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IDENTIFICATION OF PROBIOTIC STRAINS FROM HUMAN MILK IN BREASTFED INFANTS WITH RESPIRATORY INFECTIONS

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Abstract: Isolation and industrial exploitation of probiotics from human milk is a goal for worldwide milk biotechnology centres because of their modulation effect on the immune system in infants and adults. In the proposed study we have analysed fermentation patterns of *Lactobacilli* isolated from human milk, the reliability of API 50 CH carbohydrate fermentation system and a possible link between lactose concentrations and fermentation profiles on carbohydrates. We had succesfully identified three species of *Lactobacillus (paracasei ssp paracasei, fermentum, acidophilus)* and one unsatisfactory identification of *Lactoccocus lactis ssp lactis*. These strains had different carbohydrate fermentation patterns but with common characteristics and showed no statistically significant correlations between their carbohydrate metabolic trends and lactose concentrations in the milk samples.

Keywords: human milk, *lactobacilli*, API 50CH identification, lactose concentrat

INTRODUCTION

Probiotics are "living microorganisms which in adequate amounts bring

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benefits for the host" (FAO/WHO, 2002). Their roles correlate with the type of strain (Burgain et al., 2011),(Agrawal 2005). Probiotics are represented by many microorganisms in the genus *Lactobacillus* prokaryotic bacteria, about 150 species so far (O'Donnell et al., 2013) and the genus *Bifidobacterium* about 30 species, but also in the field of fungi (some yeasts) (Burgain et al., 2011).

Isolation and industrial development of probiotics from human milk is a real challenge and a goal for milk microbiology and biotechnology centres from around the globe. Human milk is an important source of probiotic strains for infant intestinal microbiota development (Malek et al. 2010), (Yavuzdurmaz et al., 2011). Clinical studies have showed unequivocally a hospitalization rate three times higher in infants fed with milk powder formula compared with breastfed infants admitted with respiratory infections. It seems that microbiota from human milk modulates the immune response in infants(Chirico et al., 2008). Most probably these strains use lactose as a principal substrate for energy and growth since lactose concentrations are much higher compared with oligosaccharides in human milk. Nevertheless, genus *Lactobacillus* has broad metabolic capacity in many cases with specific traits for each strain(O'Donnell et al., 2013). Current research projects related to different species of *Lactobacillus* investigate the impact of milk milieu on the bacterial genome expression especially the promotion of probiotic characteristics and metabolic variability (Klaenhammer et al., 2014)**.** More research data is needed in analysing this complex subject. Probiotic conventional phenotypic identification by carbohydrates fermentation method reflects in fact the proportion of the carbohydrate catabolic genes expressed by each type of strain under particular cultivation conditions **(**Leuschner et al., 2002). Sugars fermentation method is widely used to identify probiotics in isolates from various sources: 1. Human milk (Malek et al., 2010),(Yavuzdurmaz et al., 2011); 2. Animal milk kefir (Zanisic et al., 2012),(Hyun jue Kim et al., 2006); 3. Dairy-yogurt (Karna et al., 2007), (Kizerwetter et al., 2005); 4. Fermented products like meat, dairy, vegetables, fruit, pastry, drinks; 5. Animal or human digestive tracts (Ashraf et al., 2009),(Zanisic et al., 2012),(Khalil et al., 2007); 6. Genital Tracts (Herbel et al., 2013).

In our study we have analysed fermentation patterns of *lactobacilli* species isolated from human milk and the reliability of API 50 CH carbohydrate fermentation system as a first step identification method in the process of industrial exploitation of these strains. We have hypothesized that constant exposure to breast milieu, particularly human milk lactose levels could affect in a specific manner carbohydrate catabolic genes expression of these

lactic acid bacteria (metabolic capability).In this connection it was interesting to examine the possibility of a link between lactose concentrations and fermentation profiles on carbohydrates with a direct implication in the percentage of identification, T index and validation on a apiweb™ database.

MATERIALS AND METHODS

In the proposed study we have collected breast milk samples from the mothers admitted in our clinic whose infants were hospitalized for acute infections of respiratory pathways. Samples were harvested using sterile gloves with respect to local antisepsis (areola disinfected) following the methodology described by Yavuzdurmaz et al.(2011): 52 control samples from the skin without desinfection, 52 samples of breast milk from healthy mothers nursing infants with lower respiratory infections, in duplicates. Evaluation of human milk lactose levels was made using an ultrasonic analyzer. Milk samples were centrifuged at 3.000 rpm for 20 minutes. Supernatant represented by the fatty substance was separated and the levels of lactose were determined on ultrasonic analyzer Ekomilk®. Samples from each milk probe were added in the specific medium for *Lactobacilli* MRS (Man Rogosa Sharpe) using streak plate technique. Isolates were examined according to the colony morphology, catalase reaction, gram staining and sugar fermentation tests (Leuschner et al., 2002). Isolates from MRS agar were passed then on MRS broth and kept in culture with glycerol 40 %(v/v) at -80 °C in freezer. We have selected from 52 strains, 10 strains who have survived to multiple freeze-thaw cycles. These strains were evaluated as potential candidates for industrial exploitation because of their resistance.

Bacterial identification was carried out by means of galleries API 50 CH (BioMerieux, France), a standardized system which combines 50 biochemical assays for the study of the microorganisms carbohydrate metabolic profile. API 50 CH galleries were used in conjunction with the API 50 CHL medium to identify the genus and the species of *lactobacilli*. The prepared inoculum(density of the bacterial suspension 2 Mc Farland) was incubated in each tube of the 50 galleries at 37ºC for 48 hours for each strain (10 tests of 50 tubes for each of the 10 strains). After 48 hours of incubation , acid production in the tubes turned color from blue/purple to yellow. In the tube containing the substrate esculin the color turned black. The summary results were noted for each tube in part: positive tubes at which the color turned yellow, meaning that the strain ferments sugars. A color with shade between green and yellow was considered as unsatisfactory (Kos

et al., 2008). Negative reactions were assigned for those tubes that have kept blue, meaning that in the respective probes the strain didn't ferment sugars. Bacterial identification was performed using the dedicated software apiweb version 5.1 (Biomerieux, France) which compared biochemical profile obtained for each strain with existing data.
We have evaluated for each strain the percentage of profile's identification relative to other similar taxa in the database. T index estimated how close was the profile from the typical reaction set for each taxon listed in the API 50 CHL Analytical Profile Index (number of ranges between 0 and 1) and was inversely proportional to the number of atypical tests. An excellent identification required a rate of over 99 % and T index more than 0.75. Computer cluster analysis of the fermentation profiles was performed based on the calculation of Pearson corelation's coefficient and average linkage analysis using Apriori algorithm on SPPS modeler version 14.2 (IBM). Pearson's correlations between lactose concentration, identification rate and T index were realised on SPSS version 19 (IBM).

RESULTS AND DISCUSSIONS

In our study we have identified 10 strains of lactic acid bacteria to survive to multiple freeze-thaw cycles , namely: 7 strains of *Lactobacillus paracasei ssp paracasei*, 1 strain of *Lactobacillus fermentum* , 1 strain of *Lactobacillus acidophilus*, one strain of *Lactococcus lactis ssp lactis* (Figure 1).
All the isolates belonging to *Lactobacillus paracasei ssp paracasei* strains (L6, L8, L15, L16, L18, L21, L22) were able to ferment ribose, galactose, glucose, fructose, mannose, lactose, mannitol, sorbitol, n-acetyl-glucosamine, amygdaline, arbutine, esculine, salicine, cellobiose, maltose, saccharose, trehalose, melezitose and gluconate, showing some differences with the findings published by Kadere et al., (2012) regarding inuline, turanose, tagatose, sorbose (Figure 1).
Lactobacillus fermentum strain (L1) fermented arabinose, ribose, raffinose, galactose, glucose, fructose, mannose, lactose, melibiose, esculine, salicine, maltose, saccharose. Maroki et al., (2011) described for this species also the ability of fermenting rhamnose and tagatose. *Lactobacillus acidophilus* strain (L3) has shown a carbohydrate utilisation pattern including salicine, n-acetyl-glucosamine, inuline, saccharose, raffinose, mannose, galactose, glucose, fructose, lactose, arbutine , cellobiose, maltose. Unfortunately available data from other studies suggest that the use of the API 50 CH database for identification of commensal *Lactobacillus* species could lead to misidentification or

uninterpretable results. Boyd et al(2005) have found that over half of the *Lactobacillus jensenii* and *Lactobacillus gasseri* isolates were erroneously identified as *Lactobacillus acidophilus.*

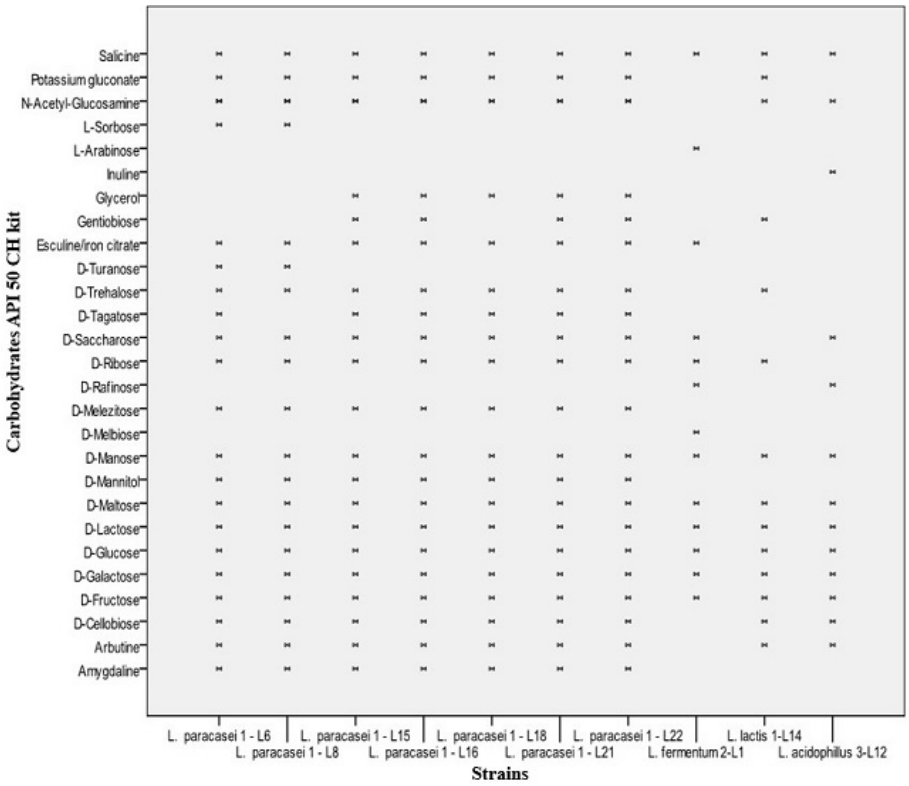
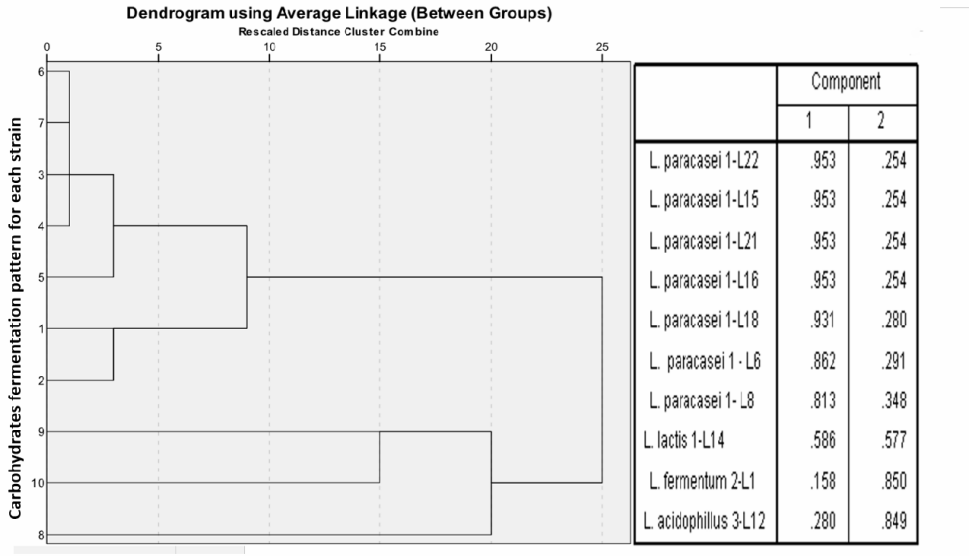


Figure 1. Strain's carbohydrates fermentation patterns on API 50 CH panel

Lactoccocus lactis ssp lactis strain (L14) fermented salicine, n-acetyl-glucosamine, gluconate, gentiobiose, trehalose, ribose, mannose, galactose, glucose, fructose, lactose, arbutine , cellobiose, maltose. Doutoum et al., (2013) have identified multiple *Lactococcus lactis* strains on API 20 CH kit with different percentages of confidence (SLA 1 – 43.8%, SLA6- 59.9 %, SLA3- 95.5%, SLA4-97.5%, SLA5-99.8%). Carbohydrate fermentation pattern for our strain on API 50 CH revealed affinity also for rhamnose, mannitol, sorbitol, esculine, sucrose hence there is also a variability in the biochemical characteristics of *Lactoccocus lactis ssp lactis*.

Applying hierarchical clustering method (Figure 2) we estimated the similarity among the ten strains carbohydrate fermentation profiles. Pearson's correlations coefficients values on strain pairs were studied regarding these patterns. We have used average linkage between groups with Apriori clustering algorithm to build the dendrogram for *Lactobacillus*

isolates according to similar approaches described by other authors Dimitonova et al., (2008). Cluster analysis indicated two main components when classifying the isolates: the first cluster included *Lactobacillus paracasei* strains (L6, L8, L15, L16, L18, L21, L22), the second one comprising *Lactobacillus fermentum* strain(L1), *Lactoccocus lactis ssp lactis* strain (L14), *Lactobacillus acidophilus* strain (L12).



	Component	
	1	2
L. paracasei 1-L22	.953	.254
L. paracasei 1-L15	.953	.254
L. paracasei 1-L21	.953	.254
L. paracasei 1-L16	.953	.254
L. paracasei 1-L18	.931	.280
L. paracasei 1 - L6	.862	.291
L. paracasei 1- L8	.813	.348
L. lactis 1-L14	.586	.577
L. fermentum 2-L1	.158	.850
L. acidophillus 3-L12	.280	.849

Figure 2. Dendrogram among the ten isolates from human milk (Apriori clustering algorithm)

Despite similar patterns in carbohydrates fermentation and statistically significant Pearson coefficients we could notice some differences between *Lactobacillus paracasei ssp paracasei* (L6, L8, L15, L16, L18, L21, L22). Glycerol was utilised as substrate for L15, L16,L18,L21,L22, trehalose for all the members, gentiobiose only for L15, L16, sorbose and turanose for L6, L8. There is a considerable metabolic capability for *Lactobacillus paracasei* species according to Kadere et al., (2012). The affinity for glycerol and trehalose for *Lactobacillus paracasei ssp paracasei* could explain the survivability to multiple freeze-thaw cycles.

In the other group, besides the common carbohydrates fermentation characteristics, *Lactobacillus fermentum* biochemical capabilities revealed catabolism of sorbose, esculine, melbiose catabolism whereas *Lactobacillus acidophilus* expressed affinity for inuline, raffinose and *Lactoccocus lactis ssp lactis* preferences for trehalose, gentiobiose, gluconate and ribose. Taxonomic identification by API 50 CH kits is still widely used, because it is

practical, easy to perform despite the increasing number of lactic acid bacteria with small differences in biochemical traits (Herbel et al., 2013). Our results overlapped with other studies findings. We have set the confidence interval for identification between 75% (acceptable) and 100% (excellent).

Seven probes have been identified as *Lactobacillus paracasei ssp paracasei* (Figure.3), five strains with a percentage of 99.9% and a T index of 0.88 . These strains had a heterofermentative metabolism type. The remaining two isolates were also identified at an excelent rate of 99.8% and a T index of 0.94 . In current literature identtification rates for this species are 92.5% (Karna et al., 2007), 98.15 % (Hyun jue Kim et al., 2006), and 99.9 % (Karna et al., 2007)**.**

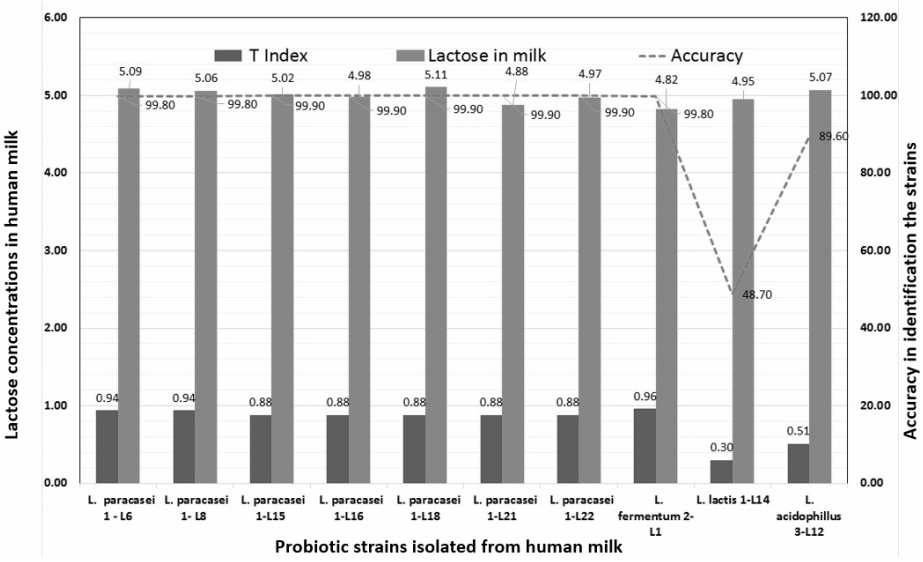


Figure 3. Sinopsis of data among the ten milk probes: lactose concentrations, strain species, strain's identification rate (accuracy), T index.

Lactobacillus fermentum strain was isolated with a rate of 99.8% and a T index of 0.96. In a study regarding infant's gut microbiota, *Lactobacillus fermentum* was isolated from the faeces of infants with an identification rate of 63% from 55 isolates (Khalil et al., 2007).

Lactobacillus acidophilus strain had a percentage of 89.6% and a T index of 0.51, implying a lower taxonomic significance compared with *Lactobacillus paracasei ssp paracasei* or *Lactobacillus fermentum*. Sources of isolation and API 50 CH identification of *Lactobacillus acidophilus* (described in the papers) with good values regarding accuracy (more than 93%) are represented by : 1. Commercial milk (Karna et al., 2007)**;** 2. Yogurt

(*Lactobacillus acidophilus LA15,* Zheng et al., 2013)**;** 3. Faeces of breast-fed infants (Khalil et al., 2007)**;** 4. Digestive tract of sacrificed chickens, *Lactobacillus ssp acidophilus 1 and 3* (Ashraf et al., 2009),(Kizerwetter et al., 2005); 5. Collections of reference for example *Lactobacillus acidophilus M 92* (Kos et al., 2008), (Çakmakci et al., 2012). Not all the studies on *Lactobacillus acidophilus* strain identification rate revealed good percentages. Kizerwetter's findings in species isolated from the digestive tract of sacrificed chickens have shown an unsatisfactory identification percentage of 51% (Kizerwetter et al., 2005). For this species our identification rate (89.6%) is placed between acceptable and good and is close but superior to other results. *Lactobacillus acidophilus* strains for example, isolated from the faeces of infants were identified with an acceptable value of 84.7% (Khalil et al., 2007). *Lactobacillus acidophilus* is described as a reactive or nonreactive strain for all 50 tests in many published papers (Herbel et al., 2013). *Lactoccocus lactis ssp lactis* identified in our study had a rate below 50% and was considered unacceptable. There are also reports similar with our results (43.8%) (Doutoum et al., 2013) although other authors, Kizerwetter et al(2005) reported admissible values of 84.2%.

Lactobacilli have the posibility of genome expansion or contraction with the deprivation of certain carbohydrate transporters types useful in the process of adapting to the specific environments. It is well established that *lactobacilli* select one main carbohydrate substrate and lactose from human milk is the most appropriate candidate (Klaenhammer et al.,2007) Regulation of carbohydrate metabolism has been identified in multiple genome studies including *Lactobacillus acidophilus NCFM* and *Lactoccocus lactis* (O'Donnell et al.,2013). The authors demonstrated in their review that multiple studies have indicated an increase in the abundance of pyruvate kinase in the presence of lactose. Pyruvate kinase concentrations suggest carbon catabolite repression, promoting the downregulation of genes for nucleotide metabolism. Constant exposure to lactose could influence catabolic genes expression of these lactic acid bacteria by amplifying carbon catabolite repression, possibly causing genome contraction.

We have used Pearson's Correlations applied between lactose levels in human milk, and accuracy in identification or T index. Pearson's correlations coefficients were neither statistical significant nor showing any tendency in this respect (table 1). Most probably these species use lactose as the most important carbohydrate source in breast milieu (Leloir pathway) but it is likely to possess an important load of pseudogenes (environment specific

genes) in their genomes which could explain phenotipic variability on the fermentation profiles. In our study all of isolated strains have presented affinity for maltose utilisation which according to O'Donnel et al., (2013) is internalised in the bacteria in a phosphorylated form by an enzyme (encoded by a gut activated specific gene).

Table 1. Pearson's Correlations applied between lactose levels in human milk, accuracy in identification and T index

Pearson's Correlations

		T index	Lactose in milk	Accuracy
T index	Pearson Correlation	1	-.033	.908**
	Sig. (2-tailed)		.929	.000
	N	10	10	10
Lactose in milk	Pearson Correlation	-.033	1	.112
	Sig. (2-tailed)	.929		.757
	N	10	10	10
Accuracy	Pearson Correlation	.908**	.112	1
	Sig. (2-tailed)	.000	.757	
	N	10	10	10

This enzyme works with the maltose phosphotransferase system. We could extrapolate considering that similar mechanisms would explain preferences for a particular carbohydrate substrate for each strain we have isolated. It seems that all of the isolates have shown an important metabolic flexibility being prepared to survive in infant's gut environment.

CONCLUSIONS

Lactobacilli species from human milk possess considerable adapting capability to survive infant's gut environment. This work has succesfully identified 3 subspecies of *Lactobacillus* (*ssp paracassei 1, ssp acidophilus 3, ssp fermentum 2*) with *paracassei 1* subspecies as the dominant ones (70%). Carbohydrate fermentation panel API 50 CHL has shown its reliability in the case of *Lactobacillus* species identification however due to genetically conditioned strain variances in metabolic performances we suggest that other studies should have an integrated approach (carbohydrates metabolism and functional genomics). Furthermore we believe that API database should be regularly updated based on these studies. Regarding lactose concentrations

influencing the expression of the genes, their regulatory networks and finaly fermentation behaviours on different carbohydrate substrates, we couldn't evidentiate any link between lactose concentrations in the milk probes and carbohydrate metabolic trends. More research data are needed on functional genomics concerning carbon catabolite repression.

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