Fermentation of whey-derived matrices by *Kluyveromyces marxianus*: alcoholic beverages development from whey and fruit juice mixes

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SUPLEMENTARY FILE

Materials and Methods

Microbiological analysis

Biomass was determined as described by Zafar and Owais (2006) with some modifications: 10 mL samples were centrifuged twice (10 min, 1000 g), before and after sediment washing with distilled water. Sediments were dried (80°C, 24 h) on a lab oven (Dalvo MCM2, Dalvo, Argentina), weighted and referred to sample volume, final biomass values being expressed in g/L units. For mould and yeast counts determination, tenfold dilutions in 0.1% (w/v) peptone solution (10^{-1} – 10^{-7}) were prepared and pour plated in duplicate onto moulds and yeast agar (HyL medium, Laboratorios Britania, Buenos Aires, Argentina). Plates were incubated under aerobic conditions (28 °C, 5 days). Colonies were counted on plates, final results expressed as CFU/mL.

Physicochemical analysis

For ethanol determination, a commercial enzymatic-spectrophotometric assay kit (Ethyl alcohol, Roche Cobas) was adapted: kinetic measurement of NAD^+ absorbance at λ=340 nm is proportional ethanol concentration in the medium assayed. A calibration curve was constructed with determinations in triplicate using ethanol solutions in a linear range (0.5 – 3.5 g/L), acceptable coefficient of determination (R^2 = 0.9913) and p-value (P<0,001) being obtained. For lactose determination, a commercial kit for enzymatic-colorimetric glucose quantification (Wiener Lab, Rosario, Argentina) was employed (λ=505 nm), after a first step of complete lactose hydrolysis into glucose and galactose using commercial β-galactosidase (Lactozym 3000 LHPG, Novozymes A/S, Bagsværd, Denmark). A calibration curve with pure lactose solutions in the range 0 – 8.15 g/L was made with determinations in triplicate, acceptable coefficient of determination (R^2 = 0.9937) and p-value (P<0,001) being obtained. For all spectrophotometric measurements a Genesys 10S UV-VIS (Thermo Scientific, Germany) equipment was used.

Growth and fermentative characteristics of *Kluyveromyces marxianus* LFIQK1 in different whey and whey–derived matrices

Preparation of starters and fermenting conditions

Pure *K. marxianus* LFIQK1 on malt extract agar (MEA) slant was inoculated in sterile YPL broth, containing 0.5% (w/v) yeast extract, 1% (w/v) meat peptone and 4% (w/v) lactose and incubated statically (30°C, 24 h). A broth volume was reinoculated (25% v/v) and incubated in fresh YPL (30°C, 24 h). Fermented medium was then subdivided in 40 mL portions and centrifuged (10 min, 1000 g). Cells were resuspended in 5 mL of 0.9% sterile sodium chloride solution, and each of these portions added as inoculum to corresponding media in borosilicate glass bottles. After homogenization, bottles sealed

with rubber stoppers equipped with an airlock (filled with water-alcohol 30:70), were cultured (24 h, 30°C) in static conditions. Initial concentration of *K. marxianus* in all fermented media was 2.5 x 10^7 CFU/mL. Samples were taken at 0, 8, 16 and 24 h, a portion immediately processed for biomass and pH determination, the remaining stored at -18°C for ethanol and lactose determination.

Development of alcoholic beverages obtained by fermentation from whey and fruit juices

Preparation of fermentation media

The alcoholic beverages were obtained by mixing fruit media (orange juice or strawberry pulp) fermented by *S. cerevisiae* SafAle S-04 and whey, fermented by *K. marxianus* LFIQK1. Thus, the three different fermentation media employed were:

i. Whey medium (W): prepared from whey powder as described previously. Whey solutions were stored at 2°C until fermentation the following day.

ii. Orange medium (OR): Oranges (*Citrus sinensis*) with mean SS level of 12.5 ± 0.3°Bx, bought at a local market. After washing and 100 ppm chloride solution decontamination, a total of 46 fruits (7.12 kg), were manually squeezed under hygienic conditions. After filtering with a household strainer, 2.9 kg of juice was obtained and subdivided in four 1000-mL plastic bottles, each containing 600 mL of juice. Initial pH and SS values were 3.6 ± 0.1 and 12.9 ± 0.2°Bx, respectively.

iii. Strawberry medium (ST): Strawberries (*Fragaria* x *ananassa*) produced in the zone were bought (4.3 kg) at a local market. After calyxes and peduncles removal, fruits were washed, crushed and thoroughly homogenised. The pulp obtained (3.4 kg) was stored at −18 °C until use. The day of fermentation,

strawberry pulp was unfrozen and subdivided in five 1000-mL plastic bottles, each containing 600 mL of pulp. Initial pH and SS values were 3.4 ± 0.1 and 8.8 ± 0.0°Bx, respectively.

Sensory evaluation of the alcoholic beverages obtained by fermentation from whey and fruit juices

A total of 101 consumers (aged between 18 and 65 years, 57% female participants) were recruited from the consumer database of the Sensory Analysis Department of Universidad Nacional del Litoral (Santa Fe, Argentina) based on their consumption of fermented alcoholic beverages (at least twice a month), availability and interest to participate, giving written informed consent for their participation. The sample comprised varying household compositions, income and education levels, but was not representative of the population of Santa Fe. Consumers were not given any additional information about beverages prior to tasting. Participants evaluated the fermented beverages samples using overall liking hedonic scale and Check-All-That-Apply questions. Chi-square tests indicated that differences in gender (female and male), age (between 18-40 and 41-65), and fermented beverages consumption frequency distributions (at last once a week and more than once a week) were not significant (XLSTAT 2014, Addinsoft).

Testing took place in a sensory laboratory in individual sensory booths, designed in accordance with ISO 8589:2007 and after Ethics Committee of Universidad Nacional del Litoral approval (Res. 379-2020|NP-19012020). Artificial daylight type illumination, constant ambient temperature (22°C) and air circulation were controlled. Still mineral water was available for rinsing before testing each beverage. Samples were given to consumers in 100 mL plastic cups, each containing 10 mL of the fermented beverage served at 10°C. Each beverage, W-OR and W-ST, was coded with 3 digit random

numbers. The order in which consumers tested the beverages was randomised and subdivided in equal parts, so that one half of participants tested samples in one sequential order, and one half in the other.

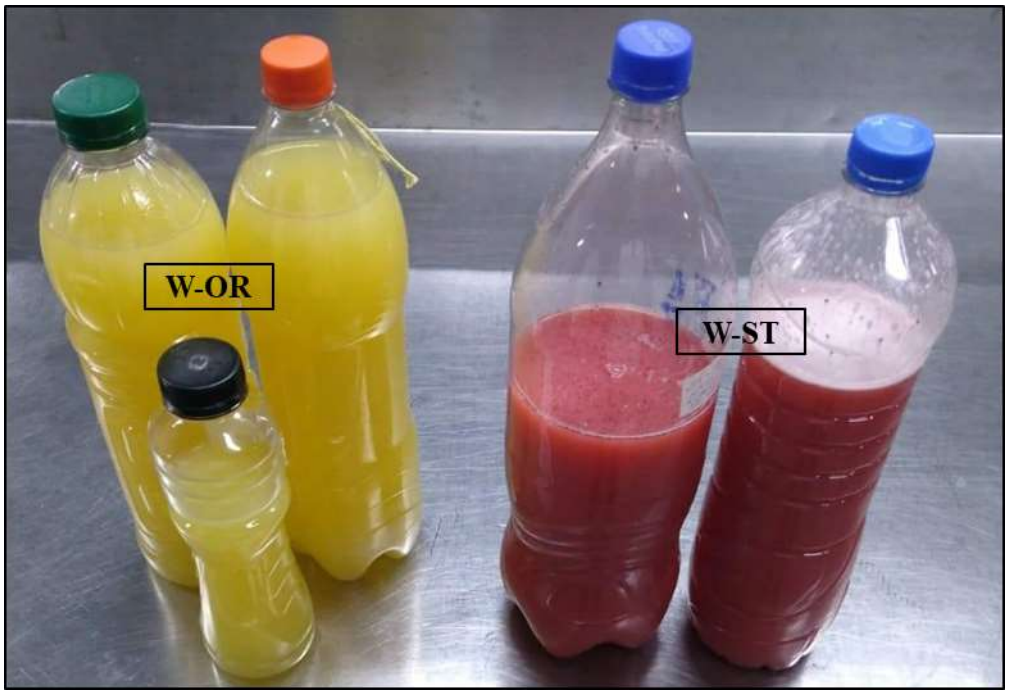
For each beverage, consumers were asked to test a first time and then rate overall liking using a horizontal hedonic score; where 9 = like extremely; 8 = like very much; 7 = like moderately; 6 = like slightly; 5 = neither like nor dislike; 4 = dislike slightly; 3 = dislike moderately; 2 = dislike very much; and 1 = dislike extremely. Verbal anchors of the scale were selected so that psychological distance between successive scale points is equal (Wichchukit and O'Mahony, 2015).

After rating overall liking of each sample, consumers were asked to re-taste samples and complete a CATA question with 24 terms related to sensory characteristics of fermented beverages. Consumers were asked to check all the terms they considered appropriate to describe each beverage. Terms were selected based on published data (Ares *et al.*, 2015; Farah *et al.*, 2017) and considering the attributes selected by trained assessors in preliminary studies. Based on recommendations by Ares *et al.* (2014), the order in which the sensory terms were listed was balanced within and across consumers, following William's Latin Square experimental design. Frequency of use of each term was determined by counting the number of consumers selecting that term to describe each sample.

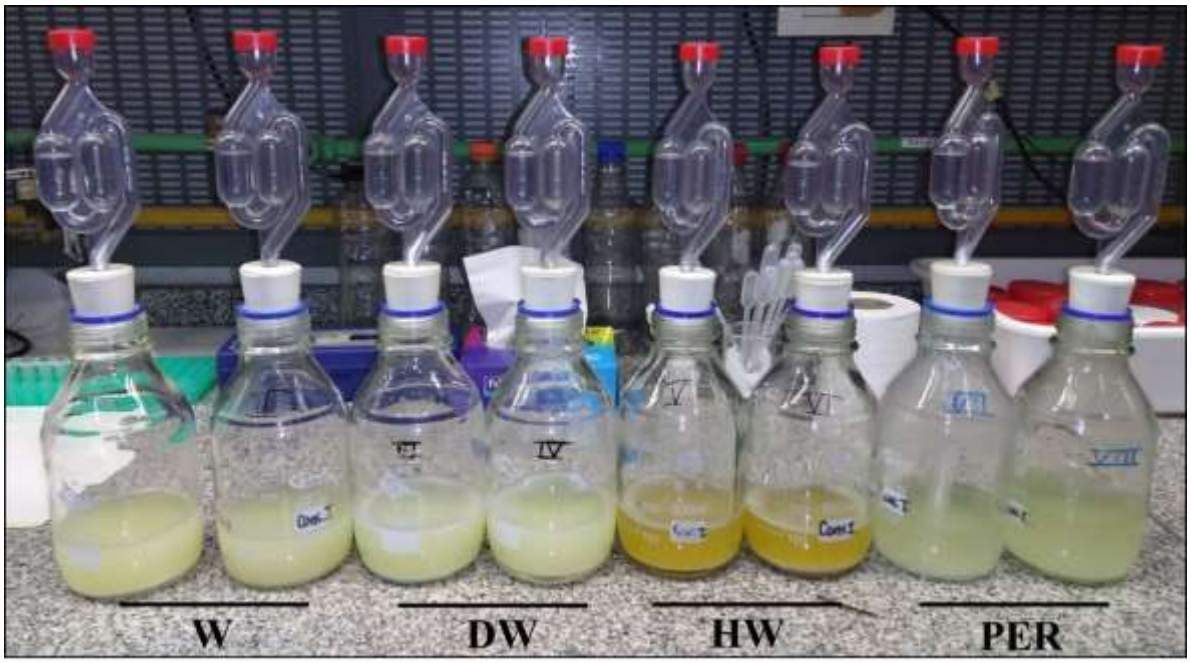
Supplemental Figures

Supplementary Figure S1. Devices employed for growth and fermentation of *K. marxianus* in reconstituted whey (W), reconstituted demineralised whey (DW), heat-treated reconstituted whey (HW) and milk permeate (PER) media.

Supplementary Figure S2. Visual appearance of beverages prepared by mixing fermented whey with fermented orange juice (W-OR) or fermented strawberry juice (W-ST).



Supplementary Figure S1



Supplementary Figure S2

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