Screening and selection of lactic acid bacteria from calves for designing a species-specific probiotic supplement

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ABSTRACT: Probiotic supplementation to animal feeds has become a standard practice in the feed industry especially since European Union banned the use of antibiotics as growth promoters. The aim of this study was to isolate and characterize mainly lactic acid bacteria (LAB) from calves, that can be used as feed additive. For this purpose, bacterial strains were recovered from calf faecal samples and characterized using MicroLog™ system, 16s rRNA gene sequencing and Riboprinter™ system.

Major representative strains were evaluated for their potential probiotic activity in vitro. Of 145 strains isolated, 3 clonal strains were selected for their potential probiotic activity, namely Lactobacillus animalis DUP5009, Lactobacillus paracasei ssp. paracasei DUP13077 and Bacillus coagulans RiboGroup 189-444-S-1. In light of this result these clonal strains can be considered for develop new probiotic products for calves.

Keywords: Calves, Lactic acid bacteria (LAB) identification, Probiotic characterization.

INTRODUCTION – The removal of antibiotics as growth promoters in animal feed from January 2006, completed the EU’s drive to phase out all antimicrobial growth promoters (AGPs) from livestock production. Since 18 October 2004, applications for authorising the placing and use of feed additives in the market, are regulated in the European Union under Regulation (EC) No 1831/2003.

There are a number of alternatives to AGPs that can be used strategically in order to improve animal growth and performance, including probiotics (Savoini et al., 2005). A probiotic is a culture of one or more microbial strain, which benefits the host by stimulating the positive properties of its natural occurring microflora in the gut (Fuller, 1989). In modern veal calf production, animals are faced with major stress events like transportation, marketing, dietary changes and exposure to a variety of infectious agents. These factors could affect the protective potential of the microbial gut flora with a successive high incidence on intestinal and respiratory disease in veal calves (Timmerman et al., 2005). It is now recognized that the most effective probiotic bacteria should be isolated from animal gut and have a high degree of host specificity. In light of these issues, the aim of the research is to select potential probiotic bacteria isolated directly from calves.

MATERIAL AND METHODS – Bacterial strains were isolate from faecal samples collected from 40 newborn calves allocated in four different farms located in Northern Italy (Brescia). Standard culture techniques using different media were applied for isolating microorganisms by serial dilution methods in buffered peptone water (Oxoid, Milan, Italy). Diluted samples were plated on MRS agar (DeMan, Rogosa and Sharpe), M17 and Modified Columbia Agar (Beeren’s media) for the determination of Lactobacillus spp., Lactococcus spp. and Bifidobacterium spp. respectively, which are known as potential probiotic bacteria.
A total of 145 colony forming unit (CFU) were selected based on colonies morphology. Microorganisms were then characterised by metabolic profiles (MicroLog™ System, Biolog Inc. 3938 Trust Way, Hayward, CA 94545, USA) and by 16S rRNA gene sequencing to attempt species identification. Clonal identification of microorganisms were performed by comparison of the Riboprinter™ pattern with an identification database of EcoRI Riboprinter™ patterns created by E. I. DuPont de Nemours and Company (Qualicon Inc.), at “Istituto Zooprofilattico Sperimentale di Brescia (IZS)” (Ripamonti et al., 2006). On the most representative clones individuated within each identified strain, the following probiotic characteristics (FAO/WHO, 2002) were considered: resistance to gastric acidity, bile acid resistance, adherence to human epithelial cells, and antimicrobial activity against potentially pathogenic bacteria (Rebucci et al., 2007). The effects of low pH and bile salts were examined by the method of Fernández et al. (2003). Briefly, aliquots of bacteria from pure cultures (10⁹ CFU/ml) were added to artificial gastric fluid (NaCl, 0.72 g/l; KCl, 0.05 g/l; NaHCO₃, 0.37 g/l; pepsin, 3 g/l) at pH 3.0 and also at pH 7.0 as control condition. The number of surviving cells was determined by colony counting on MRS agar after 0, 90 or 180 min of incubation with agitation to simulate peristalsis. To examine resistance to bile salts, after 180 min of incubation in artificial gastric fluid, the bacteria at pH 3.0 and 7.0 were harvested by centrifugation (2000 g for 10 min) and the pellet resuspended in artificial intestinal fluid [0.1% (w/v) pancreatin and 0.15% (w/v) Oxgall conjugated bile salts (Sigma Chemical Co., St. Louis, MO, USA) in water; pH 8.0].

Total viable counts were determined by colony counting on MRS agar after incubation of extracted samples as above for 0, 90 and 180 min. An adhesion assay was performed according to Lee et al. (2000), employing the human embryonic intestine line cell INT407 kindly provided by the IZS. INT407 cells were cultured in Chamber Slide™ System (Nunc Lab-Tek™ II; Nunc Inc., Naperville, IL, USA). Approximately 10⁸ CFU/ml, diluted in DMEM, were incubated for 1 hour at 37°C, 5% CO₂. Monolayer cells were washed 4 times with sterile PBS, fixed with methanol, stained with Giemsa (Merck, Darmstadt, Germany).

The slides were then examined microscopically. Images were acquired by Image Vision software (Media Cybernetics) at x100 magnification under oil immersion. The mean and standard deviation of adherent bacteria was determined on 20 random microscopic fields. Each assay was performed in triplicate. The production of effective pathogen antagonist such as ethanol, butyric, isobutyric, propionic, acetic and lactic acids was determined by gas chromatography as described by Annuk et al. (2003).

RESULTS AND CONCLUSIONS – Percentages of microorganisms identification based on biochemical analysis, 16S rRNA gene sequence and Riboprinter™ characterisation were: Bifidobacterium longum 44.6%, Streptococcus bovis 13.4%, Streptococcus macedonicus /bovis 7.1%, Lactobacillus animalis 8.9%, Streptococcus macedonicus 4.5%, Pediococcus spp. 3.5%, Lactobacillus paracasei ssp. paracasei 2.7%, Lactobacillus salivarius ssp. salicinius 2.7%, Lactobacillus ruminis 1.8%, Lactobacillus fermentum 1.8%, Lactobacillus reuteri 1.8%. Lactobacillus delbruckii, Lactobacillus delbruckii ssp. lactis, Lactobacillus paracasei ssp. tolerans, Lactobacillus salivarius ssp. salivarius, Lactobacillus murinus and Streptococcus hominis were identified with a percentage of identification <1%.

In vitro evaluation of probiotic activity were performed on: Bifidobacterium longum DUP 13305, Lactobacillus animalis DUP 5009, Bacillus coagulans Ribgroup 189-444-S, Lactobacillus paracasei ssp. paracasei DUP 13077, Lactobacillus salivarius ssp. salicinius DUP 16951, and Streptococcus macedonicus. After artificial gastric and intestinal fluid addition, Lactobacillus animalis and Bacillus coagulans showed no significant decrease in viability. The number of viable cells (0 min) of Lactobacillus paracasei ssp. paracasei and Lactobacillus salivarius ssp. salicinius decreased slightly after the addition of artificial gastric (180 min, pH 3.0) and intestinal fluid (180 min, pH 8.0): 8.39 log CFU/ml to 7.06 log CFU/ml and 8.65 log CFU/ml to 7.95 log CFU/ml respectively. The most sensitive were Streptococcus macedonicus and Bifidobacterium longum therefore they weren’t selected for further tests. Bacteria must be able adhere to the intestinal wall in order to colonize the gut. Lactobacillus animalis adhered to confluent INT407 cells two times more frequently than Lactobacillus salivarius ssp salicinius (60 ± 10 vs 30 ± 12). Lactobacillus paracasei ssp. paracasei and Bacillus coagulans adhered conspicuously (pH 5, pH 7) to the monolayer (>100 mean bacteria number per random microscopic field).

All clonal strain tested produced trace amounts of ethanol, butyric acid and iso-butyric acid, and that all produced similar amounts of propionic acid (0.277 ± 0.067 mmol/l). Acetic acid production was similar in Lactobacillus animalis and Bacillus coagulans (mean 42.903 ± 1.214 mmol/l) in anaerobic condition. Lactic acid production were more copious in Lactobacillus paracasei ssp. paracasei and Lactobacillus salivarius ssp. salicinius in microaerobic condition (222.059 ± 3.429 mmol/l). Lactobacillus animalis yielded a higher amount of lactic acid in anaerobic condition (228.808 ± 5.639 mmol/l). Bifidobacterium longum resulted the most represented microorganism isolated from calf faecal samples (44.6%), however tests performed indicated a limited potential as probiotic strain compared to the other considered in the present study. Results obtained from microorganisms id-
Identification and *in vitro* evaluation of probiotic activity, indicated that *Lactobacillus animalis* DUP 5009, *Lactobacillus paracasei* ssp. *paracasei* DUP 13077 and *Bacillus coagulans* RiboGroup 189-444-S-1 represent promising specific probiotics candidates for calves. Further steps of this project will be fermentation of selected strains and their test *in vivo* by administration in newborn calves.

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