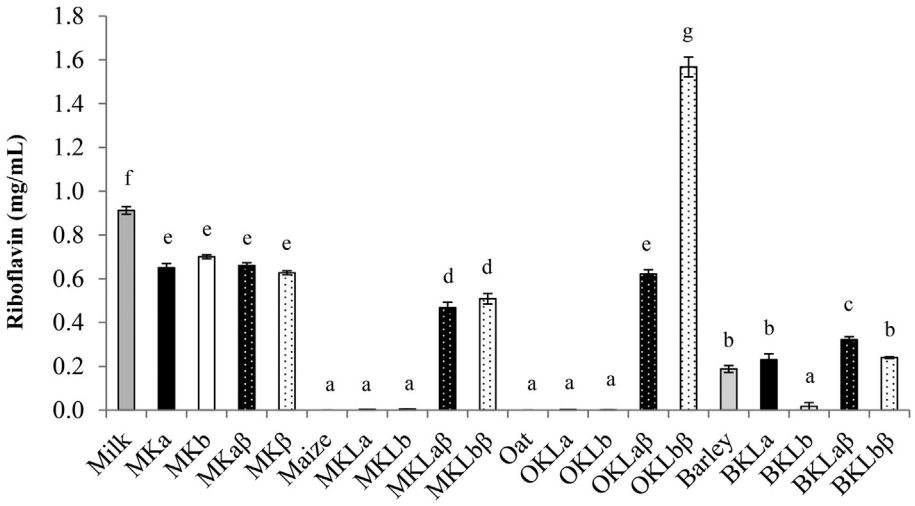

Fig. 2. Quantification of riboflavin in not-fermented matrices (grey bars); or milk kefir (MK), maize kefir-like (MKL), oat kefir-like (OKL) and barley kefir-like (BKL) fermented with "a", kefir water (black bars), or "b", kefir milk (white bars) commercial starters; and co-inoculated (β) with the riboflavin overproducing strain *L. plantarum* M5MA1-B2 (dotted bars). An ANOVA analysis was carried out and values with different letters are significantly different according to the Tukey HSD test (α = 0.05).

final product. Therefore, a further investigation will be necessary in order to evaluate if the impact is positive or negative on consumers acceptability.

4. Discussion

Functional food is a growing market with a wide range of target populations to direct its benefits. Fermented milk products like kefir are among the most popular and well recognized as healthy beverages. However, due to the quite extended milk intolerances, nowadays there is a consumer demand on non-dairy fermented functional food with similar healthy properties. Cereals are a good alternative as fermentable matrices but might need vitamin enrichment to nutritionally resemble milk. In the present study, we aim to develop new cereal-based

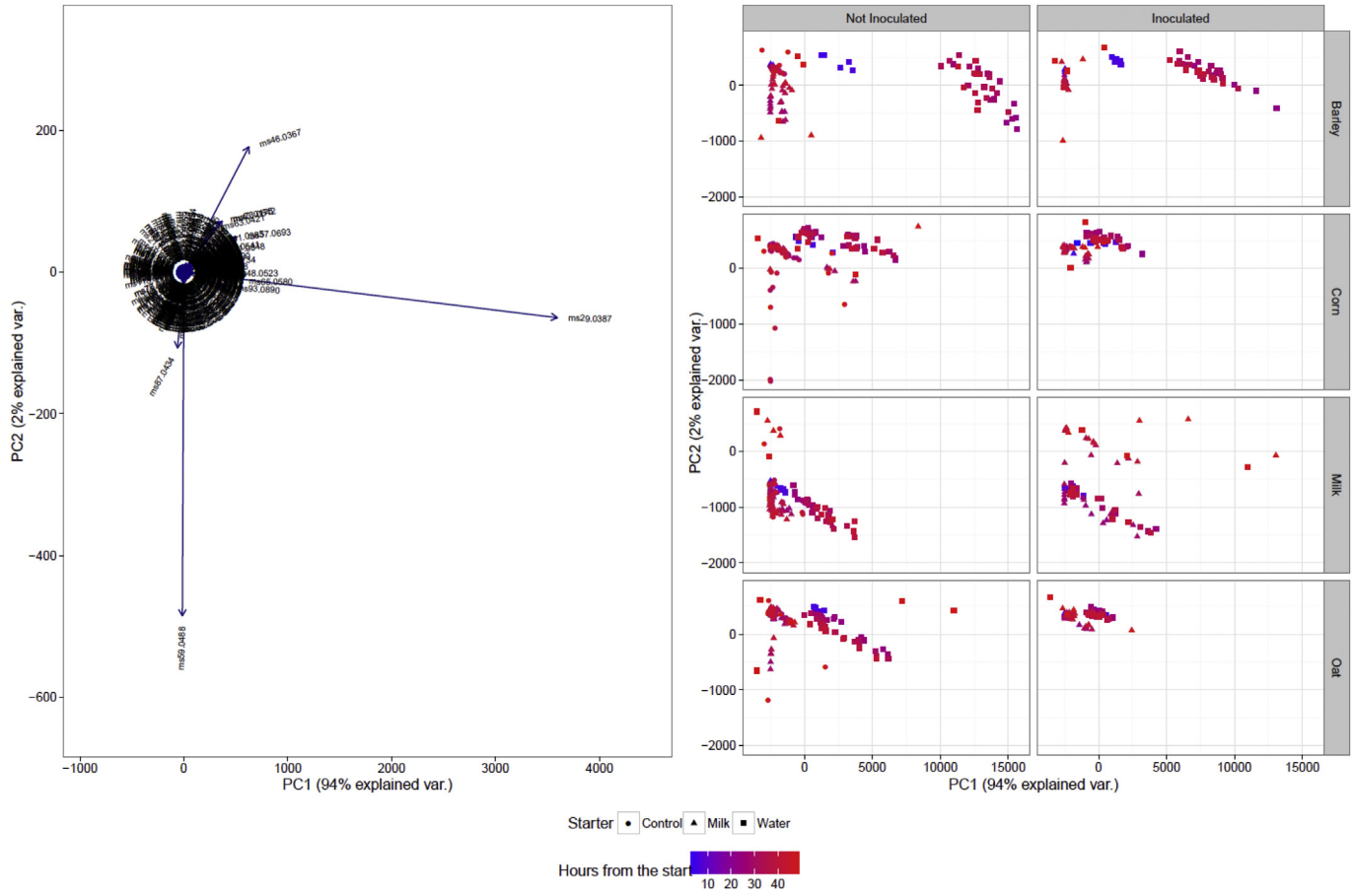

Fig. 3. Loading plot (left) and score plot (right) of principal component analysis of VOC emission evolution considering all the kefir products and unfermented samples during 11 days of experiment. Data are logarithmically transformed and centered. Different shapes indicate starter culture managements (control: uninoculated; milk: starter culture for milk kefir; water; starter culture for water kefir). Different colours indicate different time evolution. 'Not inoculated' and 'Inoculated' indicate samples not inoculated and inoculated with the riboflavin overproducing strain, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

beverages that mimic the manufacture of kefir and to improve nutritional quality of the new beverages using riboflavin overproducing LAB strains. These new products can well fit on typical Latin American diet where the low intakes of riboflavin have been observed along last decades (Singh et al., 2017; Berti et al., 2014; Bressani, 2000). Major cereal crops in South America are rice, oat, barley, sorghum, wheat, maize (Ferrero et al., 2018). Among these, we selected maize for relevance, while oats and barley where selected for their potential as cheap source of functional ingredients. For beverages fermentations, we used commercial starter cultures designed for milk kefir and water kefir, with and without the riboflavin overproducing strain *L. plantarum* M5MA1-B2. Even though that in the literature works that describe the elaboration of kefir-like vegetal-based beverages already existed (Randazzo et al., 2016; Corona et al., 2016; Cui et al., 2013; Puerari et al., 2012; Liu and Lin, 2000), information on kefir-like cereal-based products are still very scarce. We suggest to name the new products kefir-like beverage since, as for the other kefir-like vegetal-based beverages, the fermentations were carried out with commercial starter cultures containing a complex consortium of both yeasts and LAB responsible of kefir production. Microbial counts suggested that the fermentescible properties of the tested cereal matrices were comparable to other from vegetal origin previously investigated (Randazzo et al., 2016; Corona et al., 2016; Cui et al., 2013; Puerari et al., 2012; Liu and Lin, 2000). Our findings corroborated the potential of cereal ingredients in the formulation of dairy-like fermented beverages (Peyer et al., 2016; Coda et al., 2012), a field of increasing interest to develop tailored solutions for specific segments of population such as vegans and people with special dietary needs (Mäkinen et al., 2016). In general, milk starter cultures displayed a better aptitude to produce lactic acid and to increase viscosity in all cereal-based kefir-like beverages. The same microbial formulation was found to increase acetic acid content in barley-based product. From this point of view, milk starter cultures seemed to be more appropriate in order to manufacture cereal-based kefir-like beverages. Intriguingly, *L. plantarum* M5MA1-B2 strain demonstrated capacity to improve acetic acid content in maize-based beverage and lactic acid concentration in oat-based product (this last phenomenon has been slightly noticed in barley). In addition, *L. plantarum* M5MA1-B2 enhanced the viscosity when coupled with water kefir starter. For all these reasons, our findings suggest that this strain might be used to improve the performances of water kefir starter to obtain a kefir-like product through the fermentation of cereal matrices.

Some studies have developed microbial-based strategies to enrich kefir products with other group B vitamins (Van Wyk et al., 2011). Nevertheless, this is the first time that kefir and kefir-like products are bio-fortified in riboflavin. For the selection of the overproducer strain, we considered that starter cultures must survive under food process conditions (Laureys et al., 2017; Serrazanetti et al., 2009) and that kefir is an acidic-alcoholic fermented milk (Prado et al., 2015). Among the seven strains analysed, differences in response to generic food-related stresses were observed between species and strains. For instance, *Lc. mesenteroides* strains were less salt and acid tolerant than *L. plantarum* strains, in accordance to evidences reported in the literature (Hammes and Hertel, 2006). Variations in acid tolerance between *Lc. mesenteroides* and *L. plantarum* were addressed to a more pronounced capacity to reduce cellular internal pH in response to an external pH decrease (McDonald et al., 1990). In the present work, the application of selected strains belonging to the species *L. plantarum* testify the relevant interest of these versatile LAB which possess technological and functional properties, such as thickening agents (Zannini et al., 2016), and their use in the improvement of riboflavin content in cereal matrices (Russo et al., 2016; Juarez del Valle et al., 2014; Capozzi et al., 2011). Surprisingly, the aptitude of *L. plantarum* M5MA1-B2 to increase vitamin B_2 content in beverages was linked to the matrix and of the starter cultures used. For example, a reduction of riboflavin content was noted in milk when it was fermented with the commercial starters, as well as when the vitamin B_2-overproducer was added. About barley, a statistically different increase in riboflavin content was detected among milk/water kefir-like starters and the corresponding products obtained by co-fermentation with the overproducer strain. In maize and oat, we detected a clear vitamin overproduction using *L. plantarum* M5MA1-B2 in combination with either, milk kefir or water kefir starter cultures. However, in the case of oat, the overproducing phenotype of *L. plantarum* M5MA1-B2 was found markedly pronounced in association with milk kefir starter. With this regard, our study highlights the importance to select the suitable matrix and the appropriated starter cultures in order to optimize *in situ* fortification using riboflavin overproducing bacteria. By bio-fortification with *L. plantarum* M5MA1-B2 one serving oat-based kefir-like of 100 g will deliver 11.4% of Recommended Dietary Allowance (RDA) for vitamin B_2.

Finally, we report a preliminary PTR-MS-ToF analysis, which provides some differences in VOC content using different combinations of matrix/starter cultures/vitamin B_2 overproducing LAB. To our knowledge, it is the first report that applied a direct-injection mass spectrometry approach in order to characterize VOC associated with classical kefir and cereal-based kefir-like beverages. Our findings testify the potential of PTR-MS-ToF in bioprocess monitoring applied to the food sector (Capozzi et al., 2017; Benozzi et al., 2015; Soukoulis et al., 2010). A further approach will delve into the existing variability in terms of sensory properties among the analysed experimental modes.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.fm.2018.08.008.

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