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Enumeration of Lactobacilli in the Fecal Flora of Infant Usimg Two Different Modified de-Man Rogosa Sharpe Media under Aerobic and Anaerobic Incubation

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Abstract: Regarding the importance of the presence of intestinal lactobacilli and their population in infants, four different treatments were evaluated for *Lactobacillus* isolation efficiency via reduction in the growth of other groups of bacteria capable of growing on de-Man Rogosa Sharpe (MRS) medium from fecal samples of 11 Iranian infants. MRS-Vancomycin (1 mg L^{-1}) was used as a base medium and application of lactic acid and aerobic incubation of inoculated plates were performed as selective factors. Each fecal sample was cultivated as duplicate on to the base medium with or without lactic acid to reduce the pH to 5.4±0.2. Half of the plates were incubated aerobically and the rest of them incubated under 10% CO_2 concentration. Total count and *Lactobacillus* count of all samples were recorded according to the age differences of infants. The counts of false positive colonies were recorded with respect to their cell morphology and gram reaction in all treatments. Anaerobic incubation of lactic acid modified MRS-Vancomycin gave the most *Lactobacillus* percentage coverage, about 93% among the *Lactobacillus* positive samples. Using this treatment, the median *Lactobacillus* count yielded 8.29 $\log_{10}$ cfu g^{-1} in the younger and 5.70 $\log_{10}$ cfu g^{-1} in the elder group. It could be concluded that lactic acid might be a proper pH reducing agent when enumeration of lactobacilli from fecal samples is of interest.

Key words: Isolation, lactic acid, vancomycin, *Lactobacillus*, *Lactobacillus* percentage coverage, selective medium

INTRODUCTION

Lactobacilli are a heterogeneous group of microaerophilic, lactic acid producing, bacteria. They are usually present in the intestinal microbiota of healthy adults, although in substantially lower population counts than several other bacteria, through to represent 0-2% of gut microbiota (Björkstén *et al*., 2001; Mikelsaar *et al*., 2004; Ahrné *et al*., 2005). During the past twenty years researches on isolation, identification and prevalence of *Lactobacillus* flora of the gastrointestinal tract have received special interest because of their health promoting effects. The gut flora as such is essential for mucosal immune stimulation, amplification of immune-competent cells and competitive exclusion of pathogens; these actions appear correlated in infants (Ahrné *et al*., 2005; Westerbeek *et al*., 2006). In newborn infants and small children, the extent to which lactobacilli colonize the intestine and their colonization rate is controversial (Ahrné *et al*., 2005) From the physiological point of view, the *Lactobacillus* flora change by age (Mitsuoka, 1992; Mikelsaar *et al*., 2004) Some studies claim that the *Lactobacillus* colonization pattern was completely different before and after 6 months of age (Ahrné *et al*., 2005) and from the ecological point of view, the result of some studies claim lower-level presence of lactobacilli in infants and children in industrial countries rather than in developing countries (Björkstén *et al*., 2001; Ahrné *et al*., 2005). Variation in methodologies and different geographical areas may account for these differences (Ahrné *et al*., 2005). Although *Lactobacillus* selective (LBS) medium or Rogosa medium (Rogosa *et al*., 1951) provides specific condition for *Lactobacillus* growth via pH reduction by acetic acid and sodium acetate, enumeration of lactobacilli from the stool samples using this medium is not very easy because of the interference of bifidobacteria and occasional streptococci (Sharpe, 1986; Kandler and Weiss, 1986; Ahrné *et al*., 2005) with the growth of these bacteria. It has been shown that the prevalence of these microorganisms is more usual than lactobacilli in the fecal microflora of infants (Mitsuoka, 1992; Mikelsaar *et al*., 2004). Several studies have claimed

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that bifidobacteria were dominant over lactobacilli especially in breast fed infants before 6 month of age (Mountzouris *et al*., 2002; Ahrné *et al*., 2005). Bifidobacteria are strictly anaerobes (Scardovi, 1986; Mitsuoka, 1992) and aerobic incubation of *Lactobacillus* selective media improved specificity of the medium when selective isolation of lactobacilli from fecal samples was of interest (Vael *et al*., 2005). *Lactobacillus* anaerobic MRS agar with Vancomycin and Bromocresol (LAMVAB) medium was also designed for isolation and enumeration of lactobacilli from fecal samples based on low pH and Vancomycin resistance of lactobacilli which is unusual for any Gram-positive bacteria (Hartemink *et al*., 1997). The results of recent studies have shown that these media still are not efficient enough in enumeration of lactobacilli from fecal samples; Rogosa agar was more likely to support growth of non-*Lactobacillus* species (Hartemink and Rombouts, 1999; Jackson *et al*., 2002; Beasley, 2004; Vael *et al*., 2005) and LAMVAB agar could not support the growth of some strains of *Lactobacillus acidophilus* group and gave lower *Lactobacillus* counts than unspecific MRS (Jackson *et al*., 2002; Leuschner *et al*., 2003; Vael *et al*., 2005). It is known that acetic acid used in above mentioned selective media at a given pH has more growth inhibitive effect on microorganisms than lactic acid (Halm *et al*., 2004), in addition, bifidobacteria produce acetic acid and lactic acid in the molar ratio of 3:2 during fermentation (Scardovi, 1986). So they may be more resistant to acetic acid than lactobacilli in acetic acid modified medium. On the other hand, lactic acid is the main metabolite of lactobacilli (Siegumfeldt *et al*., 2000; Pieterse *et al*., 2005) and their tolerance to lactic acid may provide their selective dominance in the isolation medium as it happens in the natural fermentation process.

This study aimed to assess the application of lactic acid as the pH reducing component of the isolation media and aerobic incubation to inhibit the growth of complete anaerobes such as bifidobacteria and other inhabitants of the intestine which are capable of growth in MRS and therefore it was desirable to validate the efficacy of the methods by identification and enumeration of the false positive colonies, *Lactobacillus* count and *Lactobacillus* percentage coverage of each treatment. Because there was not any previous data of the *Lactobacillus* count in the fecal flora of Iranian infants, categorization of subjects before and after 6 month was performed to reduce the variation in the *Lactobacillus* count in the data analyzing step.

MATERIALS AND METHODS

Preparation of samples: The feces samples of eleven healthy infants (a) 3-6 month, n = 5 and (b) 12-21 month, n = 6 were used for the experiments. The experiments were carried out during March-May 2007. Fresh feces was collected from the infants by their parents and placed in tight plastic boxes at home, 8-12 h before running the experiment. The samples were kept refrigerated up until received by the laboratory, where they were processed as soon as possible.

Experimental design and inoculation of culture media: Isolation of lactobacilli was performed using four different treatments. In all treatments MRS (Scharlou, Spain)-Vancomycin (1 mg L^{-1}) (VMRS) agar was used as the base culture medium. The pH of half of the media was reduced to 5.5±0.2 by 90% lactic acid (Merck, Germany) and it was called LVMRS. For each sample, a pair of plates containing the same medium was prepared. After inoculation, one of the plates was kept aerobically and the other one was incubated under 10% CO_2 anaerobic environment, obtained with the CO_2 generating Gas pack A system (Merck, Darmstadt, Germany). Half a gram of each sample was placed in a sterilized flask, mixed with 5 mL of sterilized normal saline and centrifuged at low speed (100 rpm) for 1 min. One milliliter of the upper phase was collected and serially diluted to 10^{-8} dilution. One hundred micro liters of the diluted samples (10^{-3} to 10^{-8}) was inoculated on the MRS-Vancomycin or MRS-Vancomycin-lactic acid agar and all the plates were then incubated at 37°C for 72 h.

Bacterial counting: Plates containing 25-250 colonies were selected and representative colonies of each morphotype (differing in size, shape, color or texture from other colonies) were enumerated. Cell morphology and Gram reaction were examined using phase contrast microscopy. Cell morphotypes were categorized in four groups; Gram- positive cocci or oval shape bacteria, Gram-positive branched rods, Gram-positive rods, Gram negative rods and yeast-like morphotypes. Each cell morphotype was enumerated. Gram-positive, rod isolates were sub-cultured to purity (1-4 per sample) on MRS and tested further for catalase reaction by 3% H_2O_2 and spore formation. All rod, aerotolorant, Gram-positive, catalase-negative, non-spore-former, non-motile isolates were regarded as lactobacilli. Colony and cell morphology were recorded by a single observer. The number of *Lactobacillus* and total colonies ($\log_{10}$ cfu g^{-1}) developed on the media of each particular treatment were calculated for each stool sample.

Lactobacillus percentage coverage: The proportion percentage of the number of *Lactobacillus* count to the total colony count for each *Lactobacillus*-positive sample in each treatment was recorded as a representative data of the *Lactobacillus* percentage coverage of that treatment.

Statistical methods: Data analysis was carried out with Minitab 15 software. One way ANOVA was used to study significant differences between means, at $\alpha = 0.05$ level. Tukey's test was used to perform multiple comparisons of the means.

RESULTS

Lactobacilli isolated from 6 out of 11 total tested fecal samples using at least one of the four tested treatments. The range and median viable counts ($\log_{10}$ cfu g^{-1}) of different morphotypes containing Gram-positive cocci (enterococci, staphylococci and streptococci) and oval morphotypes, Gram-positive rods, Gram-positive-branched rods, Gram-negative rods and yeast-like cell morphotypes which regarded as false positive colonies in *Lactobacillus* isolation media of 6 *Lactobacillus* positive fecal samples, using four different treatments are shown in Table 1.

Vancomycin, at the concentration used for this study, could not induce an inhibitive effect on the growth of other groups of bacteria on MRS especially, Gram-positive cocci observed on all tested samples at the high population range of 5.37-9.3 $\log_{10}$ cfu g^{-1}, cultured on VMRS in spite of aerobic or anaerobic incubation. Gram-positive branched rods also occurred on anaerobic incubated plates containing this medium at a population range of 6-8.45 $\log_{10}$ cfu g^{-1}. They were also observed a substantially lower number on a few aerobic incubated plates containing this medium. These two cell morphotypes were also the most frequent morphotypes in *Lactobacillus* negative samples. Gram negative rods and yeast like morphotypes were detected in VMRS although they did not seem to be main disturbing microorganisms because of their rare incidence in tested agars. Addition of lactic acid to VMRS reduced the viable counts of Gram-positive cocci and Gram-positive branched rods. None of the Gram-positive branched rods were found on aerobic incubated plates containing this media anymore and no Gram-negative rod cell morphotype grew under both aerobic and anaerobic incubated condition. The counts of yeast-like microorganisms seemed unaffected by addition of lactic acid, although they were mostly detected from fecal samples belonging to the elder subjects and developed under aerobic environments. Gram positive rods primarily regarded as lactobacilli grew in VMRS at the population range of 0-8.9 $\log_{10}$ cfu g^{-1} under anaerobic and 0-8.47 $\log_{10}$ cfu g^{-1} under aerobic incubation. The minimum count of this morphotype was increased when lactic acid was added to the media but only anaerobic incubation of LVMRS gave about 1.7 $\log_{10}$ cfu g^{-1} median counts of this group more than that in VMRS under the same incubation condition.

The results of total and *Lactobacillus* counts of 11 fecal samples enumerated on examined agars using different treatments in 3-6 months infants (group a) and in 12-21 months infants (group b) have been shown in Table 2. Comparison of the total and *Lactobacillus* counts between two age groups, resulted significant differences; using four treatments total and *Lactobacillus* count of samples from younger group was more than that in the elder group. In both age groups the total anaerobes which grew in each medium out numbered the total

Table 1: Evaluation of different cell morphotypes from 6 *Lactobacillus* positive fecal samples

Cell morphotypes	VMRS (pH = 6.2)		LVMRS (pH = 5.4)	
	Anaerobic	Aerobic	Anaerobic	Aerobic
Gram-positive	6.477-9.3*	5.37-8.69	4.9-7.15	5.28-6.62
cocci or oval shape	7.9**	7.12	6.30	5.05
Gram-positive	0-8.9	0-8.47	5.46-8.92	4.54-7.50
rods	5.4	6.637	7.10	5.98
Gram-positive	6.00-8.45	0-8.00	5.2-7.3	0.00-0.00
branched rod	8.08	3.98	6.84	0.00
Gram-negative	0.00-6.90	0.00-5.5	0.00	0.00-0.00
rods	0.00	0.00	0.00	0.00
Yeast type	0.00-0.00	0.00-7.2	0.00-0.00	0.00-7.11
	0.00	5.71	0.00	5.46

*: Minimum-maximum range count (log cfu g^{-1} of the feces), **: Median count (log cfu g^{-1} of the feces)

Table 2: Counts ($\log_{10}$ cfu g^{-1}) of total and *Lactobacillus* colonies and *Lactobacillus* percentage coverage obtained by different treatments in two age groups of infant

Treatments	a (n = 6)		b (n = 5)		Coverage (%)***
	Ta	La	Tb	Lb	
VMRS -anaerobic	9.967±0.36A*	2.97±2.97	7.676±0.409A	1.83±1.83	58.6
	9.690**	0.000	7.600	0.000	
VMRS-aerobic	8.725±0.193A	3.73±2.19	6.764±0.453A	2.26±1.43	83.5
	8.839	3.23	6.949	0.000	
LVMRS-anaerobic	8.523±0.291AB	5.74±2.87	7.117±0.378A	4.63±1.58	93.6
	8.420	8.29	6.434	5.71	
LVMRS-anaerobic	7.625±0.504B	5.22±1.77	5.763±0.266B	3.88±1.33	87.1
	7.575	6.69	5.597	4.81	

Ta: Total count and La: Lactobacillus count in the younger g roup (3-6 months), Tb: Total count and Lb: *Lactobacillus* count in the elder group (12-21 months). *: Mean counts ($\log_{10}$ cfu g^{-1}) are expressed as mean±standard error of means, Means in the same column followed by different uppercase letter(s) are significantly different ($p<0.05$), **: Median counts ($\log_{10}$g cfu g^{-1}), ***: *Lactobacillus* percentage coverage of VMRS and LVMRS under aerobic and anaerobic incubation from *Lactobacillus* positive samples

aerobes capable of growth in that medium by about 1-1.4 $\log_{10}$ cfu g^{-1}. However, only aerobic incubation of LVMRS reduced the mean total colony count significantly in the younger ($p = 0.01$) and in the elder ($p = 0.036$) groups. Three fecal samples in each age group harbored lactobacilli. None of the treatments carried out, revealed a significant difference in the *Lactobacillus* count but the *Lactobacillus* count obtained in anaerobic incubation of LVMRS in both age groups was relatively higher than in other treatments, giving the median *Lactobacillus* count of 8.29 $\log_{10}$ cfu g^{-1} in the younger and 5.7 $\log_{10}$ cfu g^{-1} in the elder group in addition the most *Lactobacillus* percentage coverage about 93% was obtained by this treatment in *Lactobacillus* positive samples.

DISCUSSION

The result of this study revealed that the reduction of the pH of the medium is a main selective factor for isolation and enumeration of lactobacilli from fecal samples. lactic acid modified VMRS used in this study could effectively differentiate *Lactobacillus* colonies and other microorganisms capable of growth on VMRS medium therefore, lactic acid which is the predominant metabolite biosynthesized during the growth and fermentation of lactobacilli, could be a proper pH-reducing ingredient for *Lactobacillus* isolation from environments such as fecal samples. Acidified MRS media, by acetic acid or acetate introduced earlier for this purpose (Rogosa *et al*., 1951; Sabine and Vaselekos, 1965; Kandler and Weiss, 1986; Sharpe, 1986) but recent studies, based on molecular methods, have claimed that these media still do not have sufficient efficiency for enumeration of *Lactobacillus* species from human fecal samples (Jackson *et al*., 2002; Ahrné *et al*., 2005). In another study, plating the canine fecal samples on the LBS amended with acetic acid yielded no colonies (Beasley, 2004). As the results showed that false positive cocci and branched rods also could grow in the lactic acid modified VMRS (Table 1), comparing the *Lactobacillus* recovery of this medium, containing higher concentration of lactic acid with acetic acid modified MRS could be helpful.

The significant higher counts of total and *Lactobacillus* colonies in the younger infants in all treatments showed that paying attention to the age group of infants could reduced the variation in the *Lactobacillus* count of their fecal flora however high variation in *Lactobacillus* count was obtained in each age group because of the little size of the study. Former study showed that the intestinal *Lactobacillus* colonization pattern is different before and after 6 month of age, between these two phases, the lactobacilli reach their lowest prevalence and population (Ahrné *et al*., 2005).

Regarding incubation condition, aerobic incubation could not be a selective factor for the purpose of the study. When VMRS is used as isolation media, false positive branched rods were detected in a few aerobic plates. These plates were mostly cultivated from fecal samples belonging to the younger subjects. As these morphotypes were regarded as *Bifidobacterium* species, their growth under aerobic environments may because of their high population in these fecal samples. When the lactic acid modified VMRS was used, aerobic incubation both reduced the total colony count and false positive colonies of Gram-positive cocci. It also reduced the median *Lactobacillus* count of *Lactobacillus* positive samples in both age groups of infants. This is in agreement with previous report which claimed aerobic incubation of MRS improved its specificity and reduced its sensitivity for *Lactobacillus* recovery among different strains isolated from fecal samples (Vael *et al*., 2005).

Modification of MRS with 1 mg L^{-1} Vancomycin, could not be a selective medium for *Lactobacillus* recovery from fecal samples. This medium introduced earlier for enumeration of some probiotic *Lactobacillus* species in mixed cultures containing some other lactic acid bacteria and bifidobacteria species under anaerobic environment (Thamaraj and Shah, 2003). in the preliminary examination of this study, performed on a few fecal samples, this medium gave about 1 log cfu g^{-1} lower total counts than MRS under anaerobic environments (data was not shown) therefore, higher concentration of Vancomycin was not tested in this experiment. Concentration of Vancomycin in LAVAB medium is 20 times greater than that in this study (Hartemink *et al*., 1997). LAVAB was shown to inhibit the growth of some *Lactobacillus* species (Jackson *et al*., 2002). High concentration of Vancomycin (10-20 mg L^{-1}) also used for selective enumeration of *L. caei* group from probiotic cheese (Phillips *et al*., 2006) and fecal samples (Marzotto *et al*., 2006).

Based on former documentations, the lactoflora of infants develop during the first year of life (Mitsuoka, 1992) and the intestinal bacterial structure of infant aged 12-24 months is in a transitional state combining neonate and adult-like features (Marzotto *et al*., 2006). When a child starts to eat solid food, the fecal flora of children closely resembles that of adults where the counts of lactobacilli are usually less than 10^7 cfu g^{-1} of the feces (Mitsuoka, 1992) In this study, the types of nutrition was not the focus because tested infants in the younger group were both breast-fed and formula-fed and most of the elder infants still received breast milk in addition to bottle milk and solid foods. In this study, most of examined morphotypes seemed to be enterococci, streptococci and

bifidobacteria in both age groups with higher counts in the younger group. Yeast-like cell morphotypes developed on aerobic methodologies only from the samples belonged to the elder group. This may be because of variation in fermented foods introduced in to the elder children's diet.

As the type of nutrition is a principal factor for establishment of *Lactobacillus* flora, further studies aimed at enumeration of lactobacilli in Iranian infants concerning the impact of food in addition to age group with larger populations, will be of interest to provide more knowledge of colonization phase and pattern of these important parts of our intestinal microbiota.

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REFERENCES

Ahrné, S., E. Lönnermark, A.E. Wold, N. Aberg, B. Hesselmar, R. Saalman, I.L. Strannegard, G. Molin and I. Adlerberth, 2005. Lactobacilli in the intestinal microbiota of Swedish infants. Microbiol. Infect., 7: 1256-1262.

Beasley, S.H., 2004. Isolation, identification and exploitation of lactic acid bacteria from human and animal microbiota. Academic Thesis in Microbiology. University of Helsinki.

Björkstén, B., E. Sepp, K. Julge, T. Voor and M. Mikelsaar, 2001. Allergy development and the intestinal microflora during the first year of life. J. Allergy Clin. Immunol., 108 (4): 516-520.

Halm, M., T. Hornbæk, N. Arneborg, S. Sefa-Dedeh and L. Jespersen, 2004. Lactic acid tolerance determined by measurement of intracellular pH of single cells of *Candida Krusei* and *Saccharomyces cervisiae* isolated from fermented maize dough. Int. J. Food Microbiol., 94: 97-103.

Hartemink, R., V.R., Domenech and F.M. Rombouts, 1997. LAMVAB-A new selective medium for the isolation of lactobacilli from faeces. J. Microbiol. Meth., 29 (2): 77-84.

Hartemink, R. and F.M. Rombouts, 1999. Comparison of media for the detection of bifidobacteria, lactobacilli and total anaerobes from faecal samples. J. Microbiol. Meth., 36 (3): 181-192.

Jackson, M.S., A.R. Bird and A.L. McOrist, 2002. Comparison of selective media for the detection and enumeration of Lactobacilli in human faeces. J. Microbiol. Meth., 51 (3): 313-321.

Kandler, O. and N. Weiss, 1986. Genus Lactobacillus. In: Bergey's Manual of Systematic Bacteriology, Sneath, P.H.A., J. Halt, N.S. Nair and M.E. Sharpe (Eds.). Williams and Wilkins, Baltimore, pp: 1216.

Leuschner, R.G.K., J. Bew, V. Coeuret, J.P. Vernoux and M. Gueguen, 2003. A collaborative study of a method for the enumeration of probiotic lactobacilli in animal feed. Food Microbiol., 20 (1): 57-66.

Marzotto, M., C. Maffeis and T.H. Paternoster, 2006. *Lactobacillus paracasei* a survives gastrointestinal passage and affects the fecal microbiota of healthy infants. Res. Microbiol., 157: 857-866.

Mikelsaar, M., R. Mändar, E. Sepp and H. Annuk, 2004. Human Lactic Acid Microflora and its Role in the Welfare of the Host. In: Lactic Acid Bacteria, Salminen, S. and A. von Wright (Eds.). Marcel Dekker. New York, pp: 455-471

Mitsuoka, T., 1992. The Human Gastrointestinal Tract. In: The Lactic Acid Bacteria in Health and Disease, Wood, B.J.B. (Ed.). Vol. 1. Elsevier Applied Science. London, pp: 69-98.

Mountzouris, K.C., A.L. McCartney and R.G. Gibson, 2002. Intestinal microflora of human infants and current trends for its nutritional modulation. Br. J. Nutr., 87: 405-420.

Phillips, M., K. Kailasapathy and L. Tran, 2006. Viability of commercial probiotic cultures (*L. acidophilus*, *Bifidobacterium* sp., *L. casei*, *L. paracasei* and *L. rhamnosus*) in cheddar cheese. Int. J. Food Microbiol., 108: 276-280.

Pieterse, B., R.J. Leer, H.J. Schuren and M.J. van derWerf, 2005. Unravelling the multiple effect of lactic acid stress on *Lactobacillus plantarum* by transcription profiling. Microbiology, 151: 3881-3894.

Rogosa, M., J.A. Mitchell and R.F. Wiseman, 1951. A selective medium for the isolation and enumeration of oral and feacal *Lactobacilli*. J. Bact., 62: 132.

Sabine, D.B. and J. Vaselekos, 1965. Isolation of *Lactobacillus acidophilus* from faecal material. Nature, 206: 960-961.

Scardovi, V., 1986. Genus Bifidobacterium. In: Bergey's Manual of Systematic Bacteriology, Sneath, P.H.A., J. Halt, N.S. Nair and M.E. Sharpe (Eds.). Williams and Wilkins Baltimore, pp: 1418.

Sharpe, M.E., 1986. The Genus *Lactobacillus*. In: The Prokaryotes, Starp, M.P., H. Stolp, H.G. Trüper, A. Balows and H.G. Schlegel (Eds.). Vol. 2. Springer-Verlag. Berlin, pp: 1653-1662.

Siegumfeldt, H., K.B. Rechinger and M. Jackobsen, 2000. Dynamic changes of intracellular pH in individual lactic acid bacterium cells in response to a rapid drop in extracellular pH. Applied Environ. Microbiol., 66 (6): 2330-2335.

Thamaraj, N. and N.P. Shah, 2003. Selective enumeration of *Lactobacillus delbruckii* sp. *Bulgaricus*, *Streptococcus thermophilus*, *Lactobacillus acidophilus*, Bifidobactera, *Lactobacillus casei*, *Lactobacillus rhamnossus* and Propionibacteria. J. Dairy Res., 86: 2288-2296.

Vael, C., A. De Coninck, C. Lammens, K. Desager and H. Goossens, 2005. Evaluation of selective culture media for *Lactobacillus*, *Bifidobacterium* and *Bacteriods* to study the intestinal flora. In: Proceeding of the 15th European Congress of Clinical Microbiology and Infectious Diseases, 2-5 April, Copenhag, Denmark.

Westerbeek, E.A.M., A. Van den Berg, H.N. Lafeber, J. Knol, W.P.F. Fetter and R.M. Van Elburg, 2006. The intestinal bacterial colonization in preterm infants: A review of the literature. Clin. Nutr., 25: 361-368.