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Evaluation of Selective Media for Enumeration of *Lactobacillus acidophilus* and *Bifidobacterium animalis* Present in Probiotic Formulations

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ABSTRACT

The health benefits of probiotic organisms are well documented. A large number of pharmaceutical formulations containing probiotic microorganisms are available worldwide. These formulations are generally composed of organisms specifically from the genera *Lactobacillus* and *Bifidobacterium*. To comply with the regulatory need for pharmaceutical preparations, the label claims of these types of microorganisms have to be maintained throughout the shelf life of the product. So the quality control specifications

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demand the development of different selective media for the enumeration of each type of microorganisms present in the formulations. The selective media for *Lactobacillus acidophilus* using ofloxacin (MRS) and *Bifidobacterium animalis* using mupirocin (MRS) were already reported. In this study the efficiency of MRS-Ofloxacin and MRS-Mupirocin media were evaluated for selective enumeration of *Lactobacillus acidophilus* ATCC 4356 and *Bifidobacterium animalis* ATCC 25527 respectively from their mixed population. Performance of the selective media was also statistically validated.

Keywords: Selective enumeration, *Lactobacillus acidophilus*, *Bifidobacterium animalis*, Statistical validation

Abbreviation: WHO – World Health Organization, MRS - de Man, Rogosa and Sharpe, ATCC - American Type Culture Collection, ISO - International Organization for Standardization

1. INTRODUCTION

Probiotics are living organisms which confer a health benefit on the host (FAO/WHO, 2001). These microorganisms have a long history of safe consumption as fermented foods. The WHO recently recommended the implementation of alternative disease control measures to combat the illness caused by deficient or compromised intestinal microflora by utilizing the prophylactic and therapeutic potential of probiotic microorganisms (Bengmark, 1998; Daly and Davis, 1998). Due to its health benefits, probiotics generated considerable interest in the area of pharmaceutical formulations.

Probiotics contain live lactic acid bacteria belonging to the most typical representatives of normal human intestinal microflora (Astashkina et al, 2014). To qualify for the therapeutic segment probiotic preparations must meet strict criteria related to quality, safety and functionality (Vankerckhoven et al, 2008). A key quality criterion is that they should contain accurately defined numbers of viable cells as claimed on the product label throughout its shelf life.

It is critical to satisfy the quality criteria for the determination of total count and differential count of any probiotic formulation. Culture based enumeration of specific microorganism is the most popular approach to meet this challenge. In this method specific bacterium can replicate on a particular medium under specific conditions by following specialized and standardized methodologies (Davis, 2014).

Pharmaceutical formulations containing different probiotic microorganisms are available as therapeutics. Majority of these organisms are members specifically from genera *Lactobacillus*, *Lactococcus,* and *Bifidobacterium* (Kaur et al, 2002). Different media have been proposed for selective enumeration of *Lactobacillus acidophilus* and *Bifidobacterium* sp from mixed populations (Bhattacharjee et al, 2014; Coeuret et al, 2003; Serafini et al, 2011). In this study we have used the selective agents like ofloxacin (Bhattacharjee et al, 2014) and mupirocin (Serafini et al, 2011) supplemented in MRS media (De Man et al, 1960) for the selective enumeration of *Lactobacillus acidophilus* and *Bifidobacterium animalis* from probiotic formulations. The developed method was also statistically validated.

2. MATERIALS AND METHODS

Bacterial strains, media and growth conditions:

Freeze dried pure cultures of *Lactobacillus acidophilus* ATCC 4356 and *Bifidobacterium animalis* ATCC 25527 were procured from LGC Promochem, India.

These organisms are anaerobic. Both of these two strains (ATCC 4356 & ATCC 25527) were maintained in MRS (Difco) medium (De Man et al, 1960). The incubation was anaerobic using anaerobic gas jar and anaerobic gas pack (HI MEDIA) and incubation period was 48 hours at 37^0C. For the preparation of selective media, MRS LCys HCl medium (mMRS) was supplemented with ofloxacin (10µg/ml) or mupirocin (100µg/ml).

Preparation of reference medium:

Lactobacilli MRS (Difco) medium supplemented with L-Cystein Hydrochloride (at a final concentration of 0.05% w/v) was used as reference medium (mMRS).

Preparation of ofloxacin and mupirocin stock:

The ofloxacin (Sigma) stock solution (10mg/ml) was prepared in 0.1N hydrochloric acid. The stock solution was then sterilized by passing through 0.22 µm membrane filter (Millipore, India).

The mupirocin (HIMEDIA) stock solution (100mg/ml) was prepared in 0.1N NaOH solution. The stock solution was also sterilized by passing through 0.22µm membrane filter (Millipore, India).

Freshly prepared solutions were used for each experiment.

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Enumeration of viable count (CFU):

Single colony of each strain (ATCC 4356 & ATCC 25527) was inoculated separately in mMRS broth and incubated anaerobically at 37^{0}C for 48 hours for preparation of inoculum. The inoculum of both strains (0.1%) was further inoculated individually to fresh mMRS broth and grown for 48 hours at 37^{0}C. The broth cultures of these two organisms were then mixed at 1:1 ratio for preparation of a mixed culture. For determination of total viable count of pure culture and differential count of mixed culture each set of cultures were pour plated in reference medium and selective media (Fig 1,2,3). For each set of medium five plates were prepared. These plates were incubated anaerobically at 37^{0}C for 48 hours. The mean viable count (CFU/ml) for each strain was determined. The experiments were repeated for five times to validate the result statistically.

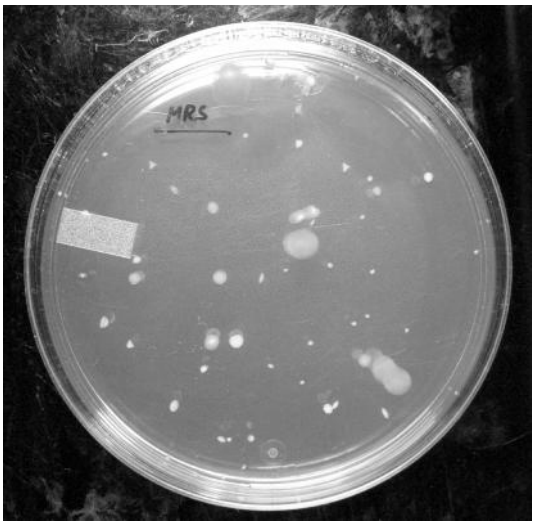

Figure 1 mMRS plate

Figure 2 mMRS Ofloxacin

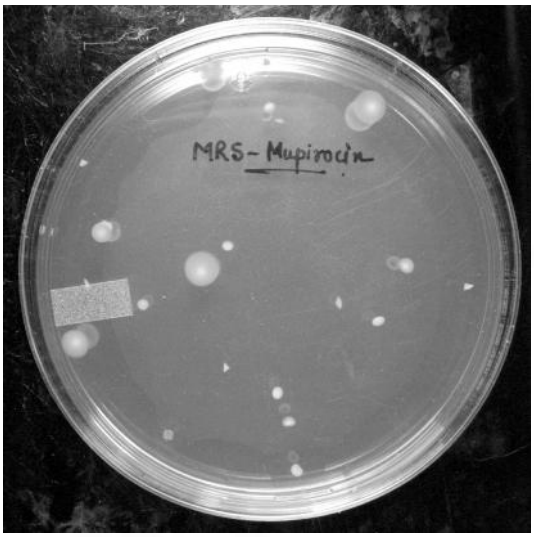

Figure 3 mMRS + Mupirocin

Statistical evaluation of the method:

To evaluate the efficiency of selective medium, the enumeration of both *Lactobacillus acidophilus* and *Bifidobacterium animalis* were performed in accordance to the recommendations of ISO/TR 13843 (ISO, 2000) and ISO/IEC 17025 (ISO, 2005) standards on validation for microbiological methods. The performances of selective media were validated on the basis of their precision, accuracy, reproducibility, selectivity and specificity characteristics.

The precision and accuracy of the method developed were calculated by relative standard deviation of differences, student's t-test and relative recovery. Reproducibility of the new method was tested by the results of two different workers who analyzed the identical samples. The selectivity of the method was determined by mixing (1:1) of broth culture of both *Lactobacillus*

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acidophilus ATCC 4356 and *Bifidobacterium animalis* ATCC 25527. Appropriate dilutions were pour-plated in selective media and reference medium. Results were expressed as percentage of presumptive target counts in selective media in relation to the reference medium (Tobasco et al, 2007). The student's t-test was performed with the log mean values (set of 5 experiments) of the total viable counts. The value of student's t-test was calculated from the formula $t = (d_{mean}/S_d)*\sqrt{n}$, where d_{mean} is the mean of differences, i.e. d_j (difference between log value of viable count in selective and reference media for each experiment). S_d is the standard deviation of d_j and n is the number of experiments (Martindale, 1999). Other parameters calculated were relative recovery = 10^{-dmean} and relative standard deviation of differences = $1\text{-}10^{-Sd}$ (Tobasco et al, 2007). Student's t-test was done by using MS Excel software (Microsoft, Redmond, WA, USA).

3. RESULTS AND DISCUSSIONS

Probiotics are living organisms. So the critical enumeration of viable counts of probiotic formulations is of much importance as this information is provided on the product label. The culture based enumeration is well accepted for organisms which enable to detect a specific organism from a mixture. There is no single culture based methodology applicable to all probiotic organisms, as there is considerable variability between species and even strains in their response towards selection (Atashkina et al, 2014). Quantification of bacteria in a given sample is routinely achieved by counting the total number of colony forming units (CFUs) grown on an agar plate from serial dilutions, expressed as CFUs per gram of original sample.

Ofloxacin (Bhattacharjee et al, 2014) and mupirocin (Serafini et al, 2011) are the two selective agents that can be used for enumeration of *Lactobacillus acidophilus* and *Bifidobacterium* sp. respectively from formulations. Here in this study we have utilized these two agents for selective enumeration of *Lactobacillus acidophilus* ATCC 4356 and *Bifidobacterium animalis* ATCC 25527 from their mixture. The statistical analyses of this method were also performed.

It was found that selective media mMRS-Ofloxacin and mMRS-Mupirocin were suited to recover closely similar viable count as compared with reference medium (mMRS) in case of *Lactobacillus acidophilus* ATCC 4356 and *Bifidobacterium animalis* ATCC 25527 (Table 1). To establish the efficiency of these two selective agents (ofloxacin and mupirocin) both the strains (ATCC 4356 and ATCC 25527) were mixed. The mixture was then plated on selective media as well as on reference medium. The viable count of mixture in reference medium was very similar to the sum total of recovery in both the selective medium (Table 1). Each experiment for determination of viable count in both reference medium and selective were repeated five times to perform the statistical data analysis of results (Table 2).

Table 1: Total and differential enumeration of *Lactobacillus acidophilus* and *Bifidobacterium animalis*

Organism	**SL. No.**	**Viable Count (CFU/ml) in Reference Medium**	**Viable Count (CFU/ml) in Selective Medium for *Lactobacillus acidophilus***	**Viable Count (CFU/ml) in Selective Medium for *Bifidobacterium animalis***
		(MRS-L-Cys-Agar)	(MRS-L-Cys--Of-Agar)	(MRS-L-Cys--Mup-Agar)
ATCC 4356	1	0.74×10^9	0.81×10^9	0
	2	0.81×10^9	0.79×10^9	0
	3	0.98×10^9	0.89×10^9	0
	4	0.92×10^9	0.85×10^9	0
	5	1.09×10^9	0.99×10^9	0
ATCC 25527	1	0.32×10^9	0	0.31×10^9
	2	0.51×10^9	0	0.56×10^9
	3	0.31×10^9	0	0.31×10^9
	4	0.45×10^9	0	0.52×10^9
	5	0.47×10^9	0	0.46×10^9
MIX of ATCC 4356 & ATCC 25527 (1:1)	1	1.06×10^9	1.06×10^9	0.44×10^9
	2	1.34×10^9	0.92×10^9	0.62×10^9
	3	1.40×10^9	1.04×10^9	0.36×10^9
	4	1.04×10^9	0.92×10^9	0.58×10^9
	5	1.28×10^9	0.98×10^9	0.46×10^9

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Table 2 Viable counts (log CFU/ml) and evaluation of selective media to enumerate *Lactobacillus acidophilus* and *Bifidobacterium animalis*

Parameters	ATCC 4356	ATCC 25527	Mixture
Mean[a] (logCFU/ml) in MRS-L-Cys-Agar Medium	8.95	8.61	9.08
Mean[a] (logCFU/ml) in MRS-L-Cys-Of-Agar Medium	8.94	0	9.17
Mean[a] (logCFU/ml) in MRS-L-Cys-Mup-Agar Medium	0	8.62	
Relative standard deviation of differences	0.08	0.07	0.15
Student's t value obtained	-1.17	1.18	2.75
Relative recovery	1.04	0.96	0.82
Selectivity (%)	99.8	100.19	100.93

[a]Means are average of total viable count (CFU/ml) from five experiments

The modified De Man Rogosa and Sharpe agar (mMRS) medium was prepared with MRS agar supplemented with L-cysteine HCl (0.05%) (Arroyo et al, 1994).This amino acid is considered as an essential nitrogen source (Shah, 1997) and has the additional function of reducing the redox potential thereby enhancing the anaerobic conditions required by these organisms (Payne et al, 1999). Several media for selective enumeration of *Lactobacillus* and *Bifidobacterium* have been proposed. There are two approaches present in this regard. First one is the selection by the use of antibiotics and other is the finding of a non antibiotic medium. Saccaro *et al*, (Saccaro et al, 2012) formulated selective plating methodologies using antibiotic (clindamycin, vancomycin) and other compounds like sorbitol in MRS medium for enumeration of probiotic bacteria in fermented milk.

The results of selective enumeration of *Lactobacillus acidophilus* and *Bifidobacterium animalis* were statistically analyzed in terms of precision, accuracy, reproducibility, and selectivity of the method developed for (ISO, 2000). For the evaluation of precision and accuracy Student's t-test, relative standard deviation of differences and relative recovery were calculated. The resulting t-values (Table 2) were lower than the tabulated value of t ($t_{0.05}$ = 2.776) which signifies that there was no significant differences between the results using the two selective media at a probability level of 95%. Relative standard deviation of differences and relative recovery were also obtained from S_d and d_{mean} (Table 2). The reproducibility of the methods was validated by analyzing the identical samples by two different analysts (data not shown). To verify the selectivity of the method cultures of *Lactobacillus acidophilus* ATCC 4356 and *Bifidobacterium animalis* ATCC 25527 were mixed (1:1) [Table 1]. It was observed that the presumptive viable count in selective media were close to 100% of viable count in reference medium (Table 2). These results revealed that the method developed for selective enumeration of *Lactobacillus acidophilus* and *Bifidobacterium animalis* from their mixture should be considered acceptable (Tobasco et al, 2007).

4. CONCLUSION

The combined use of mMRS-ofloxacin and mMRS-mupirocin as selective media for organism specific enumeration of *Lactobacillus acidophilus* and *Bifidobacterium animalis* from probiotic formulations in mixed population was found effective. Efficiency of the selection method was evaluated by statistical parameters such as precision, accuracy, reproducibility, selectivity and specificity.

DISCLOSURE STATEMENT

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