SUMMARY OF THE RESEARCH PROJECT N° LT 08/10

"EVALUATION OF THE PROBIOTIC LACTIC ACID FLORA PRESENT IN TRADITIONAL CHEESES ORIGINATING FROM THE REGIONS OF LAZIO AND TUSCANY, ITALY "

Bianca Maria Varcasia[1] and Paola De Santis[1]

e-mail: paola.desantis@izslt.it
[1]Istituto Zooprofilattico Sperimentale Lazio e Toscana, Via Appia Nuova 1411, 00178 Roma
Data 21/1/2015

Key words: Lactic acid bacteria, probiotics, traditional products, DGGE, sequencing

AIM

The aim of the project was to study the probiotic lactic flora found in traditional raw-milk cheeses produced in the Lazio and Tuscany regions of Italy, to be used by local manufacturers as lactobacillus (LAB) starter cultures. The possible use of a natural lactic flora as starters could be of benefit to small-scale local cheese producers by enhancing both the intrinsic characteristics and health properties of their products. Attention is focused on the microbiological and molecular characterization of *Lactobacillus* and *Lactococcus* species, in possession of probiotic properties beneficial to human health.

METHODOLOGY

Five traditional cheeses, produced using raw cow, sheep, buffalo and goat milk, and made without adding industrial lactic acid bacteria as starter, were analyzed. All cheeses were fully ripened, i.e. were sampled at the end of the fermenting process; two of the cheeses were marketed in the Lazio region, the remaining three in Tuscany (Table 1).
For each product, the steps in production were documented prior to its analysis.

Table 1. Cheeses analyzed and their producers

PRODUCT NAME	ADDRESS OF PRODUCER	U.O.
Pecorino di Picinisco	Caseificio di Pia Marcello - Settefrati (FR) Lazio	IZSLT01
Caciotta di capra	Caseificio Valle di Mezzo, Loc. Coppole – Anghiari (AR) Tuscany	IZSLT02
Caprino di Scilla	Azienda Agricola Angela Saba - Massa Marittima (GR) Tuscany	IZSLT03
Pecorino della Montagna Pistoiese	Consorzio Montagne e Valli di Pistoia (PT) Tuscany	IZSLT04
Provolone di bufala	Caseificio Ventre Daniele – Latina (LT) Lazio	IZSLT05

The isolation and enumeration of lactic acid bacteria (LAB) was performed using MRS and M17 specific liquid and solid culture media. The Gram + bacterial colonies reacting negatively to the Catalase test were purified in 3 consecutive passages on solid culture media, and incubated at optimal growth conditions.
To define the probiotic properties of the purified LAB, each isolate was analysed using "screening" and "specific" assays. The "screening assays" were used to check resistance in the LAB strains to the gastrointestinal tract environment, i.e. assessing the capacity of the bacterial cells to survive at a low pH (2.5); in the presence of bile salts; in the presence of a low pH and bile salts combined; and in the presence of pepsin and pancreatin. The "specific assays", were used to simulate the capacity of alleged probiotic strains, after reaching the intestinal tract, to self-aggregate and co-aggregate, and to evaluate the antibacterial activity due to the production of bacteriocins. The confirmation of the production of bacteriocins by the lactic acid bacterial strains was accomplished by PCR assays based on methods from the published scientific literature (ISTISAN Report n° 12/54).
The LAB strains that passed the screening tests, were molecularly identified to the species level, after prior extraction of the DNA using Denaturing Gradient Gel Electrophoresis (DGGE) of about 90 bp of the 16S rRNA V1 region obtained with PCR and further sequenced to confirm the DGGE results.

Among all the identified LAB strains that passed the probiotic assays, one was chosen to verify in vivo its ability to colonize the gastrointestinal tract, hence to modulate the immune system. This was a strain of *Lactobacillus lactis* (*L. lactis*) able to produce a bacteriocine, which was identified as nisin using an end-point PCR test.
CD1 outbred adult female mice (Harlan Laboratories, Italy) aged between 6 and 8 weeks, were fed with a fresh culture of the selected *L. lactis* strain, at a concentration of 10^9 ufc/ml^{-1} for 10 consecutive days. To verify the ability of *L. lactis* to stably colonize the intestine, the feaces of the mice were collected daily, during the adminstration of *L. lactis* and for an additional 7 days, and cultured in MRS media in an effort to re-isolate the strain. The extent to which the immune system was stimulated was assessed using the rate of lymphocyte proliferation in response to the mitogenic activity of an optimal and suboptimal dose of Concanavalin A (ConA). The analysis of the statistically significant differences between the results from the two groups (treated and not-treated), was performed using the Student's T test. The experiments were conducted in accordance with Italian law (D.Lgs. 116/92).

RESULTS

Microbiological assays. From the 5 traditional cheeses tested, 214 LAB strains were isolated:

- 43 strains from Pecorino di Picinisco (PP); 26 lactococcus and 17 lactobacillus;
- 77 strains from Pecorino della Montagna Pistoiese (PMP); 47 lactococcus and 30 lactobacillus;
- 12 strains from Caciotta di capra (CC), 3 lactococcus and 9 lactobacillus;
- 54 strains from Provolone bufala (PB), 36 lactococcus and 18 lactobacillus;
- 30 strains from Caprino di Scilla (CS), all lactobacillus.

Screening assays. All isolated LAB strains were subjected to screening assays. Only 51 LAB strains passed all the tests (Chart 1), in particular the 14 strains from PP, 23 strains from PMP, 4 strains from CC, 8 strains from PB and 2 strains from CS.

Chart 1. Strains of LAB positive and negative to the screening assays

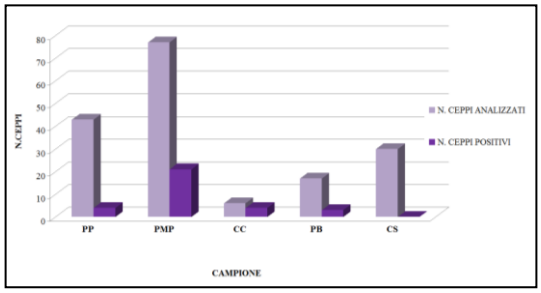


Chart 2. Potencially probiotic LAB strains

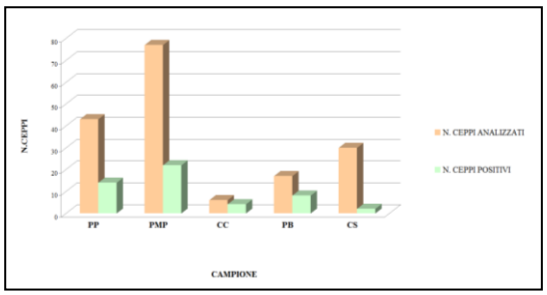
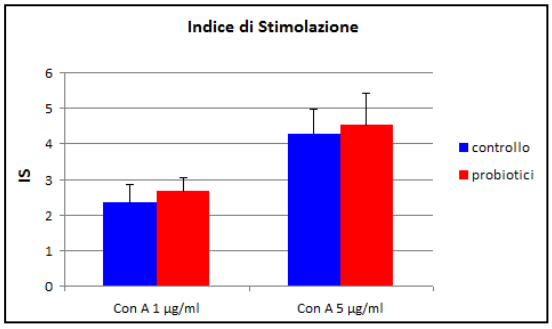


Chart 3: Immunostimulation index using ConA



Specific assays. The 51 LAB strains, positive in the screening assays, have been tested for their aggregation and co-aggregation properties and for the production of bacteriocins; of these, only 31 passed the specific assays and therefore are considered "potentially probiotic" (Chart 2). The LAB strains are distributed as follows: 4 strains from PP, 20 strains from PMP, 4 strains from CC and 3 strains from PB.

Molecular identification. The molecular analysis of the 31 potentially probiotic strains performed with DGGE and sequencing allowed the characterization to species of the LAB strains as follows: 2 strains of *Lactococcus lactis* sub. *lactis*, 20 strains of *Lactobacillus casei*, 1 strain of *Lactobacillus paracasei*, 1 strain of *Staphylococcus* spp. and 2 strains of *Enterococcus* spp.

***In vivo* assays**. From the feces of the CD1 mice, during and after administration of the *Lactococcus lactis* sub *lactis* strain, it was not possible to re-isolate the strain and the immunostimulation test (Chart 3) did not provide statistically significant results (Student's T test) between the treated and untreated groups ($p < 0.05$). Therefore, in order to limit the use of animals,

it was decided not to proceed with the administration to mice of more LAB strains.

CONCLUSIONS

The number of candidate probiotic strains, compared to the large number isolated, was relatively low, with significant differences from one product to the next. Nevertheless, only a few of the isolated strains could survive the simulated conditions of the gastrointestinal tract. Furthermore, it was not possible to assess the ability of the candidate strains to colonize the intestine *in vivo*, in spite the *in vitro* tests showing promise with regard to their aggregation and coaggregation characteristics. Most of the potentially probiotic strains were obtained from the "Pecorino della Montagna Pistoiese" (65%), followed by Caciotta di capra" (13%), "Pecorino di Picinisco" (12%) and "Provolone di bufala" (9%). With reference to the *in vivo* tests, the inability to isolate the strain of *Lactococcus lactis* sub. *lactis* orally administered to CD1 mice, is probably due to the challenges this strain faces during colonization of the intestine, in spite of the potentially probiotic characteristics it displays and the presence of nisin production, factors that make it the ideal candidate during animal experimentation. Because the ConA test was negative, it rendered it not possible to measure cytokine production, due to the immune system, in the mice not being stimulated by the LABs. The optimal amount of live probiotic bacteria to be administered, to produce a beneficial effect on health, is not easy to determine as it is strain-dependent and, probably, is also a function of the type of benefit sought for. However the finding, in these five traditional products, of LABs with probiotic features that show promise, reveals the presence in the production environment, of a lactic flora that deserves further scientific investigation, when considering the already well-known beneficial activity of LABs in suppressing pathogenic flora.

References

1. Aureli P., Banderali G., Capurso L., Castellazzi A.M., Clerici M., Giovannini M., Morelli L., Poli A., Pregliasco F., Rondanelli M., Salvini F., Zuccotti G. V. et al. PROBIOTICI E SALUTE UMANA - 2013: Lo stato dell'arte basato sulle evidenze. POSITION PAPER (2013).
2. Berthier F., Ehrlich S.D. "Rapid species identification within two groups of closely related lactobacilli using PCR primers that target the 16S/23S rRNA spacer region" FEMS Microbiology Letters 161, 97-106 (1998).
3. Fioramonti J., Theodorou V., Bueno L. "Probiotics: what are they? What are their effects on gut physiology?" Best Practice and Research Clinical Gastroenterology 17 (5), 711-724 (2003).
4. Kalavrouzioti I., Hatzikamari M., Litopoulou-Tzanetaki E., Tzanetakis N. "Production of hard cheese from caprine milk by the use of two types of probiotic cultures as adjuncts" International Journal of Dairy Technology 58 (1), 30-38 (2005).
5. Kimoto H., Kurisaki J., Tsuji N.M., Ohmomo S., Okamoto T. "Lactococci as probiotic strains: adesion to human enterocyte-like Caco-2 cells and tolerance to low pH and bile" Letters in Applied Microbiology 29, 313-316 (1999).
6. Kimoto H., Nomura M., Kobayashi M., Mizumachi K., Okamoto T. "Survival of lactococci during passage through mouse digestive tract" Canadian Journal of Microbiology 49, 707-711 (2003).
7. Kimoto H., Nomura M., Kobayashi M., Okamoto T., Ohmomo S. "Identification and probiotic characteristics of *Lactococcus* strains from plant materials" Japan Agricultural Research Quarterly 38 (2), 111-117 (2004).
8. Songisepp E., Kullisaar T., Hutt P., Elias P., Brilene T., Zilmer M., Mikelsaar M. "A new probiotic cheese with antioxidative and antimicrobial activity" Journal of Dairy Science 87, 2017-2023 (2004).
9. Vinderola C.G., Prosello W., Ghiberto D., Reinheimer J.A. "Viability of probiotic (*Bifidobacterium*, *Lactobacillus acidophilus* and *Lactobacillus casei*) and nonprobiotic microflora in Argentinian Fresco cheese" Journal of Dairy Science 83, 1905-1911 (2000).