WASTES OF AGROINDUSTRY AN ALTERNATIVE TO DEVELOP BIOPREPARATES WITH PROBIOTIC CAPACITY[1]

[RESIDUOS DE LA AGROINDUSTRIA UNA ALTERNATIVA PARA DESARROLLAR BIOPREPARADOS CON CAPACIDAD PROBIOTICA]

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SUMMARY

The objective of this study was to obtain and characterize three candidate microbial cultures for veterinary probiotics, developed in different substrates on agro-industry wastes. A selection of lactic acid bacteria (*Lactobacillus acidophilus, L. bulgaricus, Streptoccus thermophillus*) and yeasts of *Kluyveromyces fragilis* (L-4 UCLV) were used to develop probiotic preparations. The substrates chosen were: molasses (as a source of carbohydrates) and soy milk, whey, torula yeast and orange vinasse (as a source of protein) to obtain a high number of microorganisms and levels of organic acids. The substrates under study were composed of: T1, molasses and soy milk. T2, buttermilk more torula yeast and molasses. T3, orange vinasse and molasses. All the variables were incubated for 24 at 37 ° C. Dry matter, crude and true protein, ether extract, ash, viability and microbial concentration were determined in all treatments (T1, T2 and T3). At the time of incubation, the pH values of all the treatments were lower than 4.22 and at 24 hours it was reduced to 3.86. The values of the dry material and ether extract were higher ($P<0.05$) in T1; the crude and true protein was higher ($P<0.05$) in T2; whereas, ash was higher ($P<0.05$) in T3. But for all the variables the microbial concentration and the viability was higher than 93% and $7.7x10^7$ cfu/mL respectively. The results of the present study showed that byproducts such as: molasses, soy milk, whey, milk yeast and orange vinasse are suitable substrates to develop microorganisms with probiotic capacity and obtain an acceptable probiotic for veterinary use

Keywords: lactic bacteria, molasses, nutritious composition, vinasse, yeasts.

RESUMEN

El objetivo de este estudio fue obtener y caracterizar tres cultivos microbianos candidatos para probióticos veterinarios, desarrollados en diferentes sustratos sobre residuos agroindustriales. Una selección de bacterias láctico (*Lactobacillus acidophillus, L. bulgaricus, Streptoccus thermophillus*) y levaduras de *Kluyveromyces fragilis* (L-4 UCLV) se usaron para desarrollar preparaciones probiótica. Los sustratos elegidos fueron: melaza (como fuente de carbohidratos) y leche de soja, suero de leche, levadura torula y vinaza de naranja (como fuente de proteína) para obtener un alto número de microorganismos y niveles de ácidos orgánicos. Los sustratos en estudio se componen de: T1, melaza y leche de soja. T2, suero de leche más levadura de torula y melaza. T3, vinaza de naranja y melaza. Todas las variables se incubaron durante 24 horas a 37 °C. Se determinaron materia seca, proteína bruta y verdadera, extracto de éter, ceniza, viabilidad y concentración microbiana en todos los tratamientos (T1, T2 y T3). En el momento de la incubación, los valores de pH de todos los tratamientos fueron inferiores a 4.22 y a las 24 horas se

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redujo a 3.86. Los valores del material seco y el extracto de éter fueron mayores (P<0.05) en T1; la proteína bruta y verdadera fue más alta (P<0.05) en T2; mientras que la ceniza fue mayor (P<0.05) en T3. Pero para todas las variables, la concentración microbiana y la viabilidad fueron superiores al 93% y $7.7x10^7$ ufc/mL, respectivamente. Los resultados del presente estudio mostraron que los subproductos tales como: melaza, leche de soja, suero de leche, levadura de leche y vinaza de naranja son sustratos adecuados para desarrollar microorganismos con capacidad probiótica y obtener un probiótico aceptable para uso veterinario.

Palabras clave: bacterias lácticas, composición química, levaduras, melaza, vinaza

INTRODUCTION

The world population is increasing rapidly. And with this, the demand for food of animal origin such as meat, milk and eggs (FAO, 2016) implies that livestock systems must increase production to meet this demand in a sustainable and respectful way with the environment. An alternative to increase the productive performance in animals is the use of additives in the daily intake, for example; biocatalysts, enzymes, essential oils and bioactive compounds of plants and seeds (Sathyabama *et al.,* 2014). These have the ability to: a) improve the health of the digestive tract, b) break the polymers present in food into smaller molecules, increasing the degradation and digestibility of nutrients, c) anthelmintic effect in the gastrointestinal nematodes, d) reduction of ruminal methanogenesis (Pedroso *et al.,* 2014), and as a consequence of the above, greater productivity in the animal (Haffner *et al.,* 2016). However, the availability and cost of these compounds in developing countries can limit their use and minimize gains for small and medium producers.

However, the use of agro-industrial waste could be efficient and economical to develop biopreparations with probiotic capacity obtained from cultures derived from lactic acid bacteria and yeasts. In this sense, the species most commonly used as probiotics or prebiotics are *bifidobacterium, Lactobacillus, Streptococcus, Saccharomyces, Kluyveromyces* and others (Miranda *et al.,* 2017). Based on the above, preparations with probiotic capacity have been evaluated and used for different animal species with positive results, an example of this is the reduction of diarrheal disorders in piglets. In the critical stages of the life of the animal it has been seen that it improves the immune system, likewise, it has been used to treat pigs affected by *E. coli* and *Salmonella* spp., with positive results (Pajarillo *et al.,* 2014), also, has observed an increase in weight gain and average daily gain (Giraldo *et al.,* 2015), consequently, a higher feed conversion. While in the birds there has been an increase in the laying of eggs and the reduction of the content of triglycerides in the blood. On the other hand, Ranadheera *et al.* (2013) with the use of microbial cultures managed to control and prevent chickens affected with *C. jejuni, Listeria monocytogene*s, pathogenic *E. coli*, *Yersinia enterocolitica* and *C. perfrigens.* The objective of this study was to obtain and characterize: physics, chemistry and microbiology to three candidate microbial preparations for veterinary probiotics, developed in different substrates of agro-industrial wastes.

MATERIAL AND METHODS

Site of study

The study was conducted in the laboratory of bromatology and microbiology, Faculty of Sciences, Escuela Superior Politecnica de Chimborazo, Riobamba, (Ecuador) and laboratory of microbiology, Faculty of Agricultural Sciences, Universidad Central "Marta Abreu" de Las Villas, Santa Clara, (Cuba)

Selection, activation of strains and obtaining biomass mother

The strains used were: *Kluyveromyces fragilis* (L-4 UCLV) from the Bank of strains of the Universidad Central "Marta Abreu" de Las Villas (Villa Clara, Cuba) and three strains ATCC (American Type Cultures Collection, USA) Gender; *Lactobacillus acidophillus, L. bulgaricus* and *Streptococcus thermophillus*. The strains, in freeze-dried format, were individually activated in 120 ml of tryptone soy broth (BD Trypticase™, BD BBL™, 211768, USA) at 37 °C for bacteria and 30 °C for yeast, in an stove with orbital shaker (Inkubationshaube TH 15, Germany) at 60 rpm for 6 h. Subsequently, they were seeded with medium agar MRS (Man, Rogosa and Sharpe M6411-500G, HEMEDIA®, India) and Nutritive (Nutr, 213000-BD DIFCO™, USA) for *L. acidophillus, L. bulgaricus* and *S. thermophillus*, respectively. Sabouraud Agar (Sabrd 211584-BD BBL™, USA) used in yeast. The lactobacilli were grown under anaerobic conditions using the container (GasPak Plus™).

Once the strains were activated, we proceeded to obtain a microbial biomass using a standard medium for the growth of the strains. The culture was composed of 5 mg (analytical balance, Radwag AS 220/C/2, Switzerland) of each of the strains *L. acidophillus, L. bulgaricuc, S. thermophillus* and *K.*

fragilis (L-4 UCLV). Subsequently, the microorganisms were mixed in 250 mL of inoculum (skimmed milk) at 30±2 °C and incubated at 37 °C for 24 h. Finally, the initial count was performed on the plate to verify the viability of the strains.

Obtaining microbial preparations with probiotic capacity

Agro-industrial wastes were used as microbial growth substrate distributed in the following substrates (Treatments), in each treatment 12.5% ($7.1x10^7$ colony forming units cfu/mL bacteria and $7.2x10^8$ cfu/mL yeasts) of the biomass of previously obtained microorganisms was inoculated (see above). T1, 54.5% soybean milk and 33% cane molasses. T2, 48.5% fresh whey (cow) more 32% sugarcane molasses and 7% torula yeast. T3: 30% sugarcane molasses and 57.5% orange vinasse. Independently, all the variables were homogenized at 150 rpm with a magnetic stirrer (JOAN or OEM, MS001, CN, Switzerland) at 28 ° C for 10 minutes. Table 1 shows the bromatological characteristics of the agro-industrial wastes used as substrates.

Table 1. Bromatological characteristic of the byproducts of agro-industry to develop biopreparations

Agro-industrial waste	DM	CP	TP	EE	Ash	ºBrix
Soy milk	20	15.5	11.52	2.62	3.34	-
Buttermilk	18	4.55	2.25	2.82	2.85	-
Torula yeast	87.62	43.85	32.32	1.02	3.5	-
Sugar cane molasses	78.65	2.8	0.8	-	1.1	º84
Orange vinasse	20	16	10.85	-	1.85	º7.32

DM= dry matter, CP= crude protein, TP= true protein, EE = ether extract.

Response variables

Physical and chemical characterization

Color; it was compared with the HTML code described by (Miranda *et al.,* 2017), sensory evaluation; It was evaluated by the sensorial senses of the researcher following the methodology described by (Acevedo *et al.,* 2009). The determination of crude protein (CP) and true protein (TP) was carried out following the methodology described by Dadvar *et al.* (2015), dry matter, ash and ether extract, using the AOAC (2012) methodology.

pH

The same methodology as the previous one, a parallel sample was analyzed the behavior of the pH from the moment of the inoculation of the biomass in different substrates (Treatments), was measured at the beginning and during the first 24 hours in the interval of 60 minutes of incubation at 37 ° C, using a pH meter (HANNA®* H 110).

Microbiological analysis

Of each treatment, 10 mL were inoculated in 50 mL of physiological saline and incubated for 24 h at 37 ° C. Subsequently, the samples were centrifuged in a centrifuge (BD DYNAC ™ III) at 600 rpm for 5 minutes, all separately. Then, different concentrations were prepared up to the 0.5 scale of the MacFarland for viability, using the technique described by Rodríguez (2009) and Sourav & Arijit (2010). The number of CFU was quantified by visual counting of the colonies.

Statistical analysis

A completely randomized design was used, with three treatments and five repetitions. All variables were analyzed using STATGRAPHIC Plus version 15.1. The mean comparison of the treatments was carried out using the Duncan test (1955). In the case of microorganism counts, the data were transformed according to log-^{10} to guarantee the normality of variance.

RESULTS

In Table 2 it is observed that the treatments show different color tonality T1: (SADDLERBROWN), T2: (SADDLERBROWN), T3: (SIENNA). The aroma and taste did not show differences (P>0.05) between the treatments. As for the texture in all the treatments, it was creamy.

The pH at the beginning of the incubation showed no differences (P>0.05) between the treatments. However, from hour 1 to 18 and hour 20 was higher in T1 (P<0.05), oscillating between 4.20 and 3.94 respectively. Regarding hours 19 to 24, there were no differences between treatments (P>0.05, Table 3).

The MS was higher (P= 0.0125) in the T1 (25.1%) compared to the other treatments. Crude and true protein was higher (P>0.05) in T2 (42.5 and 32.2% respectively). The ethereal extract showed differences

(P= 0.0454) among the treatments, with the highest percentage being T1 (3.23%). With respect to the ashes, the highest percentage (P<.0001) was observed in T3 (2.88) (Table 4).

Table 2. Physical characteristics of the biopreparations developed in the different substrates coming from the byproducts of agro-industry

Indicators	Variants			EE	P
	T1	T2	T3		
Color (code HTML)	#8B4513	#6e2c00	#A0522D	-	-
Aroma	4	4	4	1.21	0.1112
Flavor	4	4	4	0.12	0.1254
Texture	2	2	2	1.23	0.5721

[a, b, c] distinct letters in the same row differ p<0,05 (Duncan. 1955). T1, soy milk and molasses. T2, buttermilk more molasses and torula yeast. T3, molasses and orange vinasse. Color: #8B4513= SADDLERBROWN; #6e2c00= SADDLERBROWN; #A0522D= SIENNA. Aroma: 0, without smell. 1, putrefied. 2, lactic. 3, bitter. 4, sweet acid. 5, Very nice. Flavor: 0, without flavor. 1, btter, 2, rancid, 3, dulcet. 4, sweet. 5, very sweet. Texture: 0 liquid, 1, fluid. 2, splashy, 3, creamy. 4, solid (Rodríguez, 2009).

Table 3. Behavior of the stability of the pH values for 24 hours

Time (hour)	Variants			EE	P
	T1	T2	T3		
Inicio	4.22	4.21	4.21	0.12	0.2412
1	4.22a	4.18b	4.17b	0,21	0.0002
2	4.22a	4.15b	4.14b	0,13	0.0241
3	4.18a	4.13b	4.14b	0.10	0.0011
4	4.18a	4.11b	4.12b	0,11	0.0127
5	4.16a	4.09b	4.09b	1.21	0.0012
6	4.15a	4.05b	4.07b	0,13	0.0025
7	4.12a	4.01b	4.05b	1.02	0.0024
8	4.09a	3.98b	4.03b	2.12	0.0214
9	4.07a	3.98b	4.01b	1.21	0.0121
10	4.07a	3.97b	3.98b	0.03	0.0245
11	4.05a	3.96b	3.97b	0.12	0.0124
12	4.03a	3.95b	3.94b	3.21	0.0012
13	4.01a	3.95b	3.94b	2.12	0.0125
14	3.98a	3.95b	3.91c	2.13	0.0001
15	3.98a	3.94b	3.91c	1.23	0.0127
16	3.96a	3.93b	3.91c	0.24	0.0012
17	3.96a	3.92b	3.89c	0.12	0.0025
18	3.96a	3.91b	3.88b	1.11	0.0024
19	3.93	3.91	3.88	1.21	0.1254
20	3.93a	3.89b	3.87b	2.14	0.0245
21	3.91	3.89	3.87	2.45	0.1375
22	3.89	3.88	3.86	0.12	0.5875
23	3.88	3.86	3.86	1.22	0.5783
24	3.87	3.86	3.85	2.31	0.0685

[a, b, c] distinct letters in the same row differ p<0,05 (Duncan. 1955). T1, soy milk and molasses. T2, buttermilk more molasses and torula yeast. T3, molasses and orange vinasse

Microbiological characteristics

The values of the microbial concentration were lower (P= 0.0012) in the T2 (7.7x107 CFU/mL) with respect to the other treatments. The percentage of viability was higher (P>0.05) in T2 and T3 (94%) compared to T1. As for lactic acid, it did not show differences (P>0.05) between treatments (Table 5).

DISCUSSION

Physical characteristics of the treatments obtained in the present study (Table 2) were given possibly due to the use of the raw material (substrates), the brown color, in its different tones is due to the molasses, the pleasant sweet aroma is due to the production of lactic acid, the creamy texture is related to the production of microorganisms in the substrate within the first 24 hours. In the studies reported by Díaz et al. (2013) and Miranda et al. (2017) when using sugarcane molasses, milk whey, soy milk and torula yeast reported similar results to those obtained in this study. These results are consistent with those reported by Flores et al. (2015). Results of the chemical parameters were possibly due to the content of the products of the agro-industry used (substrates) to develop the microbial preparations. The results of the present study are within the ranges admitted by FAO/WHO (2016) for biological products. Attached to this, Begum et al. (2015) reported that the use of byproducts from agro-industry with previous treatment could be an ideal alternative as a raw material to develop biopreparations of mixed cultures of lactic acid bacteria and yeasts.

Table 4. Nutritive composition (%) of the three biopreparations developed in different substrates

Indicators	Variants			EE	P
	T1	T2	T3		
Dry matter, m/v	25.12[a]	20.12[b]	19.53[b]	1.23	0.0125
Crude protein, m/v	15.23[b]	42.51[a]	17.23[b]	0.12	<.0001
True protein, m/v	11.80[b]	32.28[a]	11.80[b]	0.02	0.0122
Ether extract, v/v	3.23[a]	2.15[b]	2.53[b]	1.20	0.0454
Ash, m/m	2.54[b]	2.52[b]	2.88[a]	2.36	<.0001

[a, b, c] distinct letters in the same row differ p<0,05 (Duncan. 1955). T1, soy milk and molasses. T2, buttermilk more molasses and torula yeast. T3, molasses and orange vinasse

Table 5. Microbial characteristics of probiotic preparations

Indicators	Variants			EE	P
	T1	T2	T3		
Microbial concentration (cfu/mL)	$8.5x10^{7\,a}$	$7.7x10^{7\,b}$	$8.7x10^{8\,a}$	0.12	0.0012
Viability (%)	93[b]	94[a]	94[a]	1.23	0.0121
Lactic acid (mmol/mL)	0.72	0.72	0.72	0.02	0.5231

[a, b,] distinct letters in the same row differ p<0,05 (Duncan. 1955). T1, soy milk and molasses. T2, buttermilk more molasses and torula yeast. T3, molasses and orange vinasse.

The difference in the results can be attributed to the fact that treatments T1 and T3 (Table 4) would have a deficient supply of nutrients (Pajarillo *et al.,* 2014). However, the microbial preparations with probiotic capacity obtained in the present study present acceptable values for the biological products established by FAO (2016), despite having been developed in different substrates using by-products of the agro-industry for fermentation. These results are similar to those reported by Miranda *et al.* (2017).

Microbial characteristics of the different treatments could be due to the microorganisms used to develop the microbial preparations. The results present in the study are consistent with those reported by Ayala et al., (2014) and Castro and Martínez, (2015). The importance of evaluating the microbial concentration of probiotic preparations is to know the amount of microorganisms that are introduced into the animal, the dose and the microbial concentration. For being, an indicator of the useful life and its function as a probiotic (Rodríguez, 2009 and Pajarillo *et al.,* 2014). On the other hand, Pedroso et al. (2014) reported that probiotic microorganisms are producers of lactic acid above 55 mmol/mL, thus controlling the development of pathogenic microorganisms such as *Staphylococcus aureus* and *Clostridium*, mainly due to their inhibitory action and their ability to survive in anaerobic conditions. Sánchez et al. (2015) reported that the use of lactic acid bacteria has a homofermentative behavior, have the capacity to produce lactic acid with values higher than 0.64 mmol/mL, these results would be attributed to the microbiota of higher growth. In relation to the latter, Patarata *et al.* (1994) observed a production of lactic acid greater than 50 mmol/mL, with this, they managed to reduce the pH values below 3.98, which is an essential behavior for fermented products.

Stability of pH within the first 24 hours, could be by the rapid production of short chain fatty acids, especially lactic acid, produced during microbial fermentation. Similar results to those of this study were reported in different studies when developing microbial preparations from mixed cultures of lactic acid bacteria and yeasts (Díaz *et al.*, 2013; Ayala *et al.*, 2014; Castro & Martínez, 2015 and Flores *et al.*, 2015).

The reduction of the pH values in the first 24 hours, plays a very important role during the conservation of the biological means, obtained from mixed or pure cultures of the strains of the microorganisms used for their development, the rapid decrease of pH, inhibit the growth of other pathogenic microorganisms or contaminants that could be present in substrates (Rodríguez, 2009). Ortiz *et al.*, (2008) reported pH values lower than 4.25 in the milk fermentation from the Lactobacillus, likewise it was possible to conserve said products after 30 days without affecting the nutritional values. In the present study it was possible to demonstrate that the production of lactic acid in the biopreparations influences the decrease of the pH values and this in turn favors the rapid decrease thereof, obtaining a biologically healthy product for veterinary use (Heather *et al.*, 2013).

CONCLUSSION

Under the conditions of this study it can be concluded that the treatments evaluated meet the probiotic properties, so they could be used as microbial additives for veterinary use, to control their beneficial intestinal microbiota, stimulate their immune system, inhibit the growth of opportunistic pathogens and increase bioproductive indexes.

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