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UNIVERSIDAD AUTÓNOMA METROPOLITANA
UNIDAD XOCHIMILCO

DIVISIÓN DE CIENCIAS BIOLÓGICAS Y DE LA SALUD
DEPARTAMENTO DE PRODUCCIÓN AGRÍCOLA Y ANIMAL
DOCTORADO EN CIENCIAS AGROPECUARIAS

EVALUACIÓN DE CEPAS DE *Saccharomyces cerevisiae* EN DIGESTIÓN RUMINAL *in vitro* Y EN FERMENTACIÓN Y COMPORTAMIENTO PRODUCTIVO DE CONEJOS.

TESIS

PRESENTADO COMO REQUISITO PARA OBTENER EL GRADO DE

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PRESENTA:

M. Sc. AMR MOHAMED ABDELAZEEM ELMASRY

COMITÉ TUTORAL

Director:

Dr. Germán D. Mendoza Martínez

Co-Director:

Dr. Luis Alberto Miranda Romero

Asesora:

Dra. Gabriela Vázquez Silva

Ciudad de México, Diciembre de 2017

UNIVERSIDAD AUTÓNOMA METROPOLITANA
UNIDAD XOCHIMILCO

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DIVISION OF BIOLOGICAL SCIENCES AND HEALTH
DEPARTMENT OF AGRICULTURAL AND ANIMAL PRODUCTION

EVALUATION OF *Saccharomyces cerevisiae* STRAINS ON *in vitro* DIGESTION OF RUMINANTS AND FERMENTATION AND RABBIT PRODUCTIVE PERFORMANCE

DISSERTATION
PRESENTED AS A FULFILLMENT OF THE
REQUIREMENTS FOR THE DEGREE OF:

DOCTOR IN AGRICULTURAL AND ANIMAL SCIENCES

By:

M. Sc. AMR MOHAMED ABDELAZEEM ELMASRY

TUTORAL COMMITTEE

Director:

Dr. Germán D. Mendoza Martínez

Co-Director:

Dr. Luis Alberto Miranda Romero

Advisor**:**

Dra. Gabriela Vázquez Silva

Mexico City, December, 2017.

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La presente tesis fue realizada bajo la supervisión del comité tutoral indicado a continuación y aprobada como requisito en el plan de estudios para obtener el grado de: **DOCTOR EN CIENCIAS AGROPECUARIAS**

COMITÉ TUTORAL

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ABSTRACT

In vitro experiments were conducted to evaluate two *Saccharomyces cerevisiae* products using samples of wheat, barley, sorghum, maize (20%) with lucerne or oat hays (80%). Yeast doses were 0, 1.5×10^7 or 3.0×10^7 CFU/g DM. There were no grain forage or yeast interaction. Both yeast improved *in vitro* dry matter digestibility (IVDMD). There were differences in gas production among yeast strains that resulted in differences in CH_4 and CO_2. *In vivo* experiments were conducted on weaned New Zealand and California rabbits (n=240; 56 days evaluation) using similar diets treated with live yeast in experiment 1. Yeast products were either Procreatin®7 or Biosaf® SC47 at three different doses: 0, 6.4 or 12.8 $\times 10^9$ CFU per kg diet. Results demonstrated that as yeast dose was increased, rabbit's performance (final BW and feed efficiency) and nutrient digestion were improved without any difference in food intake. *In vitro* caecal gas production was reduced by yeast. Results showed that yeast is a safe alternative to improve rabbit's performance and digestibility if are dosed with an adequate number of viable cells.

Keywords: *in vitro,* caecal, digestibility, gas production, probiotic, rabbit, rumen, *Saccharomyces cerevisiae*, yeast.

INDEX

1- INTRODUCTION

Probiotics are living microorganisms used as feed additives for animals and humans. They can improve the health and performance of the host by modulating the activities of the digestive microbiota and by improving its intestinal balance (Patterson and Burkholder, 2003). Probiotics consist of one or more species of live microorganisms, with or without culture residues, which show a competitive growth against harmful microorganisms, reducing the intestinal pH by producing lactic acid and encouraging digestion by producing enzymes and vitamins. These actions strengthen the animal's own non-specific immune defense (Mountzouris *et al*., 2007; Vanderpool *et al*., 2008 and Zhang and Kim, 2014).

The biological effects of probiotics are highly related to the microorganism strains used, their ability to enhance metabolic activity of the digestive system and their cellular concentration (Fonty and Gouet, 1989). For example, the addition of a probiotic to the diet of rabbits can improve growth performance when the breeding conditions are not optimal (Falcao–E-Cunha, 2007). Moreover, recent results have confirmed the desirable effects of live yeast on rabbit's performance and health as mentioned by Kimsé *et al*., 2012, however, those positive effects of probiotics can be explained by several mecanisms of action and some of them are specific for a given microorganism.

Among probiotics, *Saccharomyces cerevisiae* is yeast that has been used for decades as a preventive and therapeutic agent against diarrhea in addition to other gastrointestinal disorders in humans. Galvao *et al*. (2005) and Timmerman *et al*. (2005) have suggested that supplementation with microbial food additives is a tool to maintain the microbial balance of the intestine and prevent diarrhea by improving the bacterial fecal community in ruminants and non-ruminant species. Yeasts are fungi whose common characteristics are the unicellular state, and since the bacteria also show resistance to antibiotics such as sulfamides and other antibacterial agents, this resistance is natural and genetic and is not susceptible to being modified or transmitted to other microorganisms. *S. cerevisiae* is of major industrial importance because of its ability to convert sugars (glucose, maltose) into ethanol and carbon dioxide (bakery,

brewery, distillery, liquid fuel industries) and is recognized as safe food by the US Food and Drug Administration (Auclair, 2000; Denev et al., 2007).

During the past 50 years, the world rabbit meat production has quadrupled. In 2010, global rabbit meat production was approximately 1.7 million tons, and this production volume showed the following distribution by continent: Asia 48%, Europe 30%, America 17%, and Africa 5%. According to data from the FAO (2012), the biggest rabbit meat producing countries in the world were China (668,980 tons), Italy (255,420 tons) and Venezuela (254,305 tons). However, the consumption of rabbit meat in Mexico is considered limited due to cultural factors such as the lack of knowledge of the virtues of this meat among consumers, the inexperience of how to process them, and the ideology of seeing the rabbit as pet, in the past 10 years, the value of rabbit meat production in Mexico has increased to 3.8 percent (FAO 2015).

Modern animal production faces many conflicting challenges in producing large quantities of high quality food at low prices and without relying too much on antibiotics and medicinal programs, maintaining animal health and welfare and reducing the environmental impacts of animal production, this situation is the same in the production of rabbits. Improving the diets of rabbits by food additives such as probiotics plays a vital role in meeting this demand. Rabbits are very prolific and good feed converters, Hasanat *et al*. (2006) assert that the rabbit is an important micro-cattle, has simple biological characteristics, short reproductive cycle, high prolificacy and better efficiency of feed conversion, accordingly, they have a brief gestation period (28-32 days) and a short generation interval (Aduku and Olukosi, 1990; Fielding, 1991).

Moreover, the rabbit meat compared to other meats has nutritional advantages like a low content of saturated lipids, high protein vitamins and mineral contents, (protein 19-25%, fats 3-8%, water 70%, cholesterol 25-50 $mg100^{-1}$, energy 160-200 and iron 3-5 $mg100g^{-1}$), high digestibility, low sodium content (Dalle Zotte, 2002; Hermida *et al*., 2006; Mexican National Rabbit Product System, 2009), these characteristics make it suitable for people with health problems related to coronary diseases and obesity and their consequences like hypertension and diabetes and it is recommended for cardiovascular diseases patients (Hu and Willett, 2002).

This type of meat represents an affordable and quality source of protein, given the scarcity of protein and the rapidly increasing world population, particularly in developing countries such as Mexico and Egypt (Oloyede *et al*., 2007). Recently, small scale rabbit projects are gaining attention day by day as a means of alleviating poverty threat. Agro-climatic conditions, religious point of view, social practices and technological aspects support the prospects and potentials of raising rabbits. Ahamefule *et al.* (2007) suggested that the development of alternative prime matter that will be relatively cheap when compared with commercial feeds or conventional feedstuffs will make rabbits rearing more viable as a small-scale business.

However, rabbits have a high susceptibility to gastrointestinal diseases and there is no doubt that there is a close and direct relationship between the type of food offered to rabbits and their infection with gastrointestinal diseases, which cause high mortality and low growth rates. Rabbits are strict herbivores, but their digestive strategy differs from those of other fermenters of the posterior gut or caecal (as, horses) and ruminants. Being a species of small prey, rabbits depend on high energy consumption while having the ability to quickly remove the fibrous waste that would otherwise have to be carried in the digestive tract. To achieve this, the rabbit's caecum and colon have a well-developed mechanism for separating digestible and easily fermentable components from the diet and allowing the crude fiber components to purge. The main driving force of this mechanism is the presence of large amounts of indigestible fiber mainly insoluble neutral detergent fiber, (NDF), caecal impaction alters cecocolonic motility, as well as diets high in indigestable fine particle fiber (such as *Psyllium sp.*), Can cause dehydration and compaction of caecal and colonic contents in hard lumps, or cecolites that are the most common cause of low bowel obstruction. This problem is frequent and chronic in rabbits with a history of anorexia, abdominal pain and lack of growth (De Blas *et al.,* 1999, Garcia *et al.,* 2002, Davies and Davies, 2003, Quesenberry and Carpenter, 2011, Olglesbee and Jenkins, 2012).

However, the resistance of rabbits to digestive disturbance can be improved by a high intake of fiber in combination with increased fermentation and lower caecal pH, which is supported by the results obtained by Gidenne and Licois (2005).

The caecum acts as a fermentation chamber and contains large populations of anaerobic organisms, such as bacteroides and anaerobic bacteria with metachromatic staining (Lelkes and Chang, 1987, Fekete, 1989) that are vital for health. The use of antibiotic-supplemented diets may resolve enteric disorders of rabbits, but because of their prohibition as growth promoters (EPC, 2005), research has focused on developing strategies with the aim of maintaining high productivity. The application of probiotics as dietary supplements could act as a possible solution. Some researchers have reported in their studies that some probiotics exert a barrier effect against pathogenic microorganisms by competitive exclusion, colonization of digestive tract and stimulation of the immune system of the host (Vanderpool *et al.,* 2008). In particular, *S. cerevisiae* yeast has been known to improve growth performance in weanling pigs and rabbits (Van-Heugten *et al.,* 2003; LeMieux *et al.,* 2010; Combes *et al.,* 2013) and immunological status in pigs (Monroy-Salazar *et al.,* 2012). It also increased nitrogen metabolism, fiber digestion and milk production in ruminants (Cole *et al.,* 1992) and enhanced growth performance with limited morbidity and mortality in growing rabbits (Maertens and De-Groote, 1992; El-Hindawy *et al.,* 1993; Kimsé *et al.* 2012).

Among the most beneficial effects of probiotic supplementation are the stimulation of digestive processes, modulation of microbial balance and improvement of rabbits performance and health, as well as having a positive impact on average daily gain (ADG), feed conversion rate (FCR) (Falcao-e-Cunha *et al*., 2007; Amber *et al*., 2004). Therefore, yeast is used as a feed additive in ruminants and non-ruminants, as they have favorable effects on digestion as well as in the amount of gas produced by fermentation, also contributing to mitigate many of the problems faced by rabbits in growth and production programs.

2- GENERAL AIM OF THIS STUDY

The main objective was to evaluate the effects of different levels of live cells of probiotics based on *Saccharomyces cerevisiae* on *in vitro* rumen or caecal fermentation and in the rabbit growth performance, digestive health status and nutrient digestibility.

2.1 Specific aims

1. Evaluate the effects of two commercial yeast products on the kinetic parameters of *in vitro* fermentation, determined by the gas production, of diets formed from alfalfa and oats with different grains (wheat, barley, sorghum and maize). It's dosed at the same colony forming unit (CFU) levels of *Saccharomyces cerevisiae*.

2. Estimate the impacts of Biosaf® and Procreatín7® on the growth performance and digestibility of rabbits with rations based mainly on alfalfa or oats hays.

3. Study effects of the two commercial yeast products used on kinetics *in vitro* gas production of caecal contents of slaughtered rabbits reared on fibrous rations.

3- HYPOTHESIS

1- Favorable changes in the parameters of *in vitro* gas production are expected with the two types of studied yeast when are dosed at the same number of viable cells and results will show a dose-dependent effect.

2- *Saccharomyces cerevisiae* has the possibility to modify the microbiota of the posterior tract of the rabbits, increase the nutrient digestibility and improve the feed efficiency utilization.

4- CHAPTER I

EFFECTS OF TYPES AND DOSES OF YEAST ON GAS PRODUCTION AND *IN VITRO* DIGESTIBILITY OF DIETS CONTAINING MAIZE *(ZEA MAYS)* AND LUCERNE *(MEDICAGO SATIVA)* OR OAT HAY

A.M.A. Elmasry[1,2], G.D. Mendoza[1], L.A. Miranda[3], G. Vázquez[1], A.Z.M. Salem[4] and P.A. Hernández[5].

[1] Doctorado en Ciencias Agropecuarias, Universidad Autónoma Metropolitana Unidad Xochimilco, 04960 México, D.F., México.

[2] Botany Department, Faculty of Agriculture, Menoufia University, Po 32511, Shebin El-Kom, Egypt.

[3] Universidad Autónoma Chapingo, Departamento de Zootecnia, 56230, México.

[4] Facultad de Medicina Veterinaria, Universidad Autónoma del Estado de México, Toluca 50000, Méxi co.

[5] Centro Universitario UAEM-Amecameca, Universidad Autónoma del Estado de México, 56900, México.

4.1- ABSTRACT

Two yeast products formulated with *Saccharomyces cerevisiae* were evaluated at the same colony-forming units (CFUs) per gram of substrate. Samples of maize, lucerne and oat hays were mixed (0.5 kg) to a proportion of 80% forage (lucerne or oat) with 20% maize (DM basis) and combined with each yeast to obtain 1.5 x 10^7 or 3.0 x 10^7 CFU/g DM. There was also a control without yeast. *In vitro* gas production was measured at 0, 2, 4, 6, 8, 10, 14, 18, 24, 30, 36, 42, 48, 60, and 72 h incubation. There was no forage/yeast interaction. Both yeast products tended to reduce the maximum volume produced quadratically and lag time linearly, while *in vitro* dry matter digestibility (IVDMD) increased linearly. Ruminal ammonia N and lactic acid were not affected, whereas methane and carbon dioxide tended to be reduced with the intermediate dose of yeast. When the mixture included oat hay, the total volume of gas increased, the lag time decreased, and there was higher IVDMD than in the lucerne-based mixtures, which were associated with lower methane production. Ammonia and lactic acid remained unchanged. The two yeast products showed the same effects on the dynamics of gas production and *in vitro* digestibility when dosed at the same number of viable cells or

CFUs, and there was no interaction with forage quality.

Keywords: forages, ruminal fermentation *in vitro*, *Saccharomyces cerevisiae.*

4.2- INTRODUCTION

Yeast products for ruminants based on *Saccharomyces cerevisiae* increase the number of cellulolytic bacteria (Wallace and Newbold, 1993; Alzahal *et al*., 2014), and are associated with a higher ruminal pH promoted by the yeast, which favours the growth of fibrolytic bacteria *(Fibrobacter and Ruminococcus)* and lactate-utilising bacteria (*Megasphaera* and *Selenomonas*; Pinloche *et al*., 2013). They have thus been regarded as ruminal pH stabilisers (Chaucheyras-Durand *et al*., 2008; Desnoyers *et al*., 2009). In most *in vivo* evaluations of commercial products that contain *Saccharomyces cerevisiae*, researchers confirmed that the amounts of live cells were described by the commercial manufactures (Crosby *et al*., 2004; Pienaar *et al*., 2012 Pinloche *et al*., 2013; Ahmed *et al*., 2015).

In a few experiments, the colony-forming units (CFUs) were corroborated (Emmanuel *et al*., 2007; Bitencourt *et al*., 2011; Vyas *et al*., 2014). In contrast, data from Arcos-García *et al*. (2000) showed that the CFU value determined in the laboratory differed from that reported on the yeast product packaging. Opsi *et al*. (2012) demonstrated that live yeast affects ruminal fermentation slightly more than inactivated yeast. Several studies have been conducted to evaluate neutral detergent fibre (NDF) levels with yeast (Plata *et al*., 1994; Miranda *et al*., 1996; Wang *et al*., 2001), but information that compares forage sources is scarce. Roa *et al*. (1997) compared lucerne and coffee hull and cornstalk with or without *Saccharomyces cerevisiae* on *in situ* digestion and rumen fermentation, and did not find forage/yeast interactions with differences among forages. However, a legume and a lignocellulosic residue differ greatly in nutritional value and the response to yeast addition in digestibility can be different. Therefore, the objective of this study was to evaluate the effects of two commercial yeast products on *in vitro* fermentation kinetic parameters, as determined by gas production, of lucerne- and oat-based diets, dosed at the same CFU levels of *Saccharomyces cerevisiae*.

4.3- MATERIALS AND METHODS

The products that were evaluated were Procreatin® 7 (7.53 x 10^9 CFU/g) and Biosaf® SC47 (1.18 x 10^9 CFU/g) (Safmex S.A. de C.V Mexico), both of which are formulated with *Saccharomyces cerevisiae.* They were dosed at the same CFUs per gram of substrate, based on the viable yeast concentration determined in the laboratory (Camacho *et al*., 2009).

Composite representative samples (n = 3) of maize, lucerne and oat hay were obtained from the experimental dairy farm at the University of Chapingo, oven dried at 55 °C, and ground to 1 mm. After this, 0.5 kg each of forage and maize grain were mixed in a proportion of 80% forage (lucerne or oat) with 20% maize (DM basis), and combined with each yeast product to obtain 1.5 x 10^7 or 3.0 x 10^7 CFU/g DM. There was also a control without yeast. The treatments were allotted in a completely randomized design with a 2 x 3 factorial arrangement, in which the factors were forage source (lucerne and oat) and yeast product (Procreatin® 7 and Biosaf® SC47), evaluated at three concentrations (0.0, 1.5 x 10^7 and 3.0 x 10^7 CFU/g). Forage and maize samples were analysed for dry matter (DM), organic matter (OM) and ether extract (EE), according to AOAC (1990), and neutral detergent fibre (NDF) and acid detergent fibre (ADF), according to Van Soest *et al*. (1991). Starch in the maize was measured enzymatically from the glucose that was released, as described by MacRae and Armstrong (1968) and modified by Wester *et al*. (1992). The compositions of the forages and maize are shown in Table 1.

Amber flasks (100 ml) were prepared with 500 mg DM from each treatment, with four tubes per treatment. The inoculums were consisted of rumen liquor obtained as described by Mendoza-Martínez *et al*. (2015) using an oesophageal probe from two sheeps (34 ± 1.6 kg bodyweight) fed a 50:50 forage : concentrate ratio. The inoculum was obtained before the morning feeding, and was mixed and strained through eight layers of cheesecloth into a flask flushed with carbon dioxide (CO_2). Then 10 ml strained ruminal fluid was added to each bottle, and 80 ml of the buffer solution, described by Goering and Van Soest (1970), was added under a continuous flow of

carbon dioxide to maintain anaerobic conditions. Each flask was closed tightly with a rubber stopper and aluminium crimp. The flasks were incubated in a water bath at 38 ºC. Gas pressure was measured with a pressure gauge (Metron, Mode: 63100, Mexico) at 0, 2, 4, 6, 8, 10, 14, 18, 24, 30, 36, 42, 48, 60 and 72 h of incubation (Blümmel and Lebzien, 2001). Head space pressure values were transformed to gas volumes by a linear regression equation: $V= (P+0.0186) (0.0237)^{-1}$.

Table 1 Chemical compositions of feeds on a dry matter basis used in the experiment (n = 3)

	Lucerne	Oat	Maize
Dry matter %	23.59	19.40	91.8011
Ash %	8.60	8.04	1.12
Crude protein %	18.22	10.51	8.80
Neutral detergent fibre %	46.00	47.67	33.30
Acid detergent fibre %	36.83	23.00	3.68
Ether extract %	3.93	3.51	4.86
Starch %	-	-	77.79

At each time fraction, three parameters of the kinetics of gas production were estimated: lag phase (*h*); maximal volume (Vm; mL g^{-1} DM of substrate) and rate (S; h^{-1}) of gas production, using the model proposed by Menke and Steingass (1988),

Vo= Vm /(1+e(2-4*s*(t-L))). At the end of incubation, the residuals from each bottle were filtered using a flask Buchner with a sintered filter (filter paper F/ fast MOD.617 Code P.V.NO.1034) to estimate DM digestibility. The fermentation residues were dried at 65 °C overnight before being weighed.

Lactate and N-NH_3 were determined by spectrophotometry using samples of residual fluid collected at 36 h incubation. Fluid samples were centrifuged (25,200 x g for 10 min) and subsamples of supernatant were used to analyse lactate (Taylor, 1996) and ammonia N (McCullough, 1967). The results were analysed according to a completely randomized design in which treatments were regarded as fixed effects, testing linear and quadratic effects for grain level (Steel *et al*. 1997). Means were compared using a Tukey's test. Differences among treatments were declared at P <0.05 and a tendency at P <0.10. Data were analysed with JMP7 software (Sall *et al*. 2012).

4.4- RESULTS

There was no forage/yeast interaction. Therefore, the main effects of yeast and forage are presented separately in Tables 2 and 3. Both yeast products tended to reduce the maximum volume of gas produced quadratically, while Procreatin®7 reduced lag time linearly, but they increased *in vitro* DM digestibility linearly (P <0.001). Ruminal ammonia N and lactic acid were not affected, whereas methane tended to be reduced (P <0.11) at the intermediate dose of yeast for Procreatin®7, while the carbon dioxide (CO_2) was increased (P <0.05). Biosaf had no effect on either gases (Table 2). In Table 3 shows that oats increased the total volume of gas produced, decreased lag time, and increased *in vitro* digestibility (P <0.0001), compared with the Lucerne. It is postulated that these effects could be associated with lower methane production (P <0.05) for oats, as ammonia and lactic acid remained unchanged.

Table 2 Main effects of two commercial products dosed at two colony forming unit levels of *Saccharomyces cerevisiae* on *i* *vitro* gas production parameters, digestibility.

		Biosaf SC® 47,				Procreatin® 7,				
	Control	CFU/g DM x 10^7		*P*-value		CFU/g DM x 10^7		*P*-value		
	0	1.5	3.0	L	Q	1.5	3.0	L	Q	SEM
Vmax, ml	415	384	398	0.22	0.07	384	399	0.26	0.07	17.0
S h^{-1}	0.034	0.035	0.035	0.41	0.57	0.035	0.034	0.77	0.20	0.0007
Lag, h	2.23	2.06	2.11	0.42	0.36	2.03	1.98	0.09	0.57	0.750
IVDMD, %	61.65	70.05	70.23	0.0003	0.02	70.27	71.27	0.0001	0.03	2.629
CH_4, ml	29.83	35.00	32.33	0.59	0.34	26.00	35.33	0.25	0.11	4.368
CO_2, ml	42.33	40.08	42.83	0.92	0.57	48.92	38.00	0.40	0.05	3.724
N-NH_3, mg h^{-1}	6.66	7.34	7.47	0.50	0.79	6.46	7.99	0.27	0.41	1.014
Lactic acid, μg^{-1}	15.41	19.62	25.44	0.16	0.89	20.40	24.66	0.19	0.95	4.768

CFUs: colony forming units; DM: dry matter; L: linear; Q: quadratic; SEM: standard error of the mean; VMax: maximu volume, S: rate of gas production, Lag: lag time, and IVDMD: *in vitro* dry matter digestibility based mixtures.

Table 3 Main effects of forage source incubated with maize and commercial yeast products on *in vitro* gas production parameters, digestibility and fermentation gases.

	Lucerne	Oat	SEM	*P*-value
Vmax ml	363[b]	429[a]	6.0	0.0001
S h^{-1}	0.035	0.034	0.0004	0.31
Lag h	3.74[a]	0.42[b]	0.065	0.0001
IVDMD %	63.62[b]	73.76[a]	0.887	0.0001
CH4 ml	37.86[a]	25.53[b]	2.458	0.03
CO2 ml	45.50	39.37	2.540	0.49
N-NH3 mg dl^{-1}	8.34[a]	6.03[b]	0.604	0.16
Lactic acid µg ml^{-1}	18.87	23.35	3.036	0.30

SEM: standard error of the mean, VMax: maximum volume, S: rate of gas production, Lag: lag time, and IVDMD: *in vitro* dry matter digestibility.

4.5- DISCUSSION

The results from this experiment indicated that the use of *Saccharomyces cerevisiae,* dosed at similar CFU levels, had the same effect on the dynamics of fermentation in two diets based on oat or lucerne. Therefore, some of the variability in the results reported in the literature, described as a yeast/diet interaction (Lascano and Heinrichs, 2007; Patra 2012), may be explained by differences in the number of viable cells that were used. Different substrate combinations, however, cannot be disregarded. Elghandour *et al*. (2016) for instance compared three commercial yeast products and observed that one strain was more effective in the stimulation of gas production. They suggested that the difference could be related to the number of active cells and other factors, such as

nutrients and carrier materials. The variation in viability is then a concern. Wallace and Newbold (1995) reported that the viability of preparations can vary from $10^9 – 10^{10}$ live cells/g to 2 x 10^7 live cells/g. Opsi *et al*. (2012) showed higher gas production with live yeast than with inactivated yeast, and concluded that live yeast affects ruminal fermentation slightly more than inactivated yeast. Since one of the mechanisms of action of yeast is oxygen consumption, related to the yeast high respiratory rate (Newbold *et al*., 1996), the numbers of viable cells tested should be reported in yeast evaluations, so that various strains that differ in oxygen consumption ability and metabolic activity can be identified (Kutasi *et al*., 2004).

Although it has been reported that yeast produces metabolites, such as malate, which stimulate lactate-using bacteria (Nisbet and Martin, 1991; Martin and Nisbet, 1992; Nisbet and Martin, 1993), no changes were detected in this metabolite, possibly because grain levels were low in the current substrate mix. Other studies with 62% forage found no yeast effect in ruminal lactate, even when the average ruminal pH in the control diet was significantly higher (Křížová *et al*., 2011).

The positive effects on digestibility have been confirmed in meta-analyses (Desnoyers *et al*., 2009; Poppy *et al*., 2012) and other studies that showed dose responses with increasing levels of CFUs in straw-based diets (Ganai *et al*., 2015). The higher digestibility values could be explained by a higher population of cellulolytic bacteria, which is one of the most consistent effects of yeast (Martin and Nisbet, 1992; Wallace and Newbold, 1993). However, the positive effects are not consistent, even in experiments with increasing doses of yeast (Crosby *et al*., 2004) where cell viability was not certified. In terms of the comparison of diets based on grasses (oat) and legumes (lucerne), Doran *et al*. (2007) observed lower digestibilities with lucerne diets compared with oats, which were associated with a higher lignin cellulose ratio in the lucerne legume than in the oats. Ghasemi *et al*. (2012) compared 0 or 5 g Biosaf®SC 47 (8 x 10^9 CFU/g) with lucerne hay or maize silage and detected only an improvement in the NDF *in situ* digestion measured after three hours' incubation. In another study, which compared several straws with increasing doses of *Saccharomyces cerevisiae*, Tang *et*

al. (2008) observed that supplementation with yeast cultures increased cumulative gas production, but digestibility was not affected. This may be explained by the lignocellulosic characteristic of the substrates, because the doses used by Tang *et al* (2008) were higher than in the current experiment. Several studies have confirmed that substrates with low digestibility do not respond to yeast supplementation *in vivo* (Roa *et al.*, 1997; Arcos-García *et al.*, 2000; Crosby *et al.*, 2004). It is possible that the variability in response to yeast supplementation in terms of forage quality is a function of the potentially digestible fraction, as has been suggested for the response to fibrolytic enzymes (Mendoza *et al.*, 2014), which is another factor that needs to be considered in yeast evaluation assays.

4.6- Conclusions

The results indicate that in order to conduct a proper comparison of yeast products, it is necessary to evaluate the number of CFUs to incubate products with the same number of viable cells. This will allow to elucidate the effects among forage quality x yeast source x dose on *in vitro* evaluations. These results show the importance of checking the CFUs of *Saccharomyces cerevisiae* in products used as feed additives for ruminants.

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5- CHAPTER II

IN VITRO METHANE AND CARBON DIOXIDE PRODUCTION OF HOGH FORAGE DIETS INCUBATED WITH LIVE YEASTS FROM TWO DIFFERENT SOURCES

A.M.A. Elmasry [1,2], G.D. Mendoza [1], L.A. Miranda [3], J.A Martínez [1]., G. Vázquez S. [1], F.X. Plata [1]

[1] Doctorado en Ciencias Agropecuarias, Universidad Autónoma Metropolitana, Unidad Xochimilco, 04960 México, D.F., México.

[2] Botany Department, Faculty of Agriculture, Menoufia University, Po 32511, Shebin El-Kom, Egypt.

[3] Universidad Autónoma Chapingo, Departamento de Zootecnia, 56230, México.

5.1- ABSTRACT

The objective of this experiment was to determine the effect of two probiotics administered at the same doses as additives to two high forage diets (alfalfa or oat hay plus sorghum grain), using the *in vitro* gas production kinetics recording CH_4 and CO_2 production and digestibility with ruminal fluid. Treatments were arranged as a 2 × 2 factorial experiment with two sources of dietary Neutral Detergent Fiber (NDF) as alfalfa and oat hay and two types of probiotics (Biosaf® SC47 and Procreatin® 7), tested at three inclusion levels (0, 1.5 x 10^7 or 3.0 x 10^7 CFU/g of DM). There was no forage by yeast interaction. *In vitro* DM digestibility and V_{max} of gas production of diets with oats were higher than diets with alfalfa. The lag time was shorter in oat diets compared to alfalfa ($P < 0.05$), and CO_2 production with alfalfa diet was higher ($P < 0.01$) than with oats. Additionally, the amount of methane produced tended to be higher ($P < 0.06$) in the alfalfa diet. The ammonia N concentration was greater in the alfalfa than in the oat diet, but lactate concentration was higher in oat diets. A linear effect was observed ($P <$

0.001) in V_{max} with Biosaf® SC47, whereas Procreatin® 7 did not modify this variable. Consequently, Biosaf® SC47 resulted in greater productions of CH_4 and CO_2 and showed a quadratic response to dose (P < 0.01). Lag time was linearly reduced (P < 0.03) with doses of Procreatin®7. Both probiotics increased *in vitro* DM digestibility linearly with dose.

Key words: *Saccharomyces cerevisiae*, gas production; DM digestibility; Probiotics and legume hay

5.2- INTRODUCTION

The effect of yeast cultures in animal production has been well documented mainly in the dairy and meat production (Chaucheyras-Durand and Durand, 2010). The most commonly used probiotics in adult ruminants are based on yeast preparations of *Saccharomyces cerevisiae*, however, commercial products differ in cell viability and metabolic activity (Kutasi *et al*., 2004). It is not clear if differences in response are due to varying cell viability or to the interaction between forage characteristics and strain types.

In vitro, the potential of probiotic yeasts to enhance growth and activity of fibre-degrading rumen microorganisms has been demonstrated with different strains (Puniya *et al.,* 2015). In addition, fungal spore germination and cellulose degradation were increased in the presence of a specific strain of *S. cerevisiae* (Chaucheyras *et al*., 1995). The effectiveness of some yeast strains to stimulate growth or/and activities of fibrolytic bacteria has also been demonstrated; a strain of *S. cerevisiae* stimulated growth of *Fibrobacter succinogenes S85* and reduced the lag time for growth of *Ruminococcus albus* 7, *Ruminococcus flavefaciens* FD1 and *Butyrivibrio fibrisolvens* D1 (Cita) Fonty and Chaucheyras-Durand (2006). Callaway and Martin (1997) showed that the same yeast could accelerate the rate, but not the extent, of cellulose filter paper degradation by *F. succinogenes S85* and *R. flavefaciens FD1*. *In vivo*, Chaucheyras-Durand and Fonty (2002) evaluated the effects of *S. cerevisiae* on the establishment of three species of cellulolytic bacteria (*F. succinogenes, R. albus* and *R. flavefaciens*) and

found an earlier establishment of cellulolytic bacteria and a bigger size of this microflora 50 days after birth in the yeast group compared with the control group, indicating that the yeast strain I-1077 can stimulate cellulolytic microflora growth.

The relationships between nutritional characteristics and *in vitro* gas production for high- and low-NDF forages have been established (Coblentz *et al*., 2013); however, the interaction with different kinds of probiotics is not clear. Tang *et al*. (2008) observed that supplementation with yeast cultures increased cumulative gas production, but digestibility was not improved. This may be explained by intrinsic characteristics of the forages and by the doses used. For this reason, the objective of the present study was to determine the effects of two probiotics of *Saccharomyces cerevisiae* dosed at the same CFU per gram of substrate using *in vitro* methane and carbon dioxide production of high forage diets (alfalfa or oat hay).

5.3- MATERIALS AND METHODS

Treatments were arranged in a 2 × 2 factorial design with two sources of dietary NDF (alfalfa and oat hay) and two types of probiotics (Biosaf® SC47 and Procreatin®7), tested at three gradual inclusion levels (0, 1.5 x 10^7 or 3.0 x 10^7 CFU/g of DM). Feeds were analysed in two replicates for proximate composition (AOAC, 1996). The NDF and the ADF were determined according to Van Soest *et al*. (1991). Ingredient compositions and nutritional value of the experimental diets are shown in Table 1.

Table 1. Nutritional composition of alfalfa and oat hay and the composed diet.

Item (%)	Alfalfa hay	Oat hay	Sorghum grain	Alfalfa sorghum	Oat sorghum
Crude Protein	18.22	10.51	11.6	16.90	10.73
Neutral Detertgent Fibre	46.00	47.00	10.9	38.98	39.78
Acid Detergent Fibre	8.6	8.33	5.9	8.06	7.84
NFC	23.22	30.66	61.5	30.88	36.83
Ash	3.96	3.50	2.0	3.57	3.2

Sample preparation

Forages samples of alfalfa and oat hay were collected from the experimental dairy farm at the Chapingo Autonomous University, Mexico. Samples were ground in a Willey miller then 0.5 kg of each sample was added at a proportion of 80% forage (alfalfa or oat) with 20% sorghum (DM basis) for the incubations.

Commercial products

The evaluated yeast products were Procreatin® 7 (P7) with 7.53 x 10^9 CFU/g and Biosaf® SC47 (Bs) with 1.18 x 10^9 CFU/g *Saccharomyces cerevisiae* Sc47, both marketed by Safmex S.A. de C.V., Mexico.

***in vitro* kinetics of gas production**

Rumen liquor was obtained from male and female sheep (34 ± 1.6 kg BW) according to the method described by Babayemi and Bamikole (2006), using a suction tube for sheep fed on 50:50 concentrate: forage ratio. Ruminal contents of each sheep were obtained immediately before the morning feeding, mixed and strained through eight layers of cheesecloth into a flask flushed with CO_2 and transported to the laboratory. Rumen liquor was flushed with CO_2 before use as inoculum. The substrate within each flask was a 0.5 g sample of oat or alfalfa mixture, substrates were freely suspended within the incubation medium in 100 ml serum bottles. Previously, each mixture was prepared with each yeast product to obtain 0 (control without yeast), 1.5 x10^7 or 3.0 x 10^7 CFU/g of DM. Subsequently, 10 ml of particle-free ruminal fluid and 80 ml of the buffer solution of Van Soest *et al*. (1991) were added to each bottle. Three bottles of each mixture sample with ruminal fluid were also included as blanks (Udén *et al*., 2012). Once all bottles were filled, they were immediately closed with rubber stoppers, shaken and placed in the incubator at 39ºC. Gas production volumes were recorded after 2, 4, 6, 8, 10, 14, 18, 24, 30, 36, 42, 48, 60 and 72 h of inoculation using a manometer. Total gas values were corrected for the blank incubation and expressed

as ml/g DM. In each measurement, the gas trapped in the syringe (VT) was injected into another sealed vial containing a solution of KOH (0.1 M), and CO_2 was estimated (Statham and Williams, 1999). CH_4 production was calculated from the methane concentration in each gas sample and the corresponding total gas production. Net methane production was calculated by subtracting the mean gas production of the blanks from the total gas production of each treatment, respectively.

Using the residual liquid fraction of each bottle, $N\text{-}NH_3$ and lactic acid concentrations were determined using the methods recommended by Searle (1984) and Taylor (1996), respectively. After incubation, the contents of each serum bottle were filtered using a Buchner flask with a Whatman filter (filter paper F/ fast MOD.617 Code P.V.NO.1034). Fermentation residues were dried at 65°C overnight to estimate potential DM loss, and non-degradable DM was defined as residual weight after drying.

Variables and statistical analysis

Gas production kinetic parameters (ml/g DM) were fitted with a nonlinear model as described by Menke y Steingass (1988):

$GP_t = v / (1+\exp (2-4*s*(t-L)))$,

Where GP_t (mL) is the cumulative gas production at incubation time t (h), v is the maximum gas production (mL/g DM) after the asymptote is reached, s is the fractional fermentation rate and L is the lag time (L/h).

The results were analysed according to a completely randomized design where treatments were considered as fixed effects, testing linear and quadratic effects of yeast levels (Steel *et al*., 1997). Means were compared using a Tukey's test, and differences among treatments were declared at $P < 0.05$ and a tendency at $P < 0.10$. Data were analysed using the JMP7 software (Sall *et al*., 2012).

5.4- RESULTS AND DISCUSSION

There was no forage/yeast type interaction. Therefore, main effects are presented and discussed.

Forages

As expected, alfalfa and oat hay had similar NDF contents, but significantly differed in their protein concentrations (Table 1). According to the NRC (2001), the nutritional value of alfalfa is high enough to consider it as mature hay; however, NDF levels of oat hay were lower than expected.

Table 2 shows the parameters of gas production kinetics and some metabolites of rumen fermentation from the mixtures with forages. The digestibility of the oat diet was higher than that of the alfalfa diet; consequently, V_{max} was greater in the oat diet, which is in agreement with the results reported by Coblentz *et al*. (2013) who stated that general gas production was greater within low-NDF oat forages compared with high-NDF forages. Hatew *et al*. (2015) obtained the same results when evaluating diets with different concentrations and degradation rates of non-fibre carbohydrates. The lag phase was shorter in oat than in alfalfa diets ($P < 0.05$). This is most likely because the oat fibre is formed by soluble and insoluble fractions or because of the higher lignin cellulose ratio in alfalfa compared to oat (Doran *et al*., 2007).

CO_2 production in the alfalfa diet was higher ($P < 0.01$) than in the oat diet; additionally, the volume of methane tended ($P < 0.076$) to be higher in the alfalfa diet. This higher production of greenhouse gases results from a greater total activity of ruminal bacteria and methanogenic archaea (Mohammadzadeh *et al*., 2014) associated with ruminal fermentable carbohydrates.

Table 2. Main effects of forage sources incubated with sorghum grain and commercial probiotics on *in vitro* gas production parameters

Parameter	Forages		EEM	P
	Alfalfa	Oat		
Vmax, ml	324.994	442.677	3.702	0.0001
S, %	0.035	0.033	0.0002	0.005
Lag, h	3.682	1.780	0.059	0.0001
DMIVD, %	68.49	73.72	2.61	0.022
CO_2, ml/g DMD	96.17	78.04	3.35	0.0002
CH_4, ml/g DM	52.33	40.00	3.45	0.007
CO_2, ml/100 ml	66.24	62.47	1.35	0.062
CH_4, ml/100 ml	33.75	37.52	1.35	0.062
N-NH_3, mg/100 ml	8.39	5.73	0.457	0.0002
Lactic Acid, µM	1.736[b]	2.693[a]	0.275	0.039

As expected, the concentration of ammonia-N was greater in the alfalfa than in the oat diet. Brito *et al*. (2014) demonstrated that the concentration of non-structural carbohydrates in alfalfa diets affects bacterial production and ammonia-N concentrations. Combination with sorghum grain could stimulate microbial protein synthesis; however, this was not evaluated in our study, although the relationship between microbial growth and the supply of fermentable carbohydrates is well known (Nocek and Russell, 1988).

Lactate production was higher in the oat diet, which may be the result of *L. Plantarum, L. reuteri* and *L. acidophilus* preferring soluble oat fibres (Kedia *et al*., 2008) and of higher fermentation rates of soluble fibre fractions, leading to a reduction of ruminal pH and an increase of Volatile fatty acids (VFA) and lactic acid (Nagaraja and Tigemeyer, 2007).

Probiotics

Table 3 shows the effects of the probiotics on *in vitro* gas production parameters. We observed a linear effect ($P < 0.001$) on Vmax with Biosaf® SC47, while Procreatin® 7 did not modify this parameter ($P < 0.21$). The higher gas production7® with Biosaf® SC47 was associated with an increased production of both methane and CO_2 according to the added probiotic quantity. Sullivan and Martin (1999) reported a similar effect with the addition of *S. cerevisiae* to the diet and showed that yeast increased the methane production; this effect was attributed to the ability of *S. cerevisiae* to stimulate overall ruminal fermentation. Several studies have shown that probiotics based on *Saccharomyces cerevisiae* increase the number of cellulolytic bacteria (Pinloche *et al*., 2013; AlZahal *et al*., 2014); however, the fact that Procreatin did not stimulate gas production suggests that its mechanism of action may be different in some microbial communities.

Lag time decreased in response to Procreatin (linear, $P < 0.03$), confirming that yeast strains may act differently in the rumen. Other studies also reported that the addition of yeast cultures decreased the lag time of in situ NDF degradation (Tang *et al*., 2008). Williams *et al*. (1991) suggested that the stimulation of cellulose degradation by the yeast culture is associated with a decreased lag time, which results in increased DM digestibility; however, this statement cannot be generalised since Biosaf addition did not affect the lag phase and positively affected digestibility. Both probiotics increased DM Digestibility linearly with dose (linear, $P < 0.001$; quadratic, 0. $P < 0.01$).

Table 3. Effects of two commercial probiotics dosed at two CFU levels *of Saccharomyces cerevisiae* on *in vitro* g as production parameters, digestibility and fermentation incubated with sorghum and forages (20:80).

		Biosaf® SC 47,				Procreatin® 7,				
	Control	mg/kg		*P*-value		mg/kg		*P*-value		
	0	20	40	L	Q	20	40	L	Q	SEM
Vmax, ml	375	388	399	0.008	0.84	378	385	0.21	0.76	26.10
S h^{-1}	0.035	0.034	0.034	0.12	0.22	0.034	0.034	0.08	0.13	0.0005
Lag, h	2.66	2.58	2.89	0.52	0.74	2.80	2.93	0.03	0.41	0.4
IVDMD, %	71.11	73.08	74.41	0.56	0.69	78.58	75.18	0.03	0.32	2.38
CH_4, ml	95.56	85.40	103.73	0.01	0.14	94.90	98.06	0.64	0.92	5.21
CO_2, ml	44.16	66.66	47.66	0.009	0.65	45.66	48.66	0.66	0.56	5.51
CO_2, %	65.99	54.18	66.07	0.98	0.003	65.80	64.46	0.70	0.87	3.0
CH_4, %	34.00	45.81	33.93	0.98	0.003	34.19	35.53	0.70	0.87	3.0
N-NH_3, mg/100 ml	6.30	7.00	7.11	0.43	0.73	6.77	8.15	0.08	0.60	1.0
Lactic acid, μg^{-1}	2.04	2.00	2.73	0.27	0.47	2.730	2.14	0.86	0.93	0.50

L: linear; Q: quadratic; SEM: standard error of the mean; VMax: maximum volume, S: rate of gas production, Lag: lag tim e, and IVDMD: *in vitro* dry matter digestibility.

About yeast supplementation in ruminants; it has been demontrasted that *S. cerevisiae* increased OM digestibility. This effect was decreased by the proportion of concentrate in the diet and increased by dietary NDF content and crude protein. Mullins *et al*. (2013) reported higher rates of ruminal starch digestion with the addition of *S cerevisiae* and hypothesised that, this was due to alterations of the microbial population in the rumen. Ghasemi *et al*. (2012) compared an addition of 0 or 5 g *S. cerevisiae* to alfalfa hay or corn silage and detected an improvement in NDF in situ digestion after three hours of incubation. Doran *et al*. (2007) observed lower digestibility of alfalfa diets compared to oats diets, explained by the higher lignin cellulose ratio in the alfalfa legume. However, we detected no interactions, and some discrepancies in the results may be explained with the fact that in several studies, we did not always add the same doses of viable cells.

Total CO_2 and methane concentrations were higher with the addition of Biosaf® SC 47 (linear; $P < 0.01$ and $P < 0.009$, respectively). In other studies, the total enteric CH_4 production expressed as grams per day was not affected by yeast feed; however, enteric CH_4 emission intensity tended to be affected by yeast supplementation (Chung *et al*., 2011). When methane estimates are made based on digestibility, the results indicate that the production of these gases will increase as consequence of adding yeast. However, results from our experiment indicate that CH_4 and CO_2 should be measured to evaluate strain effect.

Ammonia-N production tended *(*$P < 0.08$*)* to increase with Procreatin® 7 addition, but not with addition of Biosaf® SC47. In contrast, other studies reported that the addition of yeast at a suitable level was beneficial for a more efficient use of the N source for microbial protein synthesis (Mao *et al*., 2013). Hristov *et al*. (2010) also found an increment in the overall use of ammonia-N and increased microbial protein synthesis rates with the addition of yeast; however, ammonia-N concentrations in this study were low and indicate inefficiency of the system.

In contrast to other studies, in which *Saccharomyces cerevisiae* reduced lactic acid concentrations (Desnoyers *et al*., 2009), in this study, both strains did not have any effect on the production of lactic acid in a similar form to Moya *et al*. (2009). Li *et al*. (2016) suggested that yeast reduced the variation in ruminal pH during control feeding, associated to reduced lactic acid concentrations; however, in our study, lactic acid concentrations were low because of the high amount of forage in the diets.

5.5- Conclusions

Oat based diets showed higher digestibility and gas production than alfalfa based diets with sorghum grain. Digestibility responded linearly to the dose of the two probiotics; however, our results indicate that the strains differ in their ability to increase *in vitro* production of methane and carbon dioxide.

5.6- References

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6- CHAPTER III

EFFECTS OF DOSE AND TYPE OF *SACCHAROMYCES CEREVISIAE* ON *IN VITRO* DIGESTIBILITY, METHANE AND CARBON DIOXIDE PRODUCTION, OF ALFALFA AND OAT DIETS

Amr Mohamed Abdelazeem Elmasry[1,2], Germán David Mendoza[1], Luis Alberto Miranda[3], Gabriela Vázquez[1], Pedro Abel Hernández[4], Fernando Xicotencatl Plata[1], José Antonio Martínez-García[1]

[1] Doctorado en Ciencias Agropecuarias, Universidad Autónoma Metropolitana, Unidad Xochimilco, 04960 México, D.F., México

[2] Botany Department, Faculty of Agriculture, Menoufia University, Po 32511, Shebin El-Kom, Egypt

[3] Universidad Autónoma Chapingo, Departamento de Zootecnia, 56230, México.

[4] Centro Universitario UAEM-Amecameca, Universidad Autónoma del Estado de México, 56900, México

6.1- ABSTRACT

The objective of the study was to determine the effect of dose and type of probiotics on high forage diets with different types of fiber (alfalfa or oats) incubated with a fast fermenting grain (barley) evaluating *in vitro* DM digestibility, kinetics of gas production parameters, CH_4 and CO_2 production. The design consisted of a 2 × 2 × 3 factorial arrangement, using two forage sources, two products of *Saccharomyces cerevisiae* (Biosaf® and Procreatin®) at three doses of viable yeast (0, 1.5 x 10^7, 3.0 x 10^7 CFU g^{-1} DM). Oat diets produced more gas (Mvol) than alfalfa diets (442 vs. 344 ml; *p<0.001*), but the rate of gas production was faster with alfalfa (0.035 vs. 0.031 %/h; *p<0.001*). The N-NH3 concentration was similar *(p=0.16)* among forages but lactate was higher with oat (29.12 vs. 16.71 µg/g *p<0.001*). A linear effect (*P<0.03*) was

observed in Mvol and rate of gas production with Biosaf® Sc47 (p<0.006) but not with Procreatin®7. The Lag phase showed a quadratic effect only for Procreatin® ($p<0.02$). The digestibility of DM was improved with both yeast products ($p<0.01$). Procreatin® tended to reduce $(p<0.02)$ CO_2 production. As a conclusion, Oats produce more gas than alfalfa. Yeast increased the digestibility but the effects on gas production are different by yeast strain and have little effect on CH_4 and CO_2 production.

Key words: Alfalfa, oats, barley, UFC, methane, CO_2.

Abreviations used: DM (dry matter); UFC (colony forming units); CO_2 (carbon dioxide); Mvol (maximum volume); CH_4 (methane); NDF (neutral detergent fiber); AOAC (Association of Official Agricultural Chemist); ADF (acid detergent fiber): WB (weight body); °C (degrees Celsius); KOH (potassium hydroxide); PGT (gas production accumulated over time); t (time); ml (milliliter); g (gas); s (fermentation velocity); *L* (delay time); N-NH_3 (ammoniacal nitrogen); N (nitrogen); L (linear effect); C (quadratic effect); pH (hydrogen potential); AGV (volatile fatty acids), OM (organic matter).

6.2- INTRODUCION

The effect of probiotics on animal production has been documented in both milk and meat production (Chaucheyras-Durand and Durand, 2010). Of these products, the most used in ruminants, base their preparation in the inclusion of different yeast strains of *Saccharomyces cerevisiae*, but commercial products are different in terms of cell viability and metabolic activity of the strain from which they are made (Kutasi *et al*., 2004). It was observed that *S. cerevisiae* favors the establishment of cellulolytic bacteria in the rumen of newborn calves and that subsequently stimulate the ruminal growth of these bacteria (Chaucheyras and Fonty, 2002). In turn, inoculating cellulolytic bacteria from the rumen influences the increase in CH_4 production (Deng *et al*., 2017). In contrast, it has been suggested that yeasts may reduce production of CH_4 and CO_2 (Hernández *et al*., 2017). Also, the inclusion of high levels of starch with different

fermentation rates in the animals' diet modifies rumen fermentation patterns and alters the production of CH_4 (Popova *et al*., 2013).

The *in vitro* gas technique is a rapid procedure that has established the relationship between nutritional characteristics and gas production of forages with different fiber concentrations (Coblentz *et al*., 2013). Also, it allows to determine changes in the production of gas, which are associated with the changes in the speed of fermentation of the grains (Hatew *et al*., 2015), and allows to demonstrate the potential of yeasts to improve the growth and the activity of ruminant fungi, which degrade the cell wall in the rumen with different strains from an increase in spore germination and cellulose degradation (Chaucheyras *et al*., 1995; Puniya *et al*., 2015).

Therefore, the objective of the present study was to determine the effect of the different doses (colony forming units/g substrate, CFU) of two strains of *S. cerevisiae*, evaluating changes on *in vitro* dry matter digestibility and the production of (CH_4 and CO_2), in high forage diets with different types of neutral detergent fiber (alfalfa or oats) and a fast fermenting grain (barley).

6.3- MATERIAL AND METHODS

The experimental design consisted of a 2 x 2 x 3 factorial, with two sources of neutral detergent fiber (NDF) in the diet (alfalfa and oat hay), two probiotics (Biosaf® SC47 and Procreatin®7), with three levels of inclusion (0, 1.5 x 10^7, and 3.0 x 10^7 CFU g^{-1} DM). A proximal chemical analysis of the diets was done in duplicates following the recommendations of AOAC (1996). Neutral detergent fiber and acid detergent fiber (NDF and ADF) were determined according to Van Soest *et al*. (1991). The proximal composition and nutritional value of the experimental diets are shown in Table 1.

Table 1. Nutritional composition of alfalfa, oat hay, and composite diets.

Item, %	Alfalfa hay	Oat hay	Barley grain	Alfalfa Barley	Oat Barle
CP	18.22	10.51	12.4	17.06	10.89
NDF	46.00	47.00	20.8	40.96	41.76
Hemice	37.4	38.67	13.6	32.64	33.66
ADF	8.6	8.33	7.2	8.32	8.10
CS	23.22	30.66	56.7	29.91	35.87
Ashes	3.96	3.50	2.9	3.75	3.38

CP = Crude protein; NDF= Nutral detergnt fiber; Hemice = Hemicellulose; ADF = Acid detergent fiber; SC= Soluble or non-structural carbohydrates.

Sample preparation

Samples of alfalfa and oat hay were obtained frrom the experimental field of the Autonomous University Chapingo (Mexico) and processed in a Willey mill with a 1 mm sieve, 0.5 kg of each diet was prepared at a proportion of 80% forage (alfalfa or oats and 20% barley (dry basis) for use as a substrate in the incubations.

Yeast types

The evaluated yeast products were Procreatin® 7 with 7.53 x 10^7 CFU g^{-1} and Biosaf® SC47 with 1.18 x 10^7 CFU g^{-1} of S. cerevisiae, both marketed by Safmex SA d CV, México.

Kinetics of in vitro gas production

For this process, ruminal fluid was obtained from two sheep (male and female with 34 ± 1.6 kg BW according to the method described by Babayemi and Bamikol (2006), using a suction pump. These sheep had been previously cannulated, followin the protocols of Law Animal Protection of the State of Mexico, México (1985), and wit the approval of the Bioethics Committee of the Autonomous University Chaping (Mexico).

The sheep were fed on a diet of a concentrate / forage ratio of 50:50. The rumen samples of each sheep were collected before the morning feed, mixed and filtered through eight layers of gauze in a CO_2 purged flask and transported to the Animal Nutrition Laboratory of the Department of Animal Science of the Autonomous University Chapingo (Mexico).

Ruminal fluid was mixed with CO_2 prior to use as inoculum. A 0.5 g sample of each diet was placed into 100 ml incubation flasks and mixed with each yeast product to obtain 0 (unleavened control), 1.5 x 10^7 or 3.0 x 10^7 CFU g^{-1} of DM.

Subsequently, 10 ml of particle-free ruminal liquid were added to each incubation flask and 80 ml of the buffer solution of Van Soest *et al*. (1991). The flasks were prepared in triplicate and three samples of the mixture were included with ruminal fluid to be considered as blanks (Udén *et al*., 2012). The flasks were incubated at 39 °C, gas production volumes were recorded at 2, 4, 6, 8, 10, 14, 18, 24, 30, 36, 42, 48, 60 and 72 h of inoculation, by a manometer. Total gas values were corrected and expressed as ml / g DM. At each measurement, the total volume of gas was injected into another sealed vial containing a solution of KOH (0.1 M) and CO_2 (Statham and Williams, 1999). Production of CH_4 was calculated from the corresponding total gas production. The net production of CH_4 was calculated as the difference between the total gases produced minus the white flasks minus the CO_2 production for each treatment.

The gas production kinetics data (ml / g DM) were fitted with a non-linear model (Menke and Steingass, 1988): $PGT = v / (1 + \exp (2^{-4} * S * (t^{-L})))$; where PGT (ml) is the accumulated gas production at incubation time t (h), v is the maximum gas production (ml g^{-1} DM) after the asymptote is reached, S is the rate of fermentation and *L* is the delay time (*L* h^{-1}).

Lactic acid and ammoniacal nitrogen

The residual liquid fraction of each flask was used to determine the concentrations of lactic acid and N-NH_3, using the methods recommended by Searle (1984) and Taylor (1996) respectively.

Dry matter digestibility

After incubation, the contents of each incubation bottle were filtered using a Buchner flask with a Whatman filter (filter paper F / MOD.617 fast P.V.NO.1034). After that, fermentation residues were dried at 65 °C and the in vitro digestibility of DM was estimated (Elmasry *et al*., 2016).

Statistical analysis

The data were analyzed according to a completely randomized design using a 2 x 2 x 3 factorial arrangement, two forage sources, two *S. cerevisiae* isolates (Biosaf® and Procreatin®), and three inclusion levels (0, $1.5x10^7$, $3.0x10^7$ UFC g^{-1} from DM), where treatments were considered as fixed effects and the linear and quadratic effects of yeast levels were tested (Steel *et al*., 1997). The means were compared using the Tukey test, and as differences between treatments with $p<0.05$ and trend $p<0.10$. Data were analyzed using JMP7 software (Sall *et al*., 2012).

6.4- RESULTS

The nutritional composition of forages is presented in Table 1. Alfalfa hay and oat straw have a similar amount of NDF, having differences in N content. Because of these changes, the oat barley mixture has a lower concentration of N, but a greater amount of soluble carbohydrates than the mixture with alfalfa.

The effect of forages on the kinetic of gas production is recorded in Table 2. There was no interaction between NDF sources and yeasts, therefore main effects are presented. The oats showed higher Mvol *($p<0.001$)* than alfalfa and a slow rate and lag

time of gas production *(p<0.01).* Alfalfa with a faster rate and longer lag showed higher production of CH_4 and CO_2 *(p<0.01).* The N-NH_3 was higher with alfalfa but lactic acid *(p<0.01)* was higher with oats.

The effect of probiotics on *in vitro* gas production is presented in Table 3. A linear increment (*p<0.03*) was observed on Mvol with Biosaf® whereas Procreatin® did not modify this parameter (*p=0.*59). The rate of gas production increased (*p<0.006*) with Biosaf® whereas Procreatin® only showed a trend (*p<0.07*). The Lag phase had a quadratic effect on Procreatin® (*p<0.02*) whereas Bioasaf® showed no effect (*p=0.64*) on this time.

Table 2. Principal effects on forage incubated with barley and two types of probiotics, *in vitro* gas production kinetics, DM digestibility and fermentation variables.

	Substrates			
	Alfalfa	Oats	SEM	*P*
Mvol, ml	344[b]	442[a]	3.9	0.0001
S, %/h	0.035[a]	0.031[b]	0.0002	0.0001
Lag, h	2.00[a]	1.34[b]	0.066	0.0001
IVDDM, %	61.95[b]	77.36[a]	0.982	0.0001
CH_4, ml	34.96[a]	22.76[b]	2.116	0.002
CO_2, ml	41.23	37.27	1.771	0.03
CH_4, %	45.89[a]	29.87[b]	2.568	0.04
CO_2, %	54.11b	70.13[a]	2.568	0.04
N-NH_3	8.70[a]	6.63[a]	0.662	0.16
Lactic acid µ/g	16.71[b]	29.12[a]	2.313	0.004

Mvol: Max volume; GPR: Gas production rate; Lag: Time Lag; IVDDM: *In vitro* digest b of DM

Tabla 3. Effects of two probiotics (*Saccharomyces cerevisiae*), two levels of CFU on *in vitro* digestibility, and kinetics of gas production, two diets high in forage and barley in grain.

	Control	Biosaf® SC 47, mg/kg		*P*-value		Procreatin® 7, mg/kg		*P*-value		
	0	20	40	L	Q	20	40	L	Q	SEM
MVol, ml	384	396	404	0.03	0.80	394	389	0.59	0.32	22.50
S h^{-1}	0.033	0.034	0.035	0.006	0.52	0.034	0.034	0.07	0.65	0.0009
Lag, h	1.87	1.68	1.61	0.08	0.64	1.49	1.71	0.27	0.02	0.18
IVDMD, %	62.08	69.82	68.72	0.002	0.01	73.30	74.36	0.0001	0.006	3.74
CH_4, ml	25.83	37.00	26.66	0.85	0.01	29.41	25.41	0.09	0.32	4.12
CO_2, ml	45.42	33.17	42.50	0.41	0.002	36.75	38.42	0.06	0.10	2.73
CO_2, %	63.83	47.87	62.30	0.76	0.002	56.96	60.54	0.52	0.24	4.03
CH_4, %	36.16	52.13	37.69	0.76	0.002	43.03	39.46	0.52	0.24	4.03
N-NH_3, mg/100 ml	7.63	6.53	7.50	0.93	0.43	8.25	8.43	0.59	0.86	1.10
Lactic acid, μg^{-1}	31.51	22.02	22.95	0.09	0.23	19.67	18.44	0.01	0.22	4.38

L: linear; Q: quadratic; SEM: standard error of the mean; MVol: maximum volume, S: rate of ga s production, Lag: lag time, and IVDMD: *in vitro* dry matter digestibility.

The *in vitro* DM digestibility was increased (linear and quadratic effects; $p<0.01$) with both yeast products. The higher gas production observed with Biosaf® resulted in an increase in CH_4 and CO_2 production that increased their concentration according to the amount of probiotic added (quadratic effect; $p<0.05$), however, Procreatin® only tended to modify both gases ($p<0.10$). The N-NH_3 was not affected by yeast type or dose, however, Procreatin® reduced lactate linearly ($p<0.01$) while Biosaf® only tended to decrease ($p<0.10$).

6.5- Discussion

NDF type

These observed results are logical and related to the nutritional characteristics of each forage. Regarding to composition, the reduction of N in the diet due to the inclusion of oats in alfalfa replacement were previously reported by Jian *et al*. (2015), who evaluated the change of oats by alfalfa in silage, and showed an increase of soluble carbohydrates similarly in this study.

The observed value of Mvol with oats is related to its digestibility and to its contribution to the diet with soluble carbohydrates. It has been reported that there is a direct correlation between both total digestible nutrients and soluble carbohydrates and Mvol (Coblentz *et al*., 2013). The CH_4 is an indicator of the carbohydrates production with a lower rate of diet fermentation (Hatew *et al*., 2015). Singh *et al*. (2012) found higher production of CH_4 with legumes when compared with grasses as observed here.

A higher ammonia N was expected in alfalfa diet, however, the mineral solution contained an ammonia source and the alfalfa hay was elaborated from mature phenological stage and resulted in a substrate with low digestibility; this relationships between digestibility, protein and rate of protein degradation have been previuosly described (Jonker and Yu, 2016).

The higher lactate was observed with oats as associated to the soluble carbohydrates fermentation which has been described by Nagaraja and Titgemeyer,

(2007). This higher fermentation of soluble carbohydrates explains the observed preference of *L. Plantarum*, *L. Reuteri* and *L. acidophilus* for soluble oat fibers (Kedia *et al*., 2008).

Saccharomyces cerevisiae

Few evaluations have been reported comparing two yeast strains in the production of CH_4 and CO_2. In this sense, Sullivan and Martin (1999) showed that the addition of *S. cerevisiae* increased the production of methane, which was attributed to the ability of *S. cerevisiae* to stimulate general ruminal fermentation. In addition, probiotics based on *S. cerevisiae* increase the number of cellulolytic bacteria (Pinloche *et al*., 2013), therefore probiotics may increase CH_4 production (Wang *et al*., 2016), however, the fact that Procreatin® did not stimulate gas production, suggests that mechanism of action may be different in some strains and in the microbial communities, so it is not possible to generalize the effect on fermentation gases.

The observed effects in Lag phase indicate that they can affect differently microbial populations in the rumen. This effect was reported previously by yeasts (Tang *et al*., 2008). Statham and Williams *et al*. (1991) suggest that a shorter Lag phase results in increased DM digestibility, however, this assertion cannot be generalized since the addition of Biosaf® did not affect the Lag phase, but positively affect the digestibility. According to the models of Allen and Mertens (1988) the Lag phase has no relation with the digestion of NDF.

The increase in digestibility by yeast has been reported previously. Desnoyers *et al*. (2009) published a quantitative meta-analysis on yeast supplementation in ruminants, concluding that *S. cerevisiae* increased OM digestibility and the effect was decreased by the proportion of concentrate and increased by NDF and crude protein in diet. Mullins *et al*. (2013) reported increases in the rumen digestion rate of starch with the addition of *S cerevisiae* and attributed it to the increase in some groups of the microbial population of the rumen.

The results in concentrations of CO_2 and CH_4 do not coincide with the study of Elghandour *et al*. (2016) where they report that the type of yeast and the dose used reduce the enteric concentration of CH_4. This reduction was attributed to the competition of acetogenic bacteria with methanogenic to capture hydrogen, therefore, by reducing this element CH_4 synthesis is reduced (Mwenya *et al*., 2004). When evaluating the total production of enteric CH_4 expressed in grams / day, it is not affected by the administration of yeast, but if expressed as intensity of enteric emission (g CH_4 / g food consumed) this tends to be reduced or increased depending on the type of yeast (Chung *et al*., 2011).

Although in this study the concentration of N-NH_3 was not modified by yeast, research reports that addition of yeast produces a more efficient use of N and an increase in the rate of microbial protein synthesis (Hristov *et al*., 2010; Mao *et al*., 2014). Regarding to lactic acid, its reduction has been observed previously (Marden *et al*., 2008) as observed here.

6.6- Conclusion

The *in vitro* digestibility of alfalfa or oat diets can be improved with the two strains of *Saccharomyces cerevisiae* and there is a dose response. The effects of yeast on gas production are variable according to yeast strain with a minor effect on CH_4 and CO_2 production.

6.7- References

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7- CHAPTER IV

EFFECT OF YEAST TYPES AND LEVELS ON GROWTH PERFORMANCE AND NUTRIENTS DIGESTIBILITY OF GROWING RABBITS FED CORN OR BARLEY GRAIN

A.M.A. Elmasry[1,2], L.A. Miranda[3], G. Vázquez S.1[1], G.D. Mendoza[1] and A.M. Safwat[4]

[1]. Doctorado en Ciencias Agropecuarias, Universidad Autónoma Metropolitana, Unidad Xochimilco, 04960 México, D.F., México.

[2]. Botany Department, Faculty of Agriculture, Menoufia University, Po 32511, Shebin El-Kom, Egypt.

[3]. Universidad Autónoma Chapingo, Departamento de Zootecnia, 56230, México.

[4] Poultry Production Department, Faculty of Agriculture (El-Shatby), Alexandria University, Alexandria, Egypt.

7.1- ABSTRACT

A study of 56 days was conducted to determine the effect of live yeast supplementation in the diet of growing rabbits on growth performance and digestibility in addition to *in vitro* caecal fermentation gas production during 0, 2, 4, 6, 8, 12, 16, 20, at 24 h of incubation. A total of 120 New Zealand and California rabbits (five weeks old and average body weight 0.881 ±0.015kg) were housed into 30 cages (4 rabbits per cage). They were fed on either corn or barley grains with alfalfa and supplemented with Procreatin®7 or Biosaf® SC47 at two doses, 6.4 or 12.8 $\times 10^9$ CFU per kg basal diet in addition to the control diet. Based on the results obtained from this study, the higher yeast concentration, the greater positive effect and vice versa. The final body weight (BW), average daily gain (ADG) and carcass weight (CW) were significantly increases ($P<0.01$) as a result of the decreased ($P<0.01$) feed conversion ratio (FCR). There were

no significant differences in food intake rate. Significant increases (*P<0.05*) in nutrients digestibility (DM, OM, NDD and ADF) and *in vitro* DM and OM digestibility were observed with both grains. While, total gas production rate and fractional gas volume have decreased significantly (linear effect *P<0.01*). The results of this experiment showed that using of yeast, as additive in the diet of corn or barley with alfalfa, has positive effects on growth performance characteristics (ADG, FCR) and nutrients digestibility.

Keywords: Probiotic, yeast, *Saccharomyces cerevisiae*, digestive health, rabbit, *in vitro* caecal digestibility and total gas production.

7.2- INTRODUCTION

The balanced rabbit diet is one of the most important challenges that face rabbit growing and production programs. Due to the complex digestion of rabbit and the diet nature this species is very susceptible to enteric disease such as caecal impaction and enterotoxaemia (Trocino *et al*., 2005; Kritas *et al*., 2008 and Rabie *et al*., 2011). Improving the feeding and nutrition quality must aid with its role in increasing the profitability of rabbit production since feed is the major cost of the total production cost as mentioned by Cheeke (1987). Rabbit age of weaning, sanitary status and nature of carbohydrate reaching the cecum have a considerable effect on rabbit caecal microorganisms (Marounek *et al*., 2000). Alfalfa hay is a main source of fiber in rabbit diets which is offered at high concentrations without bad effects (Pote *et al*., 1980). Growing rabbit's resistance to digestive disturbance was improved to a high extent by a high fiber intake in a combination with higher caecal fermentative activity and lower caecal pH, this is supported by results of Gidenne and Licois (2005). Rabbit digestive problems are influenced mainly by insoluble natural detergent fiber (NDF) that plays a vital role in regulating the rate of passage and microbial activity (De-Blas *et al*., 1999; Garcia *et al*., 2002).

According to the commercial production, health problems related to intestinal pathology are principal cause for high mortality and low growth rate, definitely in fattening rabbits grown on condition of complete absence of antibiotics as growth promoter but focused on probiotics as alternatives to improve health and production in livestock (Maertens *et al*., 2006). The most beneficial effect of probiotics supplementation is to stimulate the digestive processes or to improve the microbial balance and enhance rabbit performance and health, in addition to the positive effect on average daily gain (ADG), feed conversion ratio, and a lower mortality (Amber *et al*., 2004; Falcao-e-Cunha *et al*., 2007). Although the mechanism underlying this improved performance and welfare remains partially unexplained. There is an evidence that probiotics act mainly by competing with enteric pathogens, balancing colonic microbiota, modulating the systemic and mucosal immune systems of the host, influencing the intestinal barrier, reduction of toxin production, stimulation of enzyme production by the host, production of some vitamins or antimicrobial substances, competition for adhesion to epithelial cells and increasing resistance to colonization and reduction of stress on rabbits (Falcao-e-Cunha *et al*., 2007; Fortun-Lamothe and Boullier, 2007; Sherman *et al*., 2008; Shehata and Tawfeek, 2010).

Yeast has the ability to travel through the digestive tract and could with stand the acidity on the stomach and digestive enzymes in the intestine up to reach the hind gut (caecum and colon) alive. Getachew (2016) and Oso *et al*. (2013) has revealed that the effective administration dosages of probiotics vary greatly and is dependent on the strains used and the clinical characteristics of subjects, such as lipid profiles. Although probiotics have been delivered in the range of 10^7 to 10^9 CFU/day in animals (Ha *et al*., 2006). Probiotic bacteria must be viable and available at high concentration, typically 10^6 CFU/gm of a probiotic. The probiotic strain in use should be resistant to stomach acidity, pancreatic secretion and bile (Czerucka *et al*., 2007)., Therefore, the objective of this study was to evaluate the effect of Biosaf® and Procreatín7® live yeast on digestive health of growing rabbits after weaning, growth performance and *in vitro* caecal fermentation.

7.3- MATERIALS AND METHODS

***In vivo* experiment**

The study was carried out at the Laboratory of Microbiology, Department of Animal Science and in the rabbitry of Chapingo Autonomous University (UACH), Texcoco, Mexico. One hundred twenty New Zealand White and California growing rabbits (35-38 days old) of 3 sequential weaning batches (40 rabbits of each weaning batch) were randomly allocated in 30 cages under similar housing and management conditions. Each fattening cage contained 4 rabbits (2 rabbits of each breed). Every cage had a central common feeder.

Two experimental diets were formulated to meet the nutrient requirements of fattening rabbits (Table 1). Each diet was divided into 5 equal portions after pelleting at the milling unit of (UACh) (Model of pelletizer Kt1 208): one portion without yeast, tow portions supplemented with yeast (0.85g Biosaf® SC47/kg diet and 2g Procreatin®7 kg^{-1} diet in 10 ml water) of basal flour (C1) equivalent 6.4 $\times 10^9$ CFU and tow portions supplemented with yeast 12.8 $\times$ 10^9 CFU/kg (1.7g Biosaf® SC47/kg diet and 4g Procreatin® 7 / kg diet in 10 ml water) of basal diets (C2). They were dosed at the same CFUs per gram of substrate, based on the viable yeast concentration determined in the laboratory by Elmasry *et al*. (2016). Yeast solutions were added to the pellets, by spraying after dissolving the solutions in tap water, feed was restricted to once daily with 100 g kg-1 rabbit weight (De Blas and Mateos, 2010), with free access to clean drinking water through stainless strel rabbit's nipple. Weighing the rabbits of each cage was performed at the beginning of the experiment (initial body weight), at the end of the study (final body weight) and the carcass was weighed hot and with its head. Total body weight gain, total feed intake and feed conversion ratio of each cage were determined every week of the 35th day trial for growth performance detection.Feces were collected for five days (on days 30 through 35 of each treatment) then samples of feces and feeds were dried at 70°C for 72 h, freeze-dried caecal contents, were all milled through a 1 mm screen.

Table 1. Ingredients and chemical composition of the experimental diets

Item and Ingredients (%)	Diets (D)	
	D1	D2
Alfalfa Hay	48.30	49.29
Barley grain	-	21.12
Corn grain	20.70	-
Soybean	13.00	12.24
Corn stover	6.00	1.03
Molasses	3.00	6.12
Soybean oil	7.00	8.16
Menirales and Vitamine*	2.00	2.04
Chemical composition on DM basis (%)		
Dry matter	86.74	84.28
Ash	9.53	10.09
Organic matter	94.37	95.08
Crude Fiber, CF	15.92	14.96
Crude protein, CP	18.00	18.00
DE kcal/kg DM	2514.13	2513.102
Neutral Detergent fiber (%)	32.31	32.05
Acid detergent fiber. (%)	22.78	20.63

* Per 32 kg mineral and vitamin premix contained: Calcium 17%, Phosphorus 3.5% and Sodium 5.5%.per 32 kg diet, Vitamin A and E -acetate, K3, D3, B1, B2, B6 and B12.1.3% per 32 kg.

DM and ash were measured as a triplicate of 1g samples of feeds, feces and caecal contents. DM was measured by oven drying at 104°C for 24 h, ash was determined by burning overnight at 550 °C.Neutral detergent fibre (NDF) and acid detergent fibre (ADF) were determined according to Van Soest and Wine (1967). The apparent nutrient digestibility coefficients of the diets were determined according to the classical formula (Perez *et al*., 1995): Apparent nutrient digestibility (%) = 100 × [(NI − NE) / (NI)]

Where NI represented the nutrient intake; and NE expressed the nutrient excreted.

***In vitro* experiment**

Rabbits were fasted with only free access to clean water and slaughtered following "Halal" method, according to Safwat *et al*. (2015), in the morning after recording pre-slaughter live weight. Rabbits were slaughtered, eviscerated and then the caecum was separated and the two extremities were tied by a plastic string to keep its anaerobic activity and prevent losses of digesta. Then the caecal content of each cage were subsequently mixed. Two samples were weighed, one for dry weight and the second for inoculum for *in vitro* indirect microorganisms activity. The caecal contents of each cage were handled separately (standard block) then mixed with mineral solution in glass flask saturated with CO_2 for about 20 minutes with rubber stopper and two glass tubes; one was adapted for the input of CO_2 injection and the second was to facilitate the inoculum diluted injection and then the solution was turned for 30 minutes until the precipitate the caecal contents and diluted by 1: 9 with reduced mineral solution (Menke and Steingass, 1988; Krishnamoorthy *et al*., 2005).

The kinetics of total gas production (TGP) was performed according to Menke and Steingass (1988) as an indirect method of microorganism activity and quantifying the gas as a product of their metabolic activity. Samples of 0.5g DM of substrate prepared by dietary fiber fraction, 50g dry matter basal diet was grinded. Prior to fermentation, it was defrosted in 5 L distilled water and boiled for an hour, then filtered and dried for 24h at 65°C in the drying oven, control and yeast at either C_1 or C_2 CFU/g DM, substrate and 90mL of inoculum were placed inside 125mL amber color bottle, then they were sealed with plastic stoppers and aluminum ring (Theodorou *et al*., 1994). Blank consist of only 90mL of the diluted inoculum, this was served to correct the values of fermentation. The samples were processed by six fold. The bottles were incubated in water bath at 39°C and the gas pressure was recorded by manometer (1 kg cm^{-2}) at 0, 2, 4, 6, 8, 12, 16, 20 and 24 h after incubation and were subsequently transformed to gas volume with the regression equation (V = p / 0.019) and the fractional gas volume

(mL g^{-1} DM) for the time intervals from 0 to 8 (Vf_{0-8}) and 8 to 24 (Vf_{8-24}) hours of incubation must be calculated (Vazquez-Mendoza *et al*., 2015). The kinetics of gas production (K): The first order slope of the polynomial in Microsoft Excel (y= 0.5089×2 + 25.49x) equation describes the kinetics of gas production.

Statistical analyses

A factorial experiment of completely randomize design was used in this study (two commercial probiotics, with three concentrations of each one and two different diets).

In vitro

The results were analyzed according to a generalized blocks design in which treatments were regarded as fixed effects, testing linear and quadratic effects for yeast level (Steel *et al*. 1997).

Rabbit experiment

The results were analyzed according to a complete random blocks design, factorial treatment arrangement (10 treatments resulting from 2 diets × 2 probiotics × 2 levels CFU + 2 controls without probiotics one of each grain), using the initial body weight as a covariate in which treatments were regarded as fixed effects, testing linear and quadratic effects for yeast level (Steel *et al*., 1997).

7.4- RESULTS

As shown in Table 2, average IBW of rabbits ranged between 865 and 915 g, without significant differences among the different experimental groups. Data indicated that there was a significant linear effect on FBW, TWG, CW (*P<0.01*), FCR and ADG (*P<0.05*) of rabbits fed on corn diets supplemented with Procreatin® 7 or Biosaf® SC47 and without any change on TFI compared to the control, while the effect was quadratic

(*P<0.05*) on TWG of rabbits fed on corn diet treated with Procreatin7®. Also, Biosaf® SC47 addition to corn diet has a significant quadratic effect (*P<0.01*) on CW.

On the same way barley diet treated with Procreatin® 7 produced a significant linear effect *(P<0.01)* on FBW, TWG, CW, FCR and ADG and quadratic effect (*P<0.05*) on TWG. In agreement, Biosaf® SC47 addition to barley has a linear significant effect (*P<0.01*) on FBW, TWG, CW and FCR.

Table 2. Growth performance of rabbits (n = 12 per treatment) fed experimental diets

	Probiotic	CFU ($x10^9$)	IBW (Kg)	Final BW (Kg)	TWG (Kg)	Carcass (Kg)	FCR	ADG (g)	Intake (g/animal/d)
Maiz	Control	0	0.82	1.818	0.983	1.08	3.62	31.62	113.39
	Procreatin 7	6.4	0.89	2.251	1.34	1.46	2.82	40.54	113.21
	Procreatin 7	12.8	0.91	2.257	1.34	1.47	2.99	38.18	114.10
P-value	L			0.0001	0.0001	0.0001	0.02	0.0401	0.43
	Q			0.0366	0.003	0.07	0.12	0.1022	0.82
Maiz	Biosaf SC47	6.4	0.88	2.074	1.19	1.297	3.07	37.75	113.67
	Biosaf SC47	12.8	0.89	2.193	1.29	1.41	3.10	36.68	113.76
P-value	L			0.0001	0.0001	0.0001	0.007	0.08	0.14
	Q			0.0304	0.02	0.0001	0.01	0.01	0.19
Barely	Control	0	0.87	1.835	0.94	1007	4.19	27.56	115.02
	Procreatin 7	6.4	0.90	2.049	1146	1.27	3.51	32.46	114.03
	Procreatin 7	12.8	0.87	2.195	1.32	1.41	3.06	37.32	114.14
P-value	L			0.0001	0.0001	0.0001	0.0001	0.0017	0.0592
	Q			0.2431	0.003	0.1372	0.31	0.6642	0.3845
Barely	BiosafS C47	6.4	0.86	2.035	1.17	1.272	3.57	32.89	114.20
	Biosaf SC47	12.8	0.87	2.162	1.28	1.378	3.14	36.36	114.09
	L			0.0001	0.0001	0.0001	0.0001	0.0007	0.0740
P-value	Q			0.27	0.44	0.35	0.54	0.99	0.18
SEM			0.008	0.02	0.02	0.02	0.08	0.82	0.12

IBW: Initial body weight; TWG: Total weight gain; ADG: Average daily weight gain and FCR: feed conversion ratio.

Table 3. *In vitro* gas production kinetics, digestibility and ammonia concentration during 24 h of different levels of two commercial Saccharomyces cerevisiae cultures (CFU/g DM) and two different fiber rations incubated with rabbit caecum content

	Probiotic	CFU	Vt,	V 0-8	V 8-24	k $ml g^{-1}$	OMD,	DMD,	$N\text{-}NH_3$,
		$x10^9$	$ml\ g^{-1}$	$ml\ g^{-1}$	$ml\ g^{-1}$	h^{-1}	%	%	mg/100 ml
	Control	0	231.1	152.5	78.6	22.3	26.7	23.6	6.7
Maiz	Procreatin7	6.4	155.7	90.4	65.3	12.4	40.3	33.6	7.7
	Procreatin7	12.8	122.8	61.2	61.7	8.4	42.6	31.0	6.5
P-value	L		0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0539
	Q		0.0001	0.0001	0.2827	0.0001	0.3827	0.8965	0.2376
Maiz	Biosaf SC47	6.4	155.8	102.1	53.8	16.0	36.6	26.6	4.9
	Biosaf SC47	12.8	163.8	91.7	72.1	13.2	41.8	44.0	9.0
	L		0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0010
P-value	Q		0.0047	0.0001	0.7451	0.0001	0.5398	0.4914	0.0953
	Control	0	241.8	146.3	95.6	21.3	29.9	25.2	8.6
Barely	Procreatin7	6.4	183.3	110.0	73.3	15.5	38.3	32.4	5.2
	Procreatin7	12.8	155.3	100.9	54.4	14.9	45.1	36.8	4.9
P-value	L		0.0001	0.0001	0.2997	0.0001	0.0001	0.0001	0.0363
	Q		0.0001	0.0001	0.0002	0.1165	0.0320	0.0006	0.0020
Barely	Biosaf SC47	6.4	158.3	89.6	68.8	13.5	34.2	30.4	6.4
	Biosaf SC47	12.8	164.1	110.6	53.5	16.0	40.7	36.1	6.5
P-value	L		0.0001	0.0001	0.0084	0.0001	0.0001	0.0017	0.8065
	Q		0.0001	0.0013	0.3733	0.0092	0.0001	0.0024	0.2106
SEM			4.679	3.534	2.064	0.564	0.776	0.889	0.278

Vmax: maximum volume of gas production, V_{0-8} is volume 0 to 8 hours of gas production, V_{8-24} is volume 8 to 24 hours of gas production; m: Gas production rate, DMD: *in vitro* dry matter digestibility; OMD: is *in vitro* organic matter digestibility; C: Without probiotics (control); C1: $6.4x10^9$CFU /kg feed; C2: $1.28x10^9$ CFU /kg feed; x: Interaction between Grains and Yeast.

Data presented in Table 3, indicate that corn diet supplemented with Procreatin®7 or Biosaf® SC47 has a linear reduction (*P<0.01*) in total, rapid and medium fractions fermentation and a quadratic (*P<0.01*) in total and rapid fractions fermentation only. Also there is a linear and quadratic significant effects on gas production rate and only a linear effect on both organic and dry matter digestibility with a linear significant effect (*P<0.01*) of only Biosaf® SC47 on ammonia production. Concerning barley diet, the addition of Procreatin®7 or Biosaf® SC47 has a linear and quadratic reduction (*P<0.01* and *P<0.05*) in total and rapid fractions fermentation and a quadratic reduction (*P<0.05*) in medium fractions fermentation with Procreatin®7. On the other hand using Biosaf® SC47 as a feed additive has a linear and quadratic significant effects (*P<0.01* and *P<0.05*) on organic and dry matter digestibility with only a linear significant effect (*P<0.01*) on gas production rate. While Procreatin®7 supplementation has a linear significant effect (*P<0.01*) on gas production rate and organic matter digestibility, although this effect is linear and quadratic (*P<0.01* and *P<0.05*) on dry matter digestibility, in addition to quadratic and linear significant effects (*P<0.05*) on ammonia production were recorded.

As illustrated in Table 4, Procreatin®7 or Biosaf® SC47 supplementation to corn diet show a linear significant effect (*P<0.01*) on dry matter digestibility, neutral detergent fiber and acid detergent fiber. Additionally, a linear significant effect (*P<0.05*) on organic matter digestibility when rabbits were fed on corn diet supplemented with Biosaf® SC47 only. On the other hand, barley diet treated with Procreatin®7 has linear and quadratic significant effects (*P<0.01*) on both dry matter and acid detergent fiber digestibility while the effect is only linear (*P<0.05*) on organic matter digestibility and neutral detergent fiber. In case of using Biosaf® SC47 as a probiotic, there is a linear effect (*P<0.01*) on dry and organic matter digestibility, while, the effect on acid detergent fiber is linear and quadratic (*P<0.01* and *P<0.05*), respectively.

Table 4. Effects of two different rations as affected by different levels of two commercial *Saccharomyces cerevisiae* cultures (CFU/kg ration) on coefficient of total tract apparent nutrients digestibility (CTTAD) at 10 weeks of rabbit age.

	Probiotic	CFU ($x10^9$)	OMD%	DMD%	NDF%	ADF%
Maiz	Control	0	58.78	60.64	24.15	22.53
	Procreatin7	6.4	64.79	66.83	30.77	25.99
	Procreatin7	12.8	69.95	72.15	35.09	34.35
P-value	L		0.0529	0.0001	0.0001	0.0001
	Q		0.4853	0.5102	0.1694	0.8417
Maiz	Biosaf SC47	6.4	64.37	65.85	29.02	25.51
	Biosaf SC47	12.8	65.77	69.50	33.88	29.32
P-value	L		0.0037	0.0001	0.0001	0.0001
	Q		0.8861	0.7124	0.2112	0.7201
Barely	Control	0	53.03	59.79	23.67	22.00
	Procreatin7	6.4	63.12	66.68	33.07	30.07
	Procreatin7	12.8	67.31	72.46	41.69	35.08
P-value	L		0.0005	0.0001	0.0126	0.0001
	Q		0.7794	0.0001	0.4608	0.0001
Barely	Biosaf SC47	6.4	60.92	71.15	34.62	28.59
	Biosaf SC47	12.8	67.13	68.54	36.65	32.59
P-value	L		0.0004	0.0001	0.0001	0.0001
	Q		0.3287	0.6405	0.1372	0.0377
SEM			1.068	0.815	0.514	0.228

L: Linear; Q: quadratic; FBW: Final body weight; TWG: Total weight gain; CW: Carcass weight; TFI: Total feed intake; TFCR: Total feed conversion ratio.

7.5- DISCUSSION

Rabbit growth

According to the results of this study, BWG, CW and FCR of rabbits were significantly affected by dietary supplementation of *S. cerevisiae* (*P<0.05*). These results are in agreement with Timmerman *et al*. (2005) and Kritas *et al*. (2008) and Banday and Risam (2002) who found that probiotics supplementation improve growth performance characteristics. Combes *et al*. (2012) and Bhatt *et al*. (2017) reviewed results in most of the experiments that probiotics enhance ADG and FCR with a decrease in mortality rate in rabbits. Others recorded higher (*P<0.05*) WG of rabbits fed on Bio-active yeast (probiotic) at 0.12 g yeast *(Saccharomyces cerevisiae*) /kg of diet than rabbits supplemented with either 0.08 or 0.16 or zero gram yeast/kg diet (Ezema and Eze, 2012). Also the results obtained by Paryad and Mahmoudi (2008) and Shareef and Dabbagh (2009) revealed that the addition of 1.5% S*. cerevisiae* yeast to the broilers diet affected positively their BWG, FI and FCR. Zhang *et al*. (2005) have recorded the same previous results.

Others reported significant effects (*P<0.05*) on the live weight of rabbits fed diets supplemented with yeast or probiotics, by 16.7-18.0% when control ration was supplemented with 1.5 and 2.0 kg probiotic/ton, respectively (Matusevicius *et al*., 2004). Kimsé *et al*. (2012) stated that growing rabbits (35 to 70 days old) supplemented with 10^6 CFU/g of *S. cerevisiae* NCYC® Sc 47 strain (Activesafs) significantly decreased the mortality rate during the fattening period, compared to the control group. The positive effect of probiotics on animals can result either from a direct nutritional effect of the probiotic, or acting as bio-regulators of the intestinal micro-flora and enhancing the host's natural defenses (Guillot, 2003). Giang *et al*. (2010) approved in their researches that a mixture of lactic acid bacteria complex and *Saccharomyces boulardii* improved overall live performance.

Impact of yeast supplementation on nutrients digestion

Kimsé *et al*. (2012) reported an increase in the survival rate of yeast in digestive tract from 90 to 97% with live yeast *Saccharomyces Cerevisiae* NCYC Sc 47 (Biosaf®) supplementation, (10 g Biosaf®/kg feed, corresponding to 107 CFU/g of DM in diet). Kimsé *et al*. (2008) reported that addition of a high level of Biosaf® (10%) to growing rabbit's diet has enhanced their digestive health with a significant reduction in mortality rate even with a limited number of rabbit this result must be confirmed using large number of rabbits. The desirable effects of yeast found here, could be related to the higher level of yeast found in the caecum of supplemented animals. In contrast to rumen, the caecum is more strictly anaerobic compartment; this difference probably supports a differential role of yeast in the digestive ecosystem.

As a result Seyidoglu and Peker (2015) reported a positive effect on digestive health due to administration of *S. cerevisiae* in either low or high doses. Additionally, Lascano *et al*. (2009), Lascano *et al*. (2012) and Ghazanfar *et al*. (2015) suggested significant effects ($P<0.05$) on dry matter (DM), organic matter (OM), crude protein (CP), NDF and ADF digestibility. Our data support the findings of Lascano *et al*. (2012) and Ghazanfar *et al*. (2015) who showed that NDF and ADF digestibility was improved in yeast supplemented groups. Chaucheyras-Durand *et al*. (2012) revealed the *in vitro* potential of probiotic yeasts to enhance growth and activity of fiber-degrading rumen microorganisms. Gobesso *et al*. (2012) showed that the addition of live yeast to animal ration is able to alter the digestion of dietary components.

Jouany *et al*. (2007) revealed that live yeast administration has also improved the digestibility of dry matter, in addition to a considerable positive effect on ADF. Beside the positive effect of *S. cerevisiae* on fungal zoospore germination and cellulose degradation as mentioned by Chaucheyras-Durand *et al*. (1995) who confirmed recently that yeasts could enhance fungal colonization of plant cell walls (Chaucheyras-Durand *et al*., 2010). Moreover, Marden (2007) and Kimsé *et al*. (2008) suggested a relationship between yeast (Biosaf®) supplementation and ruminal redox

potential (E_h) and pH of dairy cow by reducing rumenal E_h and increasing the pH, unlike our study where rabbit caecal E_h increased with yeast supplementation and the pH remained unchanged.

The improvement of nutrient digestibility might be related to increase cellulose degrading microbial biomass population inside the rumen in addition to the stable rumen pH and removal of oxygen from the rumen in the yeast supplemented animals (Ghazanfar *et al*., 2015). That stable rumen pH provides a suitable environment for rumenal microbes growth, as fungi and bacteria degrading cellulose. Consequently, these microbial species helped in better fiber digestion. The stable pH also enhanced microbial protein synthesis in the rumen. While, others reported no effect of yeast supplementation on the nutrient digestibility (Tripathi and Karim, 2010). These variations in nutrient digestibility may be related to the quality and nature of diet fed to animals as described by Desnoyers *et al*. (2009).

***In vitro* gas production**

The rabbit's digestive tract is adapted to process large amounts of fiber rich feed. Microbial fermentation of the food takes place in the caecum to ensure nutrient supply (Harcourt-Brown, 2004; Bagóné-Vántus *et al*., 2014). The products of fermentation are important for rabbit because the VFA and NH_3 are a source of energy for the host. VFA production can cover 30% to 50% of maintenance energy requirements of adult rabbits (Gidenne and Perez, 1994; Gidenne *et al*., 2008b). The concentration of VFA in the caecum of an adult rabbit is around 75% acetate, 15% butyrate and 10% propionate. However, these proportions change depending on the age of the animal, the level of intake (Bellier *et al*., 1995) and feed quality, including rapidly fermentable fibre concentration (Gidenne *et al*., 2004; Gidenne *et al.,* 2008b). Unlike most herbivores, in rabbits, the ratio of propionate: butyrate is less than 1 because of the characteristics of the microbiota (Adjiri *et al*., 1992). Gong *et al*. (2013) observed a significant decrease in *in vitro* total gas production in the yeast supplemented group which is in agreement with our results, while other researchers reported an increase in TGP (Lila *et al*., 2004; Lila

et al., 2006). This effect may be associated with the lower production of acetate in the yeast treatments, because CO_2 and H_2 are by-products of acetate production during carbohydrate fermentation (Gong *et al*., 2013) and forage proportion in the diet (Marounek *et al*., 2000b), probiotics can influence the microbial fermentation pattern in the caecum (Kermauner and Struklec, 1999).

7.6- CONCLUSION

The results of this study showed that administration of the probiotic *Saccharomyces Cerevisiae* (Biosaf® Sc47 and Procreatin®7) 6.4 $\times 10^9$ CFU and (C2) 12.8 $\times 10^9$ CFU/kg to the basal diets of fattening rabbits starting from 35-38 days post weaning up to slaughter age reduces *in vitro* caecal gas production and improves *in vitro* digestibility coefficient of total tract apparent nutrients digestibility and growth performance characteristics (ADG and FCR).

7.7- REFERENCES

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8- CHAPTER V

GROWTH PERFORMANCE OF RABBITS AND CAECAL FERMENTATION AS AFFECTED BY DIETARY GRAIN WITH DIFFERENT TYPES AND DOSES OF YEAST

A.M.A. Elmasry[1,2], L.A. Miranda[3], G. Vázquez S.1[1], P.A. Hernández[4], G.D. Mendoza[1]

[1]. Doctorado en Ciencias Agropecuarias, Universidad Autónoma Metropolitana, Unidad Xochimilco, 04960 México, D.F., México.

[2]. Botany Department, Faculty of Agriculture, Menoufia University, Po 32511, Shebin El-Kom, Egypt.

[3]. Universidad Autónoma Chapingo, Departamento de Zootecnia, 56230, México.

[4]. Centro Universitario UAEM-Amecameca, Universidad Autónoma del Estado de México, 56900, México.

8.1- ABSTRACT

An experiment of 56 days duration was carried out to evaluate the effect of supplemental live yeast culture on growth and nutrient digestibility in rabbit and *in vitro* caecum fermentation using the *in vitro* gas production technique. One hundred and twenty New Zealand and California rabbits were used in cages (4 rabbits per cage) with an initial BW of 0.869 ±0.024 kg and seven weeks old which were allotted to dietary treatments which consisted in: corn or barley grain, and two yeast types (Procreatin®7 and Biosaf®SC47) at three doses 0.0, 6.4 or 12.8 $\times 10^9$ CFU per kg in a basal diet of alfalfa. As yeast dose was increased, both yeasts improved (linear $P<0.01$) final BW, carcass weight, average daily gain (ADG) and feed conversion ratio (FCR) without affect intake with both grains. Digestibilities of nutrients (DM, OM, NDD and ADF) were improved with both yeast products ($P<0.05$) and *in vitro* DM digestibility showed the

same results with both grains. Volume of gas produced and rate of gas production were reduced with both yeasts (linear effect *P<0.01*). Results indicate that live yeast can improve rabbit performance by improving nutrient digestibility regardless of the grain used in an alfalfa based diet.

Key words: Yeast, Rabbit, Digestibility, Growth performance *in vitro* caecal fermentation.

8.2- INTRODUCTION

Rabbits have a complicated and sensitive digestive system due to their caecal microbial fermentation; therefore, it is important to include high levels of fibrous feed in their diet, which have low energy value. Fiber is fundamental to the digestive process, overall gut health and mobility, as well as, caecotrophy and appetite stimulation. Rabbits have an ability to digest fiber to some extent with the help of the bacterial populations in the hind gut. Fibrous constituents can only be digested in rabbits' caecum through microbial fermentation that considers the main fermentative area (Bjornhag, 1972; Gidenne, 1993).

Caecal retention time is limited to around 10 hours as a result of the daily production of soft faces from the caecal contents (Gidenne *et al*., 1998). Because of this short caecal fermentation time, caecal cellulolytic activity is low, however, the microflora resident in the caecum has the ability to degrade significant amount of the more soluble non-starch polysaccharides (pectins, pentosans, β-glucans, and oligosaccharides) as mentioned by Marounek *et al*. (1995).

When rabbits are fed an adequate amount of indigestible fiber or too high in carbohydrates, the gastrointestinal tract cannot function properly that leads to high incidence of enteric diseases and especially after weaning causing high losses because of their fragile gut balance; in this situation, probiotics may contribute to enhance their health status as shown results from Trocino *et al*. (2005), Kritas *et al*. (2008) and Gnikpo *et al*. (2016), which suggest that yeast can improve caecal fermentation.

Antibiotics were used to get rid of the rabbit's digestive disturbance but due to the ban on the use of antibiotics as growth promoters (EPC, 2005), probiotics as dietary supplements could serve as a possible suitable solution.

Probiotics are a live microbial feed supplement which beneficially affects the animal by improving its intestinal balance as described by Fuller (1989), Patterson and Burkholder (2003). In contrast with antibiotics, the objective of probiotics is not to destroy pathogenically bacteria but to prevent their development and colonization in order to secure optimum utility of feed (Maertens *et al*., 1994), as well as, their competitive growth against harmful microorganisms, reducing the intestinal pH by producing lactic acid and encouraging digestion by producing enzymes and vitamins these actions strengthen the animal's own non-specific immune defense as recorded by Mountzouris *et al*. (2007), Zhang and Kim (2014) and Vanderpool *et al*. (2008). Falaco *et al*. (2007), Kritas *et al*. (2008), Combes *et al*. (2012) and Bhatt *et al*. (2017) reviewed results in most of the experiments that probiotics reporting a positive effect on average daily gain (ADG), feed conversion ratio and mortality in rabbits.

Probiotics can influence the microbial fermentation pattern in the caecum (Kermauner and Struklec, 1999). Rodrigues *et al*. (2000) found that the yeast *Saccharomyces cerevisiae* (SC) reestablish the normal gut function due to its protective effects against enteric pathogens. The *SC* is rich in enzymes, vitamins, nutrients and cofactors and is highly resistant to inactivation along the human alimentary tract and may colonize the gut for restoring the gastrointestinal microbiology, also it produce a variety of beneficial production responses in animals (Bleichner *et al*., 1997). The yeast cell wall components, especially mannan oligosaccharides, are capable of adsorbing enteropathogens and improving the health status of growing animals.

Zhang *et al*. (2005) reported that administration of *SC* in broiler chicks resulted in increasing villus height of ileum, while the crypt depth was not changed. In addition, the longer villus has been reported by some researchers in turkeys (Sims *et al*., 2004) and in rabbits (Mourao *et al*., 2006). Yeast also has a positive effect on blood hematology

resulting in an improvement in health status of animals as reported by Agazzi *et al*. (2014) and Ghazanfar *et al*. (2015). The addition of yeast culture has many positive effects on the hemicelluloses degradability and some important nutrient digestibility (Lascano *et al*., 2012 and Lesmeister *et al*., 2004) and enhances the absorption of some minerals (Cole *et al*., 1992).

Abu *et al*. (1996), Galvao *et al*. (2005) and Timmernan *et al*. (2005) suggested that microbial feed additives supplementation as a tool to maintain the microbial balance of intestine, prevents diarrhea and improved fecal bacterial flora of ruminants (Kawakami, 2010).

Gobesso *et al*. (2012) showed that the inclusion level of yeast is able to alter the digestion of dietary components. There is a significant improvement in the performance of growing rabbits housed under less favorable conditions, when fed diets supplemented with Biosaf® (a live yeast of *Saccharomyces cerevisae*) at the rate of 0.15 or 1.0% to provide 7.5×10^6 and 5×10^7 CFU/g, respectively, while the response to such additive was of no value under optimal housing conditions as reported by Maertens and De Groote (1992). Rotoro *et al*. (2014) explained that the caecal population of yeast increased in the rabbits fed *Saccharomyces cerevisiae boulardii* (CNCM I- 1079 strain, LSB) supplemented diets, the supplementation at a dose of up to 600 mg/kg did not affect the productive performance and caecal fermentation of broiler rabbits reared in standard farming conditions, additionally, no significant differences were found for nutrients digestibility except for NDF and ADF values where they were lowest in animals fed 300 mg /kg supplemented diets.

Protected live yeast (*S. cerevisiae boulardii*, CNCMI- 1079 strain) was resistant to passage through the rabbit digestive tract as far as the caecum where it showed a 86% survival rate in the 600 mg/ kg supplemented diet as reviewed in the results of Rotolo *et al*. (2014). Also, Onifade *et al*. (1999) found that the concentration of *Saccharomyces cerevisiae* has linearly effect on growth stimulating. Maertens and De Groote (1992) supposed that *SC* is able to transit under a viable form all along the

digestive tract carrying the *E. coli* fixed on their membrane. Kim *et al*. (1991) revealed that live yeast administration has also improved the digestibility of dry matter, in addition to a considerable positive effect on ADF (Jouany *et al*., 2007).

In most of the studies previously described, the colony-forming units (CFUs) were not corroborated, therefore, the objective of this study was to evaluate the effects of two commercial yeast products on rabbit growth performance and nutrient digestibility and on *in vitro* caecal fermentation kinetic parameters, of alfalfa -based diets with two grains, dosed at the same CFU levels of *Saccharomyces cerevisiae*.

8.3- MATERIAL AND METHODS

An experiment of completely randomize design was conducted using one hundred twenty, 35 to 38 days old with an initial 0.869 ± 0.024 g, New Zealand White and California healthy males weaned rabbits of three weaning batches consecutively in the Experimental Rabbit Farm of Chapingo Autonomous University, Mexico (UACH), over a period of 35 days (5 Weeks). The trial was divided into 3 equal blocks and 40 rabbits per each block. All the rabbits were randomly housed in thirty wire net cages, (4 rabbits per cage (2 rabbits of each breed), equipped with an individual central feeder and nipple drinkers, under the same hygienic, environmental and housing conditions all over the experimental period.

Dietary treatments consisted of experimental basal diets of two grains (Table 1) supplemented with two commercial probiotics (Procreatin®7 and Biosaf®SC47) at three doses 0.0, 6.4 or 12.8 ×109 CFU per kg. Diets were pelleted (Pelletizer Kt1208). After adaptation, the forty animals of each block were randomly divided into three groups. The differences among the groups were not significant. The first group served as control (C0) which was maintained on basal diets (Table 1) without yeast. The second group was fed the diets supplemented with yeast Biosaf: *Saccharomyces cerevisiae* NCYC® Sc47) (C1) 6.4 $\times 10^9$ CFU and (C2)12.8 $\times 10^9$ CFU/kg basal diets. While the diets of the third group were supplemented with yeast Procreatin®7: *Saccharomyces cerevisiae*)

(C1) 6.4 ×10^9 CFU/ kg (0.85g Biosaf® SC47/kg diet and 2g Procreatin®7 / kg diet in 10 ml drinking water) and (C2) 12.8 × 10^9 CFU/kg (1.7g Biosaf® SC47/kg diet and 4g Procreatin®7 / kg diet in 10 ml drinking water) of basal diets.

Table 1. Composition of the basal diet fed to weanling rabbits

Ingredients (g kg^{-1} diet)	Experimental diets	
	Corn	Barley
Oat Hay	483	483
Barley grain	-	207
Corn grain	207	-
Soybean	250	240
Molasses	10	10
Soybean oil	30	40
Minerals and Vitamins*	20	20
Total	1000	1000
Chemical composition on DM basis (%)		
Dry matter (DM)***	87.54	88.14
Ash	9.05	8.45
Organic matter (OM)	96.18	96.01
Crude fiber (CF)	16.94	17.44
Crude protein (CP)	18.08	17.96
DE kcal/kg DM	2504.73	2546.44
Neutral Detergent fiber (%)	35.47	35.20
Acid detergent fiber. (%)	16.30	16.46

* Per 32 kg mineral and vitamin premix contained: Calcium 17%, Phosphorus 3.5% and Sodium 5.5%.per 32 kg diet, Vitamin A and E -acetate, K3, D3, B1, B2, B6 and B12.1.3% per 32 kg.

Yeast solution was sprayed on pellets immediately before feeding every morning. Yeast could not be added to the feed before pelleting because the viability of yeast cell gets reduced when exposed to high temperature. They were dosed at the same CFUs per gram of substrate, based on the viable yeast concentration determined in the laboratory (Elmasry *et al*., 2016)

Rabbits were weighed individually at the beginning and the end of the trial. Individual body weight and cage feed consumption were recorded weekly in order to calculate the daily weight gain and feed consumption. The number of dead animals was also recorded. The carcass was weighed hot and with its head.

At the last five days of each experimental period feed consumption was accurately determined and feces were collected. Samples of feces, diets and freeze-dried caecal contents were dried at 70 ˚C for 48 h then they were milled through a 1 mm screen. DM and ash were measured as triplicate samples using 1 g of feed, feces and caecal content. Ash was determined by burning at 550 ˚C overnight while, DM by oven drying at 104 ˚C for 24 h. Neutral detergent fiber (NDF) and acid detergent fiber were measured according to Van Soest and Wine (1967).

The method used for *in vitro* fermentation and gas production measurements was described by Theodorou *et al*. (1994). This method allows measurements of cumulative gas production by the use of pressure transducer under completely anaerobic conditions. About 0.5 g DM substrate (dietary fiber fraction) and yeast either C1 or C2 CFU/g DM were placed into 125 amber color bottles and sealed with rubber stopper till the inoculation. Freshly collected caecal samples were used to prepare the inoculum. At the end of each experimental block (35^{th} day), from the night before slaughter, the rabbits were fasted with free water supply then they were slaughtered in the morning “Halal” method, according to Safwat *et al*. (2015) after recording the final individual body weight at the slaughter house of (UACH). Once the gastrointestinal tract

was isolated, the caecum was cut out and tied at the two extremities with a plastic string to keep the caecum anaerobic condition and prevent losses of caecal contents.

At the Microbiology laboratory of the University the caecal contents of each cage were mixed well, then two samples of the mixture were weighted for dry weight and the inoculum preparation. Inoculum was prepared using the technique proposed by Menk and Steingass (1988) and Krishnamoorthy *et al*. (2005). The sample of the mixture was mixed with mineral solution in a glasses flasks saturated with CO_2 for 20 minutes then sealed with rubber stopper containing two glasses tubes one for CO_2 injection and the other for the injection of inoculums, after that all the flasks were turned up down for 30 minutes till the perception occurs and formation of the inoculum with the dilution of 10%. The samples were processed by six folds which contained either yeast and substrate or substrate only under a stream of CO_2, 90 ml of the inoculum were added to each bottle, which contained yeast and DM samples then resealed with plastic stopper and aluminum ring (Theodorou *et al*., 1994).

The bottles of controls were injected only with the inoculum without any additives and were used to correct the values of fermentation and to compensate for gas production in the absence of substrate. All the bottles were incubated in water bath at 39 ºC Gas pressure produced from fermentation was measured manually with Manometer (1 Kg cm^{-2}) at regular time interval at 0, 2, 4, 6, 8, 12, 16, 20, and 24 h post incubation then transformed subsequently to gas volume with the following regression equation (V=p/ 0.019). The fractional gas volume (mL g^{-1} DM) for the time intervals from 0 to 8 (Vf_{0-8}) and 8 to 24 (Vf_{8-24}) hours of incubation must be calculated (Vazquez-Mendoza *et al*., 2015). The kinetics of gas production (K): The first order slope of the polynomial in Microsoft Excel (y= 0.5089×2 + 25.49×) equation describes the kinetics of gas production.

Statistical analyses

In vitro

The results were analyzed according to a generalized blocks design in which treatments were regarded as fixed effects, testing linear and quadratic effects for yeast level (Steel *et al*. 1997).

In vivo

The results were analyzed according to a complete random blocks design Factorial treatment arrangement (two grain * two yeast * five concentration), use the initial body weight as a covariate in which treatments were regarded as fixed effects, testing linear and quadratic effects for yeast level (Steel *et al*. 1997).

8.4- RESULTS

Rabbit performance evaluated in final BW, ADG and carcass weight (Table 2) was improved (linear effect *P<0.01*) with both grains using Procreatin® 7 without affecting feed intake. Nutrient digestibility was improved (linear *effect P<0.01) as both* yeast were added in the rations with both grains (Table 3).

For the *in vitro* caecal fermentation, there was a linear reduction gas production by Procreatin®7and corn and Biosaf®SC47 expressed as total, rapid or medium fractional volumes fermentation (*P* <0.01) with both grains (Table 4). There was a linear increase in *in vitro* organic and total dry matter digestion with the two types of yeast and grains diets (*P <0.01*). Caecal ammonia concentration was not affected by grain or yeast type or dose.

Table 2. Effects of different rations with different levels of two commercial *Saccharomyces cerevi siae* cultures (CFU/g DM) on total feed conversion, feed intake, total weight gain, final body weight, and ch nnel weight of rabbits from weaning till slaughtering.

	Probiotic	CFU ($x10^9$)	Initial BW kg	Final BW kg	Carcass kg	FCR	ADG g	Intake g/animal/d
Corn	Control	0	0.887	1.953	1.195	3.79	30.00	113.76
	Procreatin 7	6.4	0.924	1.983	1.232	3.48	32.77	113.89
	Procreatin 7	12.8	0.897	2.174	1.391	3.07	37.25	114.52
P-value	L			0.0001	0.0001	0.0001	0.0001	0.3824
	Q			0.0001	0.0001	0.0001	0.0001	0.5783
Corn	Biosaf SC47	6.4	0.858	2.234	1.451	3.04	37.59	114.35
	Biosaf SC47	12.8	0.897	2.197	1.405	3.08	37.13	114.32
P-value	L			0.0001	0.0001	0.0001	0.0001	0.2369
	Q			0.0015	0.0257	0.2551	0.0303	0.6483
Barely	Control	0	0.866	1.939	1.175	3.75	30.20	113.40
	Procreatin 7	6.4	0.862	2.046	1.275	3.34	34.290	114.57
	Procreatin 7	12.8	0.874	2.133	1.363	3.15	36.37	114.78
P-value	L			0.0001	0.0001	0.0001	0.0001	0.0358
	Q			0.0001	0.0001	0.0001	0.0001	0.7584
Barely	Biosaf SC47	6.4	0.842	2.151	1.376	3.14	36.44	114.27
	Biosaf SC47	12.8	0.829	2.093	1.329	3.20	35.90	114.81
	L			0.0001	0.0001	0.0001	0.0001	0.0386
P-value	Q			0.6322	0.8058	0.0064	0.0130	0.4009
SEM				0.766	0.017	0.050	0.271	0.583

IBW: Initial body weight; TWG: Total weight gain; ADG: Average daily weight gain and FCR: eed conversion ratio.

Table 3. Effects of two different rations treated with different levels of two commercial *Saccharomyces cerevisiae* cultures (mg/g DM) on digestibility of nutrients at 11 week age.

	Probiotic	CFU (x109)	DMD%	OMD%	NDF%	ADF%
Corn	Control	0	59.62	58.65	21.64	21.82
	Procreatin7	6.4	66.52	66.55	26.36	28.08
	Procreatin7	12.8	71.42	71.90	32.08	31.62
P-value	L		0.0001	0.0004	0.0001	0.0001
	Q		0.2959	0.3875	0.1172	0.0001
Corn	Biosaf SC47	6.4	65.69	65.58	2477	25.15
	Biosaf SC47	12.8	69.01	68.83	27.17	30.00
P-value	L		0.0001	0.0001	0.0001	0.0001
	Q		0.4451	0.5495	0.0311	0.5241
Barely	Control	0	59.95	54.46	19.74	20.73
	Procreatin7	6.4	65.54	63.49	27.44	28.54
	Procreatin7	12.8	71.22	69.39	37.57	28.78
P-value	L		0.0001	0.0019	0.0001	0.0001
	Q		0.8433	0.1854	0.5852	0.0001
Barely	Biosaf SC47	6.4	64.18	61.62	27.55	26.32
	Biosaf SC47	12.8	68.91	6307	36.77	28.52
P-value	L		0.0001	0.0001	0.0001	0.0001
	Q		0.9702	0.4604	0.6841	0.0054
SEM			0.781	1.029	0.430	0.275

OMD: Organic matter digestibility; DMD: Dry matter digestibility; NDF: Neutral detergent fiber; ADF: Acid detergent fiber.

Table 4. *In vitro* caecal gas production, digestibility and ammonia concentration during 24 h of caecal contents incubation of two different fiber rations as affected by different levels of two commercial *Saccharomyces cerevisiae* cultures (CFU/g DM).

	Probiotic	CFU	Vt,	V 0-8	V 8-24	k $ml g^{-1}$	OMD,	DMD, N-N	H_3,
		$x10^9$	$ml g^{-1}$	$ml g^{-1}$	$ml g^{-1}$	h^{-1}	%	%	mg/100 ml
	Control	0	232.27	144.08	88.18	19.88	22.69	27.43	6.43
Corn	Procreatin7	6.4	162.86	93.42	69.44	13.44	39.71	41.92	4.95
	Procreatin7	12.8	133.64	69.72	63.92	12.00	44.48	49.28	7.16
P-value	L		0.0001	0.0017	0.0048	0.0018	0.0001	0.0001	0.1943
	Q		0.5329	0.7127	0.7140	0.9361	0.0001	0.0001	0.6629
	Biosaf SC47	6.4	204.54	125.55	78.99	17.86	41.28	44.40	6.72
Corn	Biosaf SC47	12.8	186.79	113.01	73.78	16.02	39.31	40.20	7.79
P-value	L		0.0001	0.0001	0.0003	0.0001	0.0001	0.0001	0.4833
	Q		0.0146	0.1022	0.2260	0.0172	0.0714	0.0285	0.0438
	Control	0	189.47	133.61	55.86	18.88	24.43	26.50	7.35
Barely	Procreatin7	6.4	112.44	53.87	58.58	6.99	39.68	40.89	5.55
	Procreatin7	12.8	119.6	49.21	70.39	7.63	43.49	46.39	5.63
P-value	L		0.0022	0.0001	0.1545	0.0001	0.0001	0.0001	0.1362
	Q		0.0003	0.0178	0.0495	0.0014	0.8854	0.6445	0.2047
Barely	Biosaf SC47	6.4	143.99	94.48	49.50	12.78	30.51	32.35	5.42
	Biosaf SC47	12.8	159.90	95.04	64.86	13.52	38.90	38.75	5.78
P-value	L		0.0001	0.0001	0.0237	0.0001	0. 0001	0.0001	0.1016
	Q		0.0001	0.0001	0.4028	0.0001	0.0259	0.0258	0.2984
SEM			5.132	4.458	1.904	0.593	1.059	1.139	0.243

Vt : total Volume of gas production, V_{0-8} is volume 0 to 8 hours of gas production, V_{8-24} is volume 8 to 24 hours of gas production; K: the kinetics of gas production, DMD: *in vitro* dry matter digestibility and OMD: is *in vitro* organic matter digestibility.

8.5- DISCUSSION

Rabbit growth

Results confirm that inclusion of viable *Saccharomyces cerevisiae* yeast in diets of rabbits improves performance associated to nutrient digestibility as observed in other strains such as *S. cerevisiae boulardii* (Kritas *et al*., 2008; Bhatt *et al*., 2017). Abdel-Azeem *et al*. (2004a) observed that New Zealand White rabbit fed on high starch diet plus 0.20 or 0.30 percent of *Saccharomyces cerevisiae* (Yea-Sacc with 7.6 to 9.83 log CFU/g of product) increased in final BW and ADG than those rabbits without probiotic supplementation. Rabbits supplemented antibiotic and *Saccharomyces cerevisiae* increased dressing and carcass weight (Abdel-Azeem *et al*. 2004b). The beneficial effects has been explained by an increase in the diversity of bacteria in the digestive flora and the production of organic acids (acetic, propionic, butyric acids) which may reduce the production of hydrogen peroxide and diacetyl which are antibacterial substances (Salminen, 1999; Krehbiel *et al*., 2003; Grajek *et al*., 2005).

Falcão-e-Cunha *et al*. (2007) summarized that dietary inclusion of feed additives containing yeast generally improve ADG in rabbits, but concerning FCR and mortality were partially contradictory which can be explained by some metabolites or sanitary conditions. Maertens (1992) evaluated Biosaf® at 0.15% (Biosaf® granules *Saccharomyces cerevisiae* concentration 5×10^{9} g^{-1}) and improved final BW only in normal conditions, but had no effect under strict sanitary conditions. Campos *et al*. (2014) reported higher mortality in volcano rabbit supplemented with yeast. Renouf (2006) pointed out that certain microbial metabolisms may produce biogenic amines by decarboxylation of some amino acids; the volatile phenols and biogenic amines can affect negatively feed conversion (Gnikpo *et al*., 2016).

Most of the studies agree that appropriate yeast and effective microorganism cultures significantly increase body weight gain, feed intake, nutrient digestibility, and carcass weight and improve feed efficiency in rabbits (Shanmuganathan *et al*., 2004).

Giang *et al*. (2010) showed that a mixture of lactic acid bacteria complex and *Saccharomyces boulardii* improved overall live performance in agreement with the data of Maertens and De Groote (1992) using the same yeast. The improvement of digestive health is associated to the high level of yeast found in the caecum of supplemented animals and to changes in caecal physico-chemical characteristics (Kimsé *et al.,* 2012).These results might be due to better health condition due to positive actions of live yeast in their gastro intestinal tract (Bontempo *et al*., 2006).

Fermentation experiment

The reduction in total gas production in the yeast treatments in this study is in agreement with the results reported by Besharati *et al*. (2015) with *Saccharomyces cerevisiae*. The low gas production is due to the forage proportion in the diet (Marounek *et al*., 2000b) and presumably the gas was produced manly from pectins and xylans fermentation. Gidenne *et al*. (2000, 2002) and Gidenne and Fortun-Lamothe (2002) reported that pectinolytic microorganisms and their activities were higher than xylanolytic or cellulolytic microbes in rabbits. Moreover, Marounek *et al*. (1999, 2000a) also observed that the *in vitro* fermentation of pectins was faster and the microorganisms produced more gas than with fermentation of hemicelluloses and xylans. In accordance with the published results it could be suggested that the majority of gas derived from pectins, which are important ingredients of compound food (Kermauner and Lavrenčič, 2005).

Others researchers found a higher production of metabolites from pectin degradation in 28 day old rabbits compared with 3 month old animals Marounek *et al*. (2000a). On the other hand, Gidenne *et al*. (2002) and Gidenne and Fortun-Lamothe (2002) reported that the ability of caecal microflora to degrade cell wall (mainly pectins and xylans) is well established at weaning and do not change with age. This supports the results we have obtained, where gas production from mixed food in weaned rabbits was high all over the fattening period and till the beginning of the last week. Gas

production depends on nutrient availability for rumen microorganisms as mentioned by Mahala and Fadel Elseed (2007).

The processes of digestion occurring *in vitro* will never be identical to those occurring *in vivo*. In first 10 hours of in vitro fermentation that correspond to the normal caecum retention time, the highest amount of gas from compound food was produced (Gidenne *et al*., 2000). Only in this substrate the time of maximum fermentation rate was short enough (TMFR<10 hours) that it could be fermented *in vivo*. Protein and fiber contents of the tested roughages affect directly the produced gases amount during fermentation as published by Paya *et al*. (2007). The ability of yeast to increase *in vitro* gas production has been reported by various authors with different roughages (Chaucheyras-Durand *et al*., 2008; Ando *et al*., 2004, 2005). The inoculums for the *in vitro* studies are normally made with the contents from a specific part of the gastrointestinal tract, such as caecum, colon or the faeces (Calabro *et al*., 1999; Bauer *et al*., 2004).

However, even if the diversity of various conditions cannot be fully reproduced by *in vitro* methods. Bauer *et al*. (2004) and Bindelle *et al*. (2007ab) used the gas production technique to characterize food fermentation in the large intestine of non-ruminant animals and to rank *in vitro* fermentative characteristics of substrates that would probably remain the same as *in vivo*. However, fermentability of protein produces relatively small amount of gas compared to carbohydrate fermentation (Makkar *et al*., 1995). This can explain how *S. cerevisiae* addition could improve GP at the time it reduced NDFD. In contrast to the results obtained by Lila *et al*. (2004, 2006), we recorded a significant decrease in total gas production in the yeast treatments in this study (Table 2). This may be partly associated with the decreased production of acetate in the yeast treatments, because CO_2 and H_2 are byproducts of acetate production during carbohydrate fermentation.

In the present study the increased *in vitro* caecal dry matter digestibility (IVDMD) in the higher and lower level of yeast supplementation groups (Table 4) is in agreement

with the results of Ayala *et al*. (1992) and who found a digestibility improvement with yeast supplementation because of the improved NDF degradability. Other *in vivo* studies showed that some yeast cultures increased the number of cellulolytic bacteria in the rumen and, in some cases, increased cellulose degradation (Dawson, 1990; Newbold, 1995). Tang *et al*. (2008) reported an increase in rate of gas production and IVRDMD from yeast supplementation with low quality cereal straws that was associated with an increase in protozoa and cellulolytic bacteria populations.

Some researchers recorded a high ruminal gas production with *Saccharomyces cerevisiae* (Martin and Nisbet, 1992), but others found no effect (Lila *et al*., 2004). The two strains of active live yeast tested in our study showed to be potential agents to decrease the enteric production of CH_4. The lower total gas values in the yeast treatment groups suggest the reduction in CH_4 was absolute and not a result of a decrease in the total gas. Lynch and Martin (2002) suggested that *Saccharomyces cerevisiae* reduces ruminal CH_4 production compared to the control group, the higher level of yeast 1 and the lower level of yeast 2 also improved DM digestibility, total VFA and propionate production and decreased the number of methanogenic bacteria (Gong *et al*., 2013).

The stimulatory effect of *S. cerevisiae* is based on the ability of *S. cerevisiae* to scavenge excess oxygen creating a more optimal environment for rumen anaerobic bacteria, and causes inhibition of cellulolytic bacteria attachment to plant cell wall components as stated by Newbold *et al*., (1996). Moreover, *S. cerevisiae* contains small peptides and other nutrients that are required by cellulolytic bacteria to induce growth (Newbold *et al*., 1996). Differences in effect of yeast on rumen microbes and fermentation patterns are mainly associated with the strain of *Saccharomyces cerevisiae* used (Ando *et al*., 2005), diet composition (Sullivan and Martin, 1999) and dose (Lila *et al*., 2006).

Digestibility

An *in vitro* fermentation study demonstrated that branched-chain fatty acids (BCFA) supplementation could increase microbial protein synthesis and DM digestion as recorded by Cummins and Papas (1985). Yeast cultures have stimulated beneficial changes in activity and numbers of the rumen microbes with special interest to cellulolytic bacteria as mentioned by Kumar *et al*. (2013) and Pinloche *et al*. (2013). Others showed that yeast supplementation impaired (*P<0.01*) the digestibility of nutrients (Campos *et al*., 2014). Guedes *et al.* (2008) reported that a live yeast strains increased NDF degradation of different corn silage samples incubated *in saco*. Fiber degradation processes would set up more efficiently in the early age of the animal, as shown by the increase in polysaccharidase and glycoside-hydrolase activities in the presence of yeast in the rumen of gnotoxenic lambs (Chaucheyras-Durand and Fonty, 2001).

Ando *et al*. (2005) also pointed out that the differences in the yeasts' metabolic functions or cell wall structures can influence their degradability of roughages, found that the yeast survival rate to rabbit digestion increased when the yeast intake increased by one Log (10^7 to 10^8) (Kimsé *et al*., 2008). Many reports have shown that live yeasts can improve the microbial balance in the hindgut of horses and stimulate the population of cellulolytic bacteria and their activity as confirmed by Medina *et al*. (2002) thus increasing the digestibility of dietary nutrients (Glade, 1991) with increasing efficiency of energy utilization by the microbiota (Lattimer *et al*., 2005).

8.6- CONCLUSION

The inclusion of Procreatin®7and Biosaf® SC47 (*S. cerevisiae*) had a beneficial effect on growth performance and nutrient digestibility in rabbit rations with a linear effect in doses between 6.4 and 12.8× 10^9 CFU/kg of diet. The yeast *S. cerevisiae* reduced *in vitro* caecal gas production and increase *in vitro* digestibility which presumably is an indicator of healthy caecal fermentation.

8.7- REFERENCES

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9- GENERAL CONCLUSIONS

1. A proper evaluation of yeast products, *Saccharomyces cerevisiae,* requires a determination of CFUs to be able to incubate the samples with the same number of viable cells. In order to clarify the results in terms of yeast strains, diets quality or dose in *in vitro* studies.

2. Oat based diets enhanced a high digestibility and gas production more than alfalfa diets. Digestibility responded linearly to the dose of the two probiotics at the same CFU moreover, the results indicate that the desirable effects on the *in vitro* production of methane and carbon dioxide depend on the used strain.

3. The probiotics derived from *Saccharomyces Cerevisiae* (Biosaf® Sc47 or Procreatin®7) added to alfalfa or oat diets improved *in vitro* digestibility with a dose response. While the effects of yeast on gas production are strain dependent with a minor effect on CH_4 and CO_2 production.

4. Administration of Biosaf® Sc47 or Procreatin®7 at doses of 6.4 $\times 10^9$ CFU or (C2) 12.8 $\times 10^9$ CFU/kg of the basal diets of weaned rabbits of 35-38 days old up to slaughtering, modified its microbiota resulting in a reduced *in vitro* caecal gas production and increased *in vitro* digestibility.

5. Yeast supplementation in weaned rabbits diets at doses of 6.4 $\times 10^9$ CFU or (C2) 12.8 $\times 10^9$ CFU/kg of the basal diets improved *in vivo* digestibility coefficient of total tract apparent nutrients digestibility and daliy gain and feed conversion ratio.

6. The response to Procreatin®7 and Biosaf SC47® in rabbit rations showed a linear response, in the doses evaluated, in growth performance.

7. The yeast *S. cerevisiae* reduced *in vitro* caecal gas production and increased *in vitro* digestibility which is an indicator of healthy caecal fermentation. This yeast could be evaluated as an alternative to reduce methane emissions from rabbits and other non ruminant herbivorous.

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