Potential of solid state fermentation products for probiotic capacity as indicated by a newly developed reliable bioassay

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SUMMARY

A fermentation kinetic study was made to evaluate the effect of a commercial probiotic and a mould based on copra cake SSF products on the growth of *Selenomonas ruminantium*, lactate consumption and volatile fatty acid production. Lactate consumption by *S. ruminantium* was higher in *A. niger* SSF samples (4.12 g/L), than in the *P. italicum* (3.06 g/L) and *Penicillium* sp (2.60 g/L) SSF samples as well as commercial probiotic (1.02 g/L). Lactate consumption of *S. ruminantium* was enhanced (2.8 fold) by the fungal SSF production in comparison to that produced by commercial probiotics. Between the probiotics of moulds, the maximum differences observed in lactate consumption rate were less than 11%. Data indicate the potential of using extracts of fungal solid state cultivation as probiotics. A newly developed reliable bioassay involving the use of lactic acid consuming rumen *S. ruminantium* HD4 also proved reliable.

Keywords: Solid state fermentation, probiotic activities, *Penicillium* spp, *Aspergillus* spp, copra cake fermentation, bioassay for probiotic activity, lactose consuming rumen bacteria, *Selenomonas ruminantium*.

RESUME

Potentilités d'utilisation comme probiotique des produits fermentés en milieu solide confirmées par un nouveau bio-essai mis au point.

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Une étude a été réalisée afin de comparer l'effet d'un probiotique commercial à celui de différents lots de tourteau de coprah fermentés en milieu solide, sur la croissance de *Selenomonas ruminantium*, l'utilisation du lactate et la production d'acides gras volatils par cette bactérie du rumen. La consommation du lactate par *S.ruminantium* a été supérieure pour les échantillons de tourteau de coprah fermentés par *A.niger* (4.12 g/L); elle a été moindre pour les échantillons fermentés en FMS par *Penicillium italicum*, *Penicillium* sp (2.60 g/L) et le probiotique commercial (1.02 g/L). La consommation du lactate par *S. ruminantium* a été 2.8 fois supérieure pour les produits fongiques par rapport au probiotique commercial. Entre les divers produits fermentés utilisés, la différence observée pour la consommation du lactate a été de 11%. Les résultats obtenus confirment les potentialités d'utilisation comme probiotique des extraits fongiques obtenus par FMS. Un Bio-essai , basé sur l'augmentation de la consommation du lactate par *S. ruminantium* cultivée en présence d'extraits de produits fermentés a été mis au point .

Mots clés: Fermentation en Milieu Solide, Activités probiotiques, *Penicillium*, *Aspergillus*, Tourteau de coprah, Biomasse avec activité probiotique, Utilisation du lactate par les bactéries du rumen, *Selenomonas ruminantium.*

INTRODUCTION

RUMEN FUNDAMENTALS

Rumen could be considered as a complex fermenter, where synergism and antagonism among bacteria, fungi and protozoa take place (Hungate, 1966). Carbohydrate metabolism leads to the formation of organic acids (lactic, acetic, propionic, butyric). Around 70% of energy required for meat and milk production is obtained from these organic acids. The proportion of different micro-organisms in the rumen depends on type of feeding. When cattles are fed with a high carbohydrate diet, lactic acid bacteria are predominant. Consequently, a high lactic acid is produced, which decreases the pH of rumen and this phenomenon is recognized as

acidosis (Slyter, 1976). A pH below 6.0 affects the activity of cellulolytic micro-organisms and, therefore, the overall efficiency of the rumen.

Nevertheless, to overcome this problem, there are lactate consuming bacteria such as *Selenomonas* and *Megasphaera* in the rumen, which allow maintenance of a dynamic equilibrium of rumen activity. Nutritional needs of *Selenomonas* species are complex. They require n-valerate and PABA, when growing on glucose, and biotin has a stimulatory effect. On the other hand, when growing on lactate, biotin is essential for growth, while n-valerate has a stimulatory effect on growth (Bryant, 1956; Kanegasaki and Takahashi, 1967).

PROBIOTICS

It is felt by different authors that rumen activity can be enhanced by some products or compounds or a mixture of them, which are called as probiotic (Williams, 1991; Males and Johnson, 1990). The role of probiotics in human nutrition is related to the built-up of intestinal microflora. In the case of animal nutrition, they are used as additives in order to increase animal yields. In this work, the term probiotic will be used exclusively for ruminant nutrition purposes.

The probiotics should be non-toxic, non-pathogenic, efficient in small doses (1-3%), with the ability to stimulate overall activity of rumen bacteria, and the micro-organisms producing probiotics should be easily cultured and conserved (Males and Johnson, 1990; Hose and Sozzi, 1991). The mechanism of action of probiotics is not well elucidated and more research work is needed in this direction.

IMPORTANCE OF BIO-ASSAY FOR PROBIOTICS

Evaluation of probiotics *in vivo* is an expensive and laborious test. Consequently, several authors have proposed the use of some rumen bacteria to test the stimulatory capacity of probiotics (*in vitro* assays), especially regarding bacterial growth and lactate consumption (Nisbet and Martin, 1990, 1991; Russell and Baldwin, 1979; Tapia *et al*, 1988).

The aim of this work was to test the potential of different solid state fermention products for their stimulatory effect on growth and lactate utilization by *Selenomonas ruminantium* HD4. In this direction, solid state fermentation (SSF) products present an interesting alternative to conventionally produced probiotics (Pandey, 1992). Newly developed simple and reliable bioassay method for probiotics is also presented.

MATERIALS AND METHODS

MICROORGANISM

A strain of *Selenomonas ruminantium* HD4, provided by Dr. Martin, was used throughout this study. The strain was kept in glucose medium (as described below) at -76°C, before activation for different trials.

CULTURE MEDIUM AND CONDITIONS

A basal medium, modified from literature data (Prins, 1971; Tinari *et al,* 1968; Hobson *et al,* 1963) was used. It contains in mg/L: Yeast extract 500, Peptone of casein 500, K_2HPO_4 292, KH_2PO_4 292, $(NH_4)_2SO_4$ 480, resarzurin 1, PABA 1, NaCl 480, $MgSO_47H_2O$ 100, $CaCl_2$ 50, cystein 600, Na_2CO_3 4000, biotin 0.1. Volatile fatty acids (VFA) were added to the culture medium as follows (mM):acetic acid 30, propionic acid 8.1, butyric acid 3.4, isovaleric acid 1, valeric acid (as required).

Glucose and lactate were used as carbon sources. Sugar was used at 2 g/L level, while maintaining valeric acid at 1 ppm. Lactate was used at 13 g/L level, keeping valeric acid concentration constant at 0.2 ppm, and adding sodium acetate at 30 mM in this case. Cultures were grown at 39°C at pH 6.5-7.0, under a reduced CO_2 atmosphere, after autoclaving at 120°C for 15 min. Inoculation was carried out at 10% (v/v). All trials were carried out in duplicate.

ANALYTICAL ASPECTS

Bacterial growth was determined by optical density at 600 nm. Glucose, VFA's, and lactic acids were measured by HPLC, as described elsewhere (Giraud *et al,* 1991).

PROBIOTIC

A commercial yeast probiotic (submerged culture, SmF) and 4 solid state fermentation (SSF) products were tested. All SSF products were obtained from copra cake fermentations. Strains used were *Aspergillus carbonarius* No.57, *Aspergillus niger* No. 2.10, *Penicillium sp* No. 1.4 and *Penicillium italicum* No. 2.25. The last three samples were tested after o and 31 hour fermentation, while the commercial product was based on *Saccharomyces cerevisiae.*

SSF fermentation products were lyophilized after fermentation. Samples (4 g) quantity were mixed thoroughly with 50 ml distilled water, filtered through Whatman No.1 filter paper, then sterilized by passing through a 0.45 µm Millipore membrane. Probiotic extracts were added to the culture media at 2% (v/v).

RESULTS AND DISCUSSION

KINETIC STUDY

Kinetics were carried out in order to test the effect of addition of an *Aspergillus carbonarius* SSF product and a commercial probiotic upon lactate utilization and growth evolution, during cultures of *S. ruminantium* HD4. Figs. 1 and 2 show the results obtained for biomass formation and lactate utilization, respectively. Higher values of biomass were obtained in the case of addition of a SSF extract, compared with the commercial product or the control (without additive) culture (Fig. 1). Maximum values were attained after 12 hour cultivation for SSF extract. In the case of the commercial product, maximum value was reached at 50 hour . Addition of an *Aspergillus carbonarius* SSF extract presented a stimulatory effect on biomass level and growth rate.

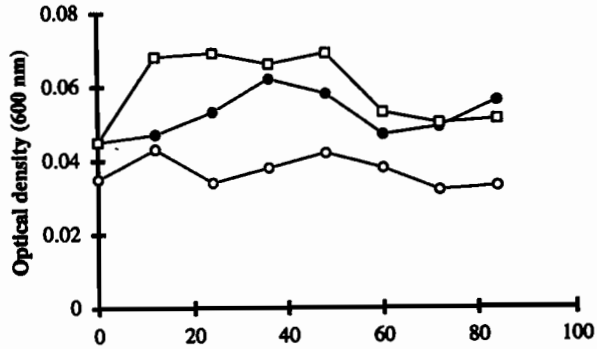

Fig. 1.- Effect of addition of a commercial probiotic (Yea-Sacc), a SSF extract on evolution of *S. ruminantium* HD4 biomass.
❑:*A.. carbonarious* SSF extract, ●: Yea-Sacc and, ○: Control (without additive).

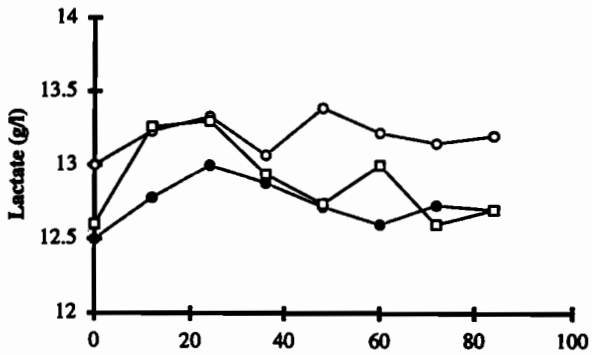

Fig. 2.- Effect of addition of a commercial probiotic (Yea-Sacc), a SSF extract on lactate evolution during cultivation of *S. ruminantium* HD4 biomass.
❑:*A. carbonarious* SSF extract, ●: Yea-Sacc and, ○: Control (without additive).

For all three treatments, maximum values of lactate concentrations were reached after 24 hour cultivation, followed by a progressively slow decrease (Fig. 2). The same pattern was observed when glucose (2 g/L) was used as a carbon source (data not shown). Higher lactate consumption was obtained using a SSF extract (89 µM/h) compared with the commercial product extract (44 µM/h) and the control (17 µM/h). These results may be considered of low magnitude. Nevertheless, the reported affinity of *Selenomonas* against lactate is extremely low. In the case of *M. eldenii,* the value of Ks was of o.37 mM (Russell and Baldwin, 1979). The rise in lactate concentration during first 24 hour cultivation (Fig. 2) can be attributed to the presence of readily utilizable nutrients, such as peptone and yeast extract.

BIOASSAY

The last trial showed that the increase in lactate concentration was followed by a culture phase, where lactate was consumed by *S. ruminantium* HD4. According to this observation, a bioassay was carried out to test the stimulatory effect of 3 SSF products (before and after 31 hour solid state cultivation) upon lactate and biomass concentrations during cultivation of *S. ruminantium* HD4. Analyses were carried out after 0, 24 and 80 hour cultivation of rumen bacteria. Fig. 3 shows the results of biomass concentration. Higher levels of biomass were found in the case of SSF extracts. Nevertheless, two unfermented inoculated SSF samples (*Penicillium* sp and *P. italicum*) presented a higher stimulatory effect, than in the case of using the fermented SSF product (Fig. 3). It can be explained based on the presence of readily utilizable nutrients in copra cake.

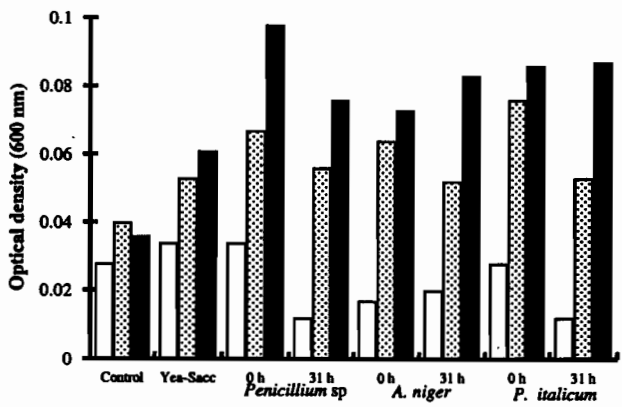

Fig. 3.- Effect of addition of a commercial probiotic (Yea-Sacc) and different SSF extracts on biomass of *S. ruminantium* HD4. Analysis were realized after 0 (□), 24 (▨) and 80 h (■)cultivation of rumen bacteria. Numbers for each fungus strain correspond to the fermentation time in SSF.

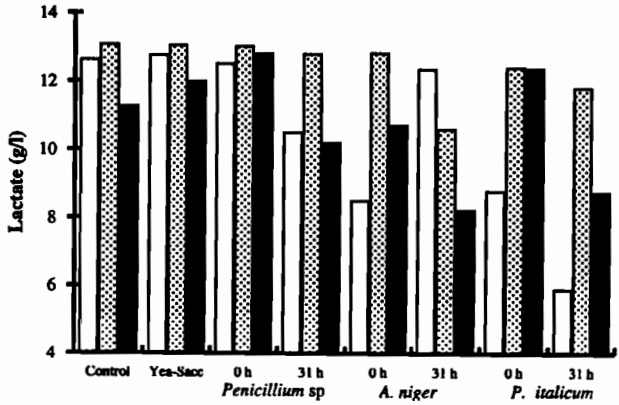

Fig. 4.- Effect of addition of a commercial probiotic (Yea-Sacc), and different SSF extracts on lactate concentration during cultivation of *S. ruminantium* HD4. Analysis were realized after 0 (❑), 24 (❑) and 80 h (■) cultivation of rumen bacteria. Numbers for each fungus strain correspond to the fermentation time in SSF.

Lactate increased from 0 to 24 hour (Fig. 4), then its consumption was observed. These results are in agreement with those reported earlier in this work. In the case of unfermented samples of *Penicillium* sp *and P. italicum*, no consumption of lactate was observed from 24 to 80 hour cultivation of the rumen bacteria. In the case of unfermented samples *of A. niger*, a consumption of 2.11 g/L of lactate was observed during the same period of time. It is probably due to the enzymatic activity of *Aspergillus* spores. After 24 hour cultivation of *S. ruminantium* HD4, a consumption of lactate was observed in fermented samples (31 hour, SSF) of *Penicillium* sp and *P. italicum*. *A. niger* extracts presented a continuous utilization of lactate from 0 to 80 hour cultivation. Lactate consumption was higher (Table 1) for *A. niger* SSF samples (4.12 g/L), followed by *P. italicum* (3.06 g/L), *Penicillium* sp (2.60 g/L) and commercial probiotic (1.02 g/L). Lactate consumption rates were higher (2.8 fold) in samples derived from fungal SSF compared with those from commercial probiotics. In the case of moulds, maximum differences of lactate consumption rate were less than 11% (Table 1).

Table 1. Effect of the addition of mould SSF extracts on consumption of lactate during cultivation of *S. ruminantium* HD4.

	Lactate consumption	
Strains	Total, (g/L)	Rate (mg/L h)
S. cerevisiae	1.02	18.2
Aspergillus niger	4.12	51.5
Penicillium sp	2.60	46.4
Penicillium italicum	3.06	54.6

In this work, evidence has been presented for the potential of using mold extracts from solid state cultivation, as probiotics in ruminant nutrition, especially taking into account the lactic acid consuming activity. It must be emphazised that the function of probiotics is to give essential elements other than carbon and nitrogen.

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