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Effect of the Yeast and Bacteria Biomass on the Microbiota in the Rumen

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Abstract: This study aims at obtaining a probiotic product based on viable biomass from 6 yeast strains and 2 strains of lactic bacteria used for nutrition of animals. The strains are subjected to some resistance tests, at temperature, pH, pepsin, pancreatin and biliary salts so as to make obvious their viability. Tests were done by comparison to the witness strain and respectively a protective solution based on mucin and casein. Based on the resulted viabilities 2 products are formulated. Their effect is tested by inoculating fresh rumen content and supervising the microbic balance for a period of 12 days. After the final tests, it resulted that the product Fp1 (20% *Saccharomyces cerevisiae* 1-29, 10% *Kluyveromyces marxianus* R-CS, 20% *Issatchenkia orientalis* R-BC, 30% *Lactobacillus paracasei* CMGB16, 20% *Enterococcus faecium* GM8) was chosen because anaerobic strains were preponderant as a consequence of the tests performed with rumen.

Key words: Probiotic, biomass, yeast, lactic bacteria, viability, rumen

INTRODUCTION

Microorganisms in the bovine rumen represent an extremely diversified and important component for the nutrition of ruminants. Their capacity to act on ingested food and transform it in assimilation compounds is their main function (Fraser *et al*., 2007a). Another role extremely aimed at by the researches is represented by the protection effect they may have against some pathogens. Thus, together with food, the type and number of micro-organisms which reach the rumen is very high. Due to the conditions in here and to the role of these strains, a favourable balance for the farm animal is kept (Cerrato-Sánchez *et al*., 2007).

The majority of bacteria strains in the rumen are Gram-negative. The Gram-positive ones reach the rumen at the same time with food (Jalc *et al*., 2002). An important input is produced when food is supplemented by such products based on viable probiotic biomass. This type of bacteria is strictly anaerobe and since a great part tolerate oxygen to a small extent, it creates a series of advantages from a trade and practical point of view (Cerrato-Sánchez *et al*., 2007). A very important aspect is constituted by resistance to pH, temperature and different acids or enzymes. Marketed products use Gram positive strains which resist at a pH between 5 and 7 and have maximum productivity at temperatures of 37-39°C. When choosing such strains, their resistance to different acids and to the enzymatic action in the digestive tube of the animal has to be considered. The strains used are carefully selected so as to use those strains with a high resistance, because, generally this is not the strongest characteristic of the lactic bacteria (Colombatto *et al*., 2003).

Products which might improve zootechnic performances and health of the animals are mainly based on yeasts and acidolactic bacteria. Strains are carefully selected so as to offer maximum protection and a quick effect by improving the health of the digestive system. (Wadhwa *et al*., 2001a,b) Thus, one uses strains which can adhere and are resistant to a low pH, digestive enzymes or various other factors, such as temperature (Vallimont *et al*., 2004). The resistance and persistency in the animal's digestive system represent some of the basic criteria because probiotic strains have to prevail over pathogen strains which can survive for a long time in unfavourable conditions. Thus, an important modality for

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prevailing is their number and their increased capacity of multiplying in the conditions of the animal's digestive tube. This is why one doesn't use only one strain or only one type of micro-organisms but a combination of several micro-organisms, different genus, in general. For example, the combination between yeast and bacteria is an adequate one taking into account the improvement of the animal's health and production increase. In this sense also, the use of different genus of each type of micro-organisms is considered (Greenfield *et al*., 2001). Due to the restrictions imposed by the latest regulations of the European Union in this domain, the use of such natural products is stimulated in the detriment of antibiotics or other synthesis substances.

The purpose of this study consists in testing some yeast and bacteria selected strains so as to formulate a probiotic product used in animal nutrition. Tests consisted in establishing viability under the influence of some physical and chemical factors. Their action is exerted either in the handling and conditioning period of the product of after it is ingested by the animal. Testing the effectiveness was done by inoculating the rumen content of a calf with the probiotic product, based on lyophylizing biomass (Silveira *et al*., 2007).

MATERIALS AND METHODS

Experimental studies were done in the laboratory of Fermenting Bio-technologies under the Biotechnological Centre in Bucharest, from January 2007-July 2007.

Biological material: Microbial strains are kept in the freezer at -82°C, in protecting environment with glycerol 20%. We use 6 yeast strains and 2 lactic bacteria strains: T_1: *Saccharomyces cerevisiae* 2-15; T_2: *Saccharomyces cerevisiae* 1-29; T_3: *Saccharomyces cerevisiae* R-BF; T_4: *Kluyveromyces marxianus* R-CS; T_5: *Issatchenkia orientalis* R-BC; T_6: *Trichosporon beigelii* R-LF; T_7: *Lactobacillus paracasei* CMGB16; T_8: *Enterococcus faecium* GM8.

Cultivation mediums and fermentations conditions: Revitalization of the yeast cells is done by using YM modified medium: yeast extract 0.3%, glucose 2%, peptone 0.5%, malt extract 0.3%, pH 6. Sowing is done with cryotube bow and the development takes places at 30°C, 48 h.

For *Lactobacillus paracasei* CMGB16, revitalization is done through cultivation on MRS medium, la 37°C, for 24 h (Novik *et al*., 2007).

For *Enterococcus faecium* GM8, the revitalization of the strain is done through the cultivation on a medium specific to the strain of *Enterococcus*, MEI noted, which contains (g L^{-1}): yeast extract 10 g, peptone 8 g, glucose 10 g. The sowing medium is introduced in the thermostat at 31°C, 24 h.

pH effect and of the temperature on the viability of the probiotic strain: The role of these tests consists in the fact that when fodder is produced, it may undergo some thermal treatments (e.g., granulation) or keeping them for a longer period of time may also imply a pH variation, mainly its decrease. Thus, viability tests at extreme values were done acid pH 1, 2, 3 and basic pH 8, 10 and 12. For temperature, viability was tested at values of 50, 70 and 90°C.

So, as to perform the tests, 2 mL of fresh culture from an Epperdorf sterile tube is put. For each strain, viability was tested at every pH interval by using NaOH 20% or concentrated HCl. After 30, the value of the pH was brought to 7 and viability was determined. So, as to test viability in the 3 intervals of temperature, the culture was introduced in a thermostat bath for 30. After this interval, the tube was put on ice bath in a special ice box and after the liquid cooled viability was determined.

Enzyme effect on probiotic strains: So, as to accomplish this experiment, the following enzymes are used: pepsin (Sigma-Aldrich), pancreatin (Fluka Biochemika) and biliary salts. For testing the protective effect of some substances on strains, the same set of tests was done but NaCl 0.5% was supplemented with casein and mucin with a concentration of 1 g L^{-1}, the procedure is the same for the rest (Perea Vélez *et al*., 2007).

After performing the tests with the two enzymes viability/mortality was determined according to Blaenka *et al*. (2000).

The effect of the probiotic biomass on the microbiota in the rumen: So, as to test the formula of probiotic product, the inoculation of the rumen recently extracted from the stomach of the calf with the mixture of strains was used. So, the following mixture was used: rumen content 500 g, 200 mL saliva, 100 mL sterile distilled water (Boguhn *et al*., 2006). The experiment was done for a period of maximum 12 days, at 37-40°C, with samples taken at 6 and 12 days. From these samples, pH, lactic acid and viability were determined. For the majority of the yeast, the following medium formula was used, YEPD (g L^{-1}): yeast extract 10 g; peptone 20 g; agar 20 g. So, as to determine the number of lactic bacteria, Rogosa medium was uses; for the determination of Enterobacteriaceae, the Istrate-Meittert medium was used (Abel *et al*., 2006; Kamra, 2005).

All the tests done were based on the consideration of a viability of 10^5 and minimum 10^6, because according to the data in the literature in the domain with respect to probiotic, these are the minimum viabilities necessary for taking into consideration a microbial strain as a possible probiotic.

RESULTS AND DISCUSSION

Upon performing experimental studies, two strains were used, in parallel, as close as possible from a phylogenetic point of view so as to better observe their evolution. Another aspect of this choice is represented by the use of one of the strains for the formula of the final product.

From Table 1 a very good viability of the 2 lactic strains at the temperature of 50°C can be observed. However, at higher temperatures, the viability of the 2 strains is 0. What needs to be observed is the resistance of the strain *Lactobacillus paracasei* CMGB16 at pH between values 2 and 12, the strain doesn't resist at pH_1. The strain *Enterococcus faecium* GM8 is not viable at a pH higher than 8. A very important fact is that both strains keep the viability of 10^6 irrespectively of the value of the pH from where the measurement was done.

In Table 2, one can observe that the strain *Issatchenkia orientalis* R-BC is viable at 50°C. For the other two intervals of temperature, viability doesn't exist in the 2 yeast strains. Although it is an important strain from a probiotic and point of view and more others, *Kluyveromyces marxianus* R-CS has a maximum viability only in the pH interval 2-3. After this interval viability decreases from 10^6 to 10^5, after which the strain is no longer viable. Moreover, *Issatchenkia orientalis* R-BC has a maximum viability in the pH interval 1-8. At a pH higher than 8, viability is 0.

Trichosporon beigelii R-LF and *Saccharomyces cerevisiae* R-BF are not viable in the 3 tested intervals of temperature (Table 3). What needs to be observed is that the strain *Saccharomyces cerevisiae* R-BF has a maximum viability in the pH interval 1-12, with a smaller number of colonies in the case of a basic pH than in the case of an acid one. *Trichosporon beigelii* R-LF is not viable at a pH higher than 8, in other cases viability is high at any tested interval. The strain tolerates very well the low values of the pH, the number of colonies exceeding the one considered to be necessary so as to validate this dilution.

In Table 4, only the strain *Saccharomyces cerevisiae* 1-29 is viable at the temperature of 50°C. The fact that the strain *Saccharomyces cerevisiae* 2-15 is not viable in the conditions of a basic pH, but is viable in the case of the acid one is made obvious. The number of colonies at maximum dilution decreases proportionally to the decrease of the pH. The strain *Saccharomyces cerevisiae* 1-29 has maximum viability in the pH interval 1-8, after which at pH 10, viability decreases up to 10^5 and becomes 0 at pH 12.

In Table 1-4 one can observe that none of the strains has a maximum viability at temperatures higher than 50°C. However, the strains *Kluyveromyces marxianus* R-CS, *Trichosporon beigelii* R-LF, *Saccharomyces cerevisiae* R-BF and *Saccharomyces cerevisiae* 2-15 are not viable at 50°C.

From the point of view of the pH, only one yeast strain is viable at all the intervals of the pH. Except for *Kluyveromyces marxianus* R-CS and *Saccharomyces cerevisiae* 1-29, for which the maximum value of the pH determines a decrease of the viability at 10^5, all the other strains have a maximum viability. Thus, we can generally consider that strains are resistant at extreme values of pH and the number of colonies at the maximum value of the viability is mostly high, more than 5. The majority of the strains with a maximum viability have more than 10 colonies at the pH tested values.

In case they are exposed to the 2 enzymatic solutions, it can be observed from Table 5 that, once the concentration of the biliary salts increases, viability decreases, but not in a uniform way. It needs to be

Table 1: Viability of the *Enterococcus faecium* GM8 strain and *Lactobacillus paracasei* CMGB16 at different intervals of pH and temperature

	pH						
Species	1	2	3		8	10	12
Enterococcus faecium GM8	$>10\times10^6$	$>10\times10^6$	$>10\times10^6$	$>10\times10^6$	-	-	
	$>10\times10^6$	-	-				
	50°C		70°C			90°C	
	$>10\times10^6$						
Lactobacillus paracasei CMGB16	-	$>10\times10^6$ >	10×10^6		$>10\times10^6$	$>10\times10^6$>	10×10^6
	50°C		70°C			90°C	
	$>10\times10^6$		-			-	

Table 2: Viability of the strain *Kluyveromyces marxianus* R-CS and *Issatchenkia orientalis* R-BC at different intervals of pH and temperature

	pH					
Species	1	2	3	8	10	12
Kluyveromyces marxianus R-CS	$>10\times10^5$	4×10^6	2×10^6	5×10^5	-	-
	-	-	-			
	50°C		70°C		90°C	
	-		-		-	
Issatchenkia orientalis R-BC	3×10^6	5×10^6	$>10\times10^6$	2×10^6	-	-
	50°C		70°C		90°C	
	$>10\times10^6$		-		-	

Table 3: Viability of the strain *Trichosporon beigelii* R-LF and *Saccharomyces cerevisiae* R-BF at different intervals of pH and temperature

	pH					
Species	1	2	3	8	10	12
Trichosporon beigelii R-LF	$>10\times10^6$	$>10\times10^6$	$>10\times10^6$	2×10^6	-	-
	50°C		70°C		90°C	
	-		-		-	
Saccharomyces cerevisiae R-BF	7×10^6	$>10\times10^6$	$>10\times10^6$	$>10\times10^6$	4×10^6	1×10^6
	50°C		70°C		90°C	
	-		-		-	

Table 4: Viability of the strain *Saccharomyces cerevisiae* 2-15 and *Saccharomyces cerevisiae* 1-29 at different intervals of pH and temperature

	pH					
Species	1	2	3	8	10	12
Saccharomyces cerevisiae 2-15	1×10^6	4×10^6	5×10^6	-	-	-
	50°C		70°C		90°C	
	-		-		-	
Saccharomyces cerevisiae 1-29	1×10^6	2×10^6	2×10^6	6×10^6	1×10^5	-
	50°C		70°C		90°C	
	4×10^6		-		-	

Table 5: The number of viable cells in case of exposure to solutions 1, 2 and 3

	The number of viable cells for exposure to solutions 1 and 2, with different concentrations of biliary salts (mg mL^{-1})				The number of viable cells for exposure	
Strain	1.5	2	3	5	to protective solution	Viability at t_0
T_1	1,000,000	1,000,000	600,000	500,000	3,000,000	5,000,000
T_2	3,000,000	3,000,000	1,000,000	1,000,000	8,000,000	12,000,000
T_3	5,000,000	1,000,000	1,000,000	1,000,000	5,000,000	6,000,000
T_4	1,000,000	100,000	100,000	-	8,000,000	10,000,000
T_5	6,000,000	4,000,000	3,000,000	1,000,000	9,000,000	15,000,000
T_6	3,000,000	2,000,000	2,000,000	-	9,000,000	11,000,000
T_7	-	-	-	-	3,000,000	20,000,000
T_8	-	-	-	-	-	14,000,000

observed that the two strains of lactic bacteria are not viable when such a treatment is applied. However, the use of protective solutions (mucin, casein) determines the preservation of a significant part of the viability of the probiotic strains. This fact is not important for the strain of *Enterococcus faecium* which is not viable in the presence of such substances. The essential conclusion is that the yeast strains are much more resistant than the

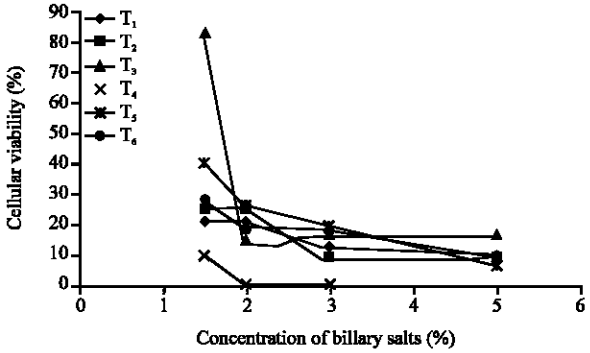

Fig. 1: The variation of the viability (%) after exposure to the solutions 1 and 2 function of the concentration of the biliary salts

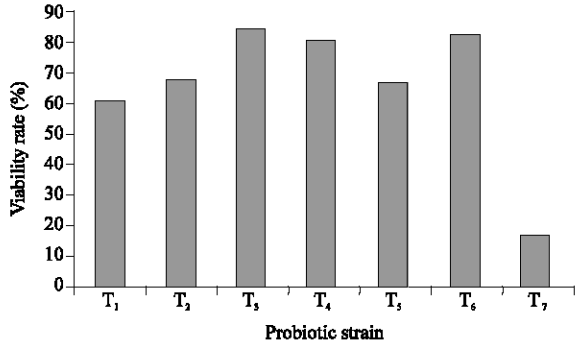

Fig. 2: The viability rate for each strain in case of exposure to the protective solution

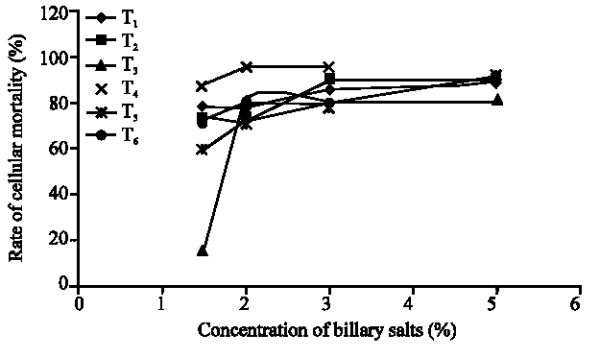

Fig. 3: Mortality variation (%) after exposure to solutions 1 and 2 function of the concentration of the biliary salts

lactic ones to the enzymatic action, but the use of support mediums or of some protections given to the probiotic cells confer additional resistance and increase viability.

Thus, from the diagrams below one can observe that viability decreases when the concentration of biliary salts

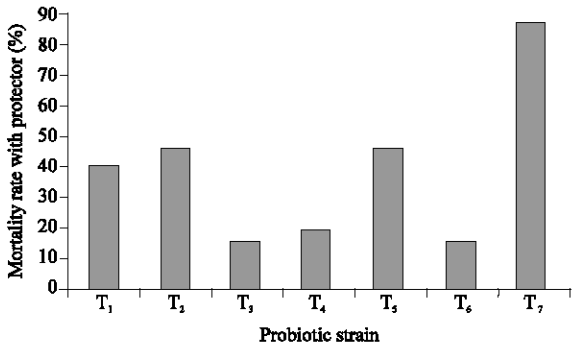

Fig. 4: Mortality rate for each strain in case of exposure to the protective solution

increases (Fig. 1). Some strains (T_2 or T_4) have a constant viability at a certain concentration. From all the analysed strains, the worst results were observed in the case of the strain T_7. From Fig. 2 one can observe that yeasts are much more resistant, in case protective solutions are used. Good results are also obtained in the case of the strain T_6, the presence of the protectors stimulating viability.

In the case of mortality, the increase of the concentration of the biliary salts determines an increase of the mortality rate also (Fig. 3). This tendency can be mainly observed for concentrations of 1.5 and 2 mg mL^{-1}. The mortality rate (Fig. 4) is very high for the strain T_7, however, for the strain T_6 it is relatively low. Strains T_1, T_2 and T_5 have the highest mortality values in the presence of the protector.

In the formula Fp_1 only the strains for which experimental results were the best are kept and those intestinal ecosystem of the animal. In the structure of the formula Fp_2 all the strains used are distributed in variable percent. The relation between them expresses the experimental results obtained in the performed tests, related to the data which exist in the literature in the domain.

Taking into account the tests performed, 2 formula of probiotic product were proposed

	Formula No. 1-Fp_1 (%)	Formula No. 2-Fp_2 (%)
T_1	-	10%
T_2	20%	15%
T_3	-	10%
T_4	10%	15%
T_5	20%	15%
T_7	-	5%
T_7	30%	20%
T_8	20%	

Thus, so as to verify viability, the number of micro-organisms developed on the designated mediums was determined: YEPD, Rogosa and Istrate-Meittert, from the rumen sowed with probiotic strains. What can be observed is that the number of yeast (41%) is identical to the one of anaerobic or strictly anaerobic micro-organisms (42%), in the case of the Rogosa medium. We conclude strains are of major importance (lactic bacteria) in that the animal from which the sample was taken had an equilibrated metabolism. Microbial balance was also equilibrated with a slight advantage in favour of the strains which tolerate air. The number of entero-bacteria is low (17%), significantly under the values of the other two categories. This small disequilibrium can be explained by the fact that the animal was fed close to the moment when the sample was taken.

In first case, one can observe that 6 days after the sowing with Fp_1, the majority of the strains is anaerobic and especially those of lactic bacteria. The number of entero-bacteria is very low, only 2%. After more 6 days from the incubation, the balance starts to equilibrate, the strains of the entero-bacteria are found in a percent of 25%. The most important aspect is that anaerobic strains are still of a majority, being those strains which ensure the decrease of the pH and inhibition of the pathogen micro-organisms.

In second case, one can observe that Fp_2 is not a viable formula because it leads to an important disequilibrium between the aerobic and the anaerobic species in the animal's rumen. Thus, after 6 days aerobic strains are preponderant (74%). The anaerobic ones are of maximum 1%. After 12 days the disequilibrium is manifested through the inexistence of the anaerobic strains, no lactic bacteria strain can be isolated. However, the strains which appear are intensely coloured and give off a heavy smell, specific to the strains of aerobic bacilli.

Selective pressures exerted by antibiotics on the digestive micro-flora lead to a disequilibrium of the intestinal microbial ecosystem. This microbial ecological equilibrium established between hundred of different bacterial populations is maintained due to a subtle game of interactions between different biotic and abiotic constituents of the ecosystem. The association of some non pathogen micro-organisms with the antibiotic therapy, known for a long period of time leads to the recovery of the microbic ecological equilibrium.

The quality of the product obtained from yeast and bacteria probiotic strains is determined first of all by the content of viable micro-organisms and by their genus. Another aspect of novelty and originality of the study is represented by the formula of the product, the mixture of micro-organisms and their association. The most important parameter is represented by the microbiological determination of the number of micro-organisms (successive dilutions and spreading on a specific agar medium). The results of the determination of the viability titre of the probiotic obtained from yeast and bacteria strains are presented in Table 6.

Table 6: Determination of the viability titre for the selected strains

Component strain	Viability titre (viable cells g^{-1})
Saccharomyces cerevisiae 2-15	2×10^{9}
Saccharomyces cerevisiae 1-29	7×10^{8}
Trichosporon beigelii R-LF	1×10^{14}
Saccharomyces cerevisiae R-BF	2×10^{14}
Kluyveromyces marxianus R-CS	7×10^{13}
Issatchenkia orientalis R-BC	4×10^{14}
Enterococcus faecium GM8	3×10^{8}
Lactobacillus paracasei CMGB16	5×10^{9}

It results from the table that all the studied strains have a very good viability, at cultivation in the given conditions. In the case of lactic bacteria strains, low viability is not determined by the low productivity of the strains but by limitative factors (pH) which manifest their presence during the lactic fermentation.

CONCLUSION

The 6 strains were tested with different physical and chemical agents. The most resistant strains were chosen and 2 formula of probiotic product were established. Tests were done in fresh rumen content in conditions similar to those in the animal rumen. Pursuant to the performed tests Fp_1 determined a positive result. In this case anaerobic strains were preponderant (lactic bacteria) and the aerobic ones had a maximum of 25%. For Fp_2 the results were negative, in this case aerobic strains which cause a heavy smell in the tested system were preponderant.

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