Science Journal of Microbiology
ISSN: 2276-626X
http://www.sjpub.org/sjmb.html

Published By
Science Journal Publication

Research Article Volume 2012 (2012).

INFLUENCE OF CASEINOPHOSPHOPEPTIDES ON PERFORMANCE OF LACTIC CULTURES IN FERMENTED MILK

*** Parisa Behbahani [1,2]**
M.Jayashankara [1]
G.S.Bhat [2]

[1,2] Mangalore university campus, Madikeri

[2] Brindavan college,Bangalore

Accepted 5th September, 2012

ABSTRACT

Casein precipitated from market milk was completely hydrolyzed (>10% DH) using trypsin at Enzyme: Substrate (1:25) ratio and the CPP was isolated by aggregation with 1% CaCl2 in 50% ethanol. The influence of CPP on performance of lactic cultures in milk was evaluated by estimation of development of acidity and total viable count during incubation of cultured milk. The CPP influenced the development of acidity to great extent in all the fermented milks. The increase in acidity due to added CPP was more in slow growing cultures like *L.acidophilus*, *Bifido.bifidum* and *S.thermophilus* rather than fast growing cultures like *L.bulgaricus*. The incubation period for development of acceptable level of 1% acidity was reduced by 6.5 hrs in the case of *L.acidophilus* and 5 hrs for *S.thermophilus* and *Bifido.bifidum* however, the CPP did not influence the incubation period in case of *L.bulgaricus* cultured milk. Addition of CPP increased the total viable count during the fermentation of milk by all the lactic cultures. The influence of CPP is highly significant in the case of *L.acidophilus* compare to other cultures; this may be of great importance in probiotic industry.

KEYWORDS: Casein, milk, fermentation, *S.thermophilus*

INTRODUCTION

Casein in milk is digested by the pancreatic enzymes in the intestine and the caseinophosphopepides (CPPs) produced could be absorbed in the body without further degradation along with the calcium phosphate attached to it. These peptides are shown to have excellent calcium binding and solubilising ability (Meisel, 2003). The CPPs could be produced through proteinases of lactic cultures (Meissel 1990). The CPPs isolated from milk did not show any bitterness even from extensively hydrolyzed casein. Efrat Semo *et al*., 2006 has reported that CPPs could also be utilized as nanocapsular vehicles to deliver other nutraceuticals

Recent studies have shown that the peptides of milk proteins can enhance the growth of probiotics considerably, thus advancing the time taken for minimum total viable count. The caseinophosphopeptides have excellent calcium binding and solubilising ability and are smaller peptides which can contribute to the growth of slow growing probiotic microorganisms since it is well established that the lactic acid bacteria would split β-casein, α-casein and whey proteins in the order of preference. There is substantial evidence those casein peptides, besides being utilized as nutraceuticals can also be exploited to enhance growth and sustainability of lactic microorganism in a variety of fermented foods. Janer *et al*., (2003) have also reported that WPC along with Caseinomacropeptide (CMP), isolated from cow's or combined ewes and goat's sweet cheese whey enhanced *Bifidobacterium lactis* growth in milk. Their study substantiated that supplementation of milk @ 2% CMP increased counts of *B. lactis* by 1.5 log cycles after 24 h incubation at 37 °C when compared with unsupplemented milk. Krissansen., (2007) suggested that casein @ 0.25 to 4 g / L along with whey enhanced growth of *S. thermophilus* and acidification, thus reducing the fermentation time, and growth of probiotic bacteria. CMP and WPC could thus be utilized to complement milk in order to increase counts of *bifidobacteria* in probiotic fermented milks (Ha E, 2003). Hence, it could be stated that whey proteins and casein can be used to enhance the Total viable Count of probiotic cultures in dairy products; however it is noteworthy to observe that these dairy based proteins not only stimulate the vitality of the cultures but manifest a concept of synergy with them.

There is substantial evidence that milk proteins like whey and casein (Shah, 2000) besides being utilized as nutraceuticals can also be exploited to enhance growth and sustainability of probiotic microorganism in a variety of fermented foods thereby synthesizing a novel variety of functional foods. Caseinophoshopeptides, are known to have

Corresponding Author: M.Jayashankara
Mangalore university campus, Madikeri.
Email: p.behbahani@yahoo.com

excellent growth inducing activity on probiotics, however its production depends on the lactic cultures used for fermentation of milk (Chandini 2005).

Lactic acid bacteria are used widely in preparation of various fermented milk products such as yoghurt, buttermilk, cheese, kumis, kefir and many other probiotic products. Slow microbial growth is major problem in probiotic products. The influence of Caseinophosphopeptides on growth of lactic culture is investigated in the present study.

MATERIALS AND METHODS

The UHT skim milk from market was used for this study. The lactic cultures were obtained from National Dairy Research Institute .MRS Agar procured from Hi Media was used for plating.

I. Isolation of CPPs

Caseinophosphopeptides were isolated from skim milk obtain from the market. The casein was prepared from milk by acidification to bring down the pH to 4.6. Trypsin was added to 4% suspension of casein to obtain an enzyme; substrate ratio of 1:25 and allowed to hydrolyze about 10% of degree of hydrolysis. The caseino phosphopeptides in hydrolysate solution was prepared by adding 1%$cacl_2$ in 50% ethanol, centrifuged at 5000 rpm for 10 minutes. The CPP was vacuum dried and stored for usage

II. Fermentation of milk with lactic cultures

The lactic cultures such as *L.acidophilus, S. thermophilus, L. bulgaricus* and *Bifido. bifidum* they obtained from NDRI (National Dairy Research Institute –Bangalore). Cultures were maintained by sub culturing regularly.

The sterilized the skim milk with CPP in different combinations was inoculated with 1% lactic cultures as shown below:

1) 100ml skim milk +1g casein (control)
2) 100 ml skim milk + 0.8g casein + 0.2g CPP
3) 100 ml skim milk + 06g casein + 0.4g CPP
4) 100 ml skim milk + 0.4g casein + 0.6g CPP
5) 100 ml skim milk + 0.2g casein + 0.8g CPP
6) 100 ml skim milk + 1.0g CPP

The cultures samples were incubated at respective incubation temperature and samples were drawn at regular intervals for estimation of titratable acidity and total viable count. And analyze for titer acidity and microbial count.

III. Estimation of total viable count

The product was serially diluted by taking 1ml of yoghourt containing Microorganism and suspending it into 9ml of 85% saline, which was considered as 10^{-1}. Further the dilution 10^{-2}, 10^{-3} 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}, 10^{-9}, 10^{-10}, 10^{-11}, 10^{-12}, 10^{-13} and 10^{-14} were prepared by suspending 1ml from each of the previous dilutions into 9 ml of saline. Care was taken to have homogenous dilution by vortexing each test tube and then moving to next test tube. 500µl of dilution was taken from 10^{-10}, 10^{-11}, 10^{-12}, 10^{-13}and10^{-14} tubes and were pour plated using sterile MRS agar. The plates were allowed to solidify and incubated for 24-48 hours at 38.5°C to observe the colonies.

RESULT AND DISCUTION

The effect of casienophosphopeptide (CPP) on performance of lactic acid bacteria was investigated by estimating the development of acidity and the growth of microorganisms in skim milk inoculated with lactic acid bacteria (*L.acidophilus , S.thermophilus,, L. bulgaricus , Bifido bifidum*).

The effect of CPP on production of acidity during fermentation of milk by lactic cultures is presented in **Table 1**. It is evident from the table that the influence of the CPP in enhancing the growth of microorganisms as represented by production of acidity is more in slow growing cultures like *L.acidophilus* and *S.thermophilus* rather than fast growing cultures like *L. bulgaricus , Bifido bifidum.* This may have industrial significant since the major problem in cultures like acidophilus is slow growths.

Table 1: Effect of CPP on production of acidity during fermentation of milk by lactic cultures
The values are average of 4 trials

Microorganism	***L.acidophilus***				***L.bulgaricus***				***S.thermophilus***				***Bifido.bifidom***			
Amount of CPP g/100 ml	**incubation time (*h*)**				**incubation time(*h*)**				**incubation time (*h*)**				**incubation time (*h*)**			
	8	**12**	**16**	**20**	**5**	**7**	**9**	**11**	**5**	**6**	**7**	**9**	**4**	**6**	**8**	**10**
0 (control)	0.713 ±0.075	0.938 ±0.075	1.098 ±0.083	1.245 ±0.048	0.73 ±0.043	1.033 ±0.039	1.13 ±0.022	1.223 ±0.031	0.603 ±0.039	0.628 ±0.017	0.655 ±0.021	0.935 ±0.034	0.668 ±0.017	0.725 ±0.026	0.848 ±0.017	0.905 ±0.034
0.2	0.81 ±0.037	1.058± 0.07	1.265 ±0.05	1.415 ±0.034	0.758 ±0.039	1.06 ±0.059	1.17 ±0.067	1.308 ±0.03	0.653 ±0.031	0.738 ±0.017	0.828 ±0.017	1.123 ±0.031	0.698 ±0.022	0.77 ±0.029	0.938 ±0.017	0.99 ±0.022
0.4	0.87 ±0.059	1.135± 0.058	1.355 ±0.056	1.51 ±0.029	0.77 ±0.022	1.11 ±0.096	1.243 ±0.046	1.35 ±0.029	0.668 ±0.031	0.765 ±0.013	0.858 ±0.017	1.145 ±0.021	0.738 ±0.017	0.85 ±0.043	0.973 ±0.017	1.04 ±0.029
0.6	0.953 ±0.123	1.195 ±0.042	1.465 ±0.042	1.608 ±0.043	0.795 ±0.034	1.135 ±0.048	1.308 ±0.038	1.408 ±0.022	0.718 ±0.025	0.805 ±0.021	0.908 ±0.03	1.188 ±0.031	0.758 ±0.017	0.875 ±0.034	1.038 ±0.017	1.135 ±0.029
0.8	0.983 ±0.11	1.245 ±0.026	1.508 ±0.049	1.65 ±0.029	0.838 ±0.042	1.148 ±0.062	1.373 ±0.039	1.468 ±0.039	0.785 ±0.021	0.858 ±0.017	1.035 ±0.026	1.318 ±0.025	0.798 ±0.017	0.908 ±0.03	1.08 ±0.022	1.168 ±0.025
1	1.045 ±0.071	1.355 ±0.048	1.535 ±0.042	1.675 ±0.029	0.908 ±0.05	1.19 ±0.029	1.433 ±0.031	1.538 ±0.022	0.838 ±0.017	0.87 ±0.018	1.063 ±0.026	1.348 ±0.017	0.845 ±0.013	0.955 ±0.021	1.123 ±0.025	1.198 ±0.025
"t" value'	15.009				19.467				14.628				13.612			
Regression coefficient	0.052				0.096				0.119				0.056			

The increase in acidity due to CPP over the control is shown in **Table 2**. The increase in acidity due to CPP during fermentation of milk by lactic cultures are presented in figure a,b,c,d it can be observed that the amount of CPP is directly co related to the development of acidity in fermented milk. It is further confirmed that the acidity development in culture milk is more in acidophilus and *S.thermophilus* cultured milk than *Bifido. bifidum* and *L. bulgaricus* cultured milks. Though the development of acidity was more with increase in CPP in some cases there is a drop in increase acidity particularly at incubation period at which the control samples has developed about 1% lactic acid .This clearly indicates that CPP has significant influence on the growth of microorganisms at initial level of fermentation and may not have much influence at higher level of fermentation.

Table 2: Effect of CPP on increase of activity level over the control during fermentation of milk by lactic Cultures.

Microorganism	***L.acidophilus***				***L.bulgaricus***				***S.thermophilus***				***Bifido.bifidom***			
Amount of CPP g/100 ml	**incubation time (*h*)**				**incubation time(*h*)**				**incubation time (*h*)**				**incubation time (*h*)**			
	8	**12**	**16**	**20**	**5**	**7**	**9**	**11**	**5**	**6**	**7**	**9**	**4**	**6**	**8**	**10**
0.2	0.1 ±0.03	0.13 ±0.0	0.16 ±0.03	0.1 ±0.02	0.03 ±0.0	0.03 ±0.02	0.04 ±0.05	0.08 ±0.0	0.05 ±0.01	0.11 ±0.0	0.18 ±0.0	0.19 ±0.0	0.02 ±0.0	0.05 ±0.0	0.09 ±0.0	0.09 ±0.0
0.4	0.17 ±0.01	0.2 ±0.01	0.25 ±0.02	0.27 ±0.02	0.04 ±0.02	0.07 ±0.06	0.11 ±0.03	0.13 ±0.0	0.07 ±0.01	0.13 ±0.01	0.21 ±0.0	0.22 ±0.01	0.07 ±0.0	0.12 ±0.01	0.13 ±0.0	0.14 ±0.0
0.6	0.25 ±0.05	0.27 ±0.03	0.36 ±0.04	0.36 ±0.01	0.06 ±0.01	0.1 ±0.01	0.17 ±0.02	0.18 ±0.01	0.12 ±0.01	0.17 ±0.0	0.25 ±0.01	0.26 ±0.0	0.09 ±0.0	0.16 ±0.0	0.19 ±0.0	0.23 ±0.0

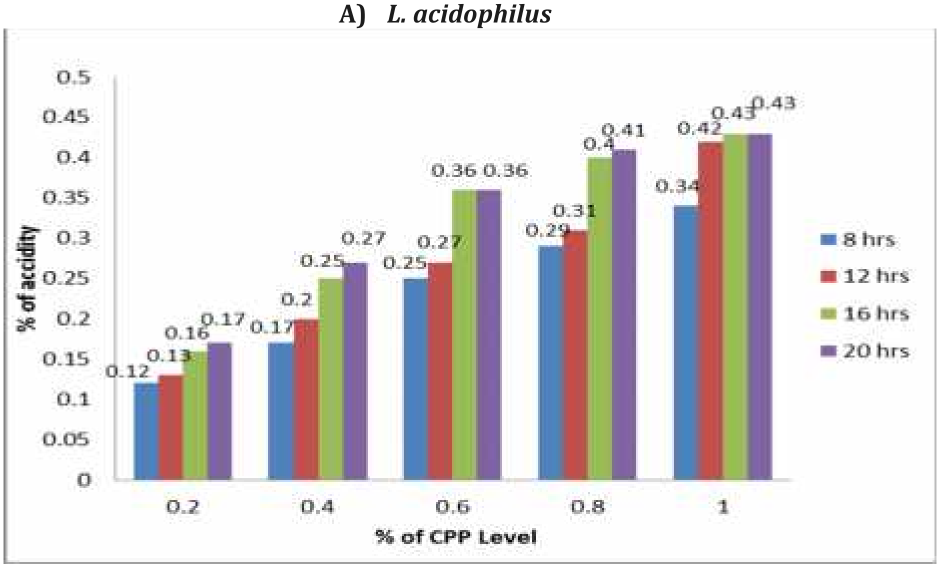
How to Cite this Article: Parisa Behbahani, M.Jayashankara, G.S.Bhat"Influence Of Caseinophosphopeptides On Performance Of Lactic Cultures In Fermented Milk" Science Journal of Microbiology, Volume 2013, Article ID sjmb-264, 7 Pages, 2012. doi: 10.7237/sjmb/264

0.8	0.29 ±0.04	0.31 ±0.04	0.4 ±0.03	0.41 ±0.02	0.11 ±0.0	0.12 ±0.02	0.24 ±0.02	0.25 ±0.01	0.18 ±0.02	0.23 ±0.0	0.38 ±0.01	0.39 ±0.0	0.13 ±0.0	0.18 ±0.0	0.23 ±0.0	0.27 ±0.0
1	0.34 ±0.0	0.42 ±0.02	0.43 ±0.04	0.43 ±0.02	0.17 ±0.01	0.18 ±0.01	0.31 ±0.01	0.31 ±0.01	0.23 ±0.02	0.24 ±0.0	0.41 ±0.01	0.42 ±0.01	0.18 ±0.01	0.23 ±0.01	0.27 ±0.0	0.3 ±0.0

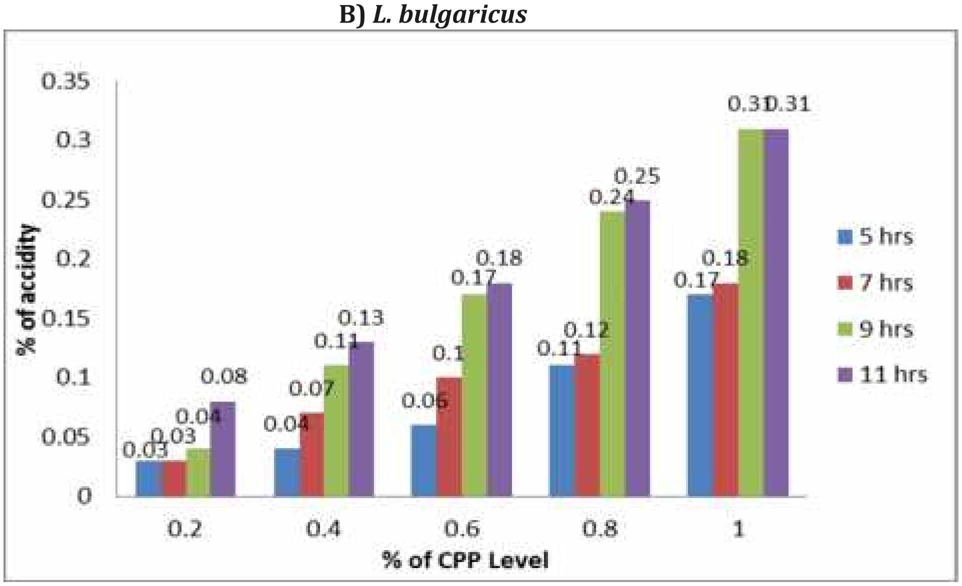
Values are average of 4 trials.

Influence of CPP on fermentation of milk (acidity) by lactic cultures

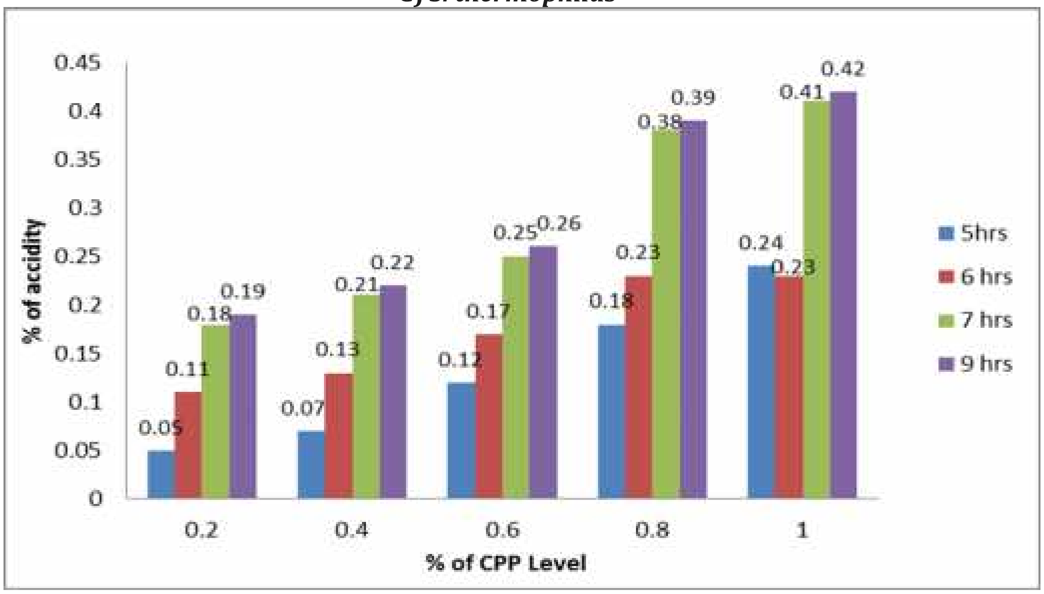
A) *L. acidophilus*



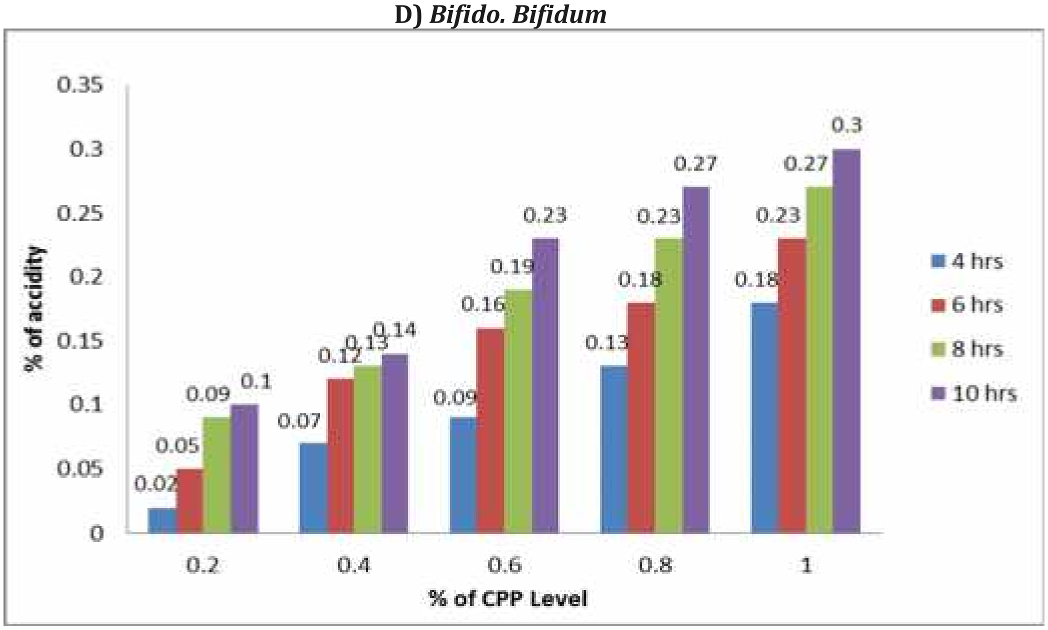
B) *L. bulgaricus*



C) ***S. thermophilus***



D) ***Bifido. Bifidum***



The normal acceptable acidity in fermented milk is around 0.8% lactic acid. The reduction in incubation period required to develop this acidity level in different cultured milks because of added CPP is presented in **Table 3**.

Table 3: Incubation period for desirable fermentation (0.8%acidity) in different lactic cultures

Amount of CPP (gr/100ml)	*L.acidophilus*	*L.bulgaricus*	*S.thermophilus*	*Bifido.bifidum*
0	14hrs	7hrs	10 hrs	12 hrs
0.2	11.5hrs	6.5hsrs	8 hrs	10.5 hrs
0.4	10.5hrs	6 hrs	7 hrs	9 hrs
0.6	9hrs	6 hrs	6.5 hrs	8 hrs
0.8	8hrs	5.5hrs	6 hrs	7.5 hrs
1.0	7.5hrs	5 hrs	5 hrs	6.5 hrs

The incubation period required to develop 0.8%acidity in *L.acidophilus* was 14 hrs in case of control while it require7.5 hrs in case of milk added with 1%CPP.indicating that the growth of this microorganism is 2times faster. The same reduction time was notice in *S.thermophilus* and *Bifido.bIfidum* cultures that is the incubation time was found to be respectively 5hrs and 5.5 hrs, indicating the influence of CPP on growth of these microorganisms about 2 times faster. However the influence of CPP was very little in case of *L.bulgaricus* since the time required for1% acidity level were 7 hrs and 5 hrs respectively in control and CPP added samples.

The *Bifid.bifidum* and *L.acidophilus* are considered to be important probiotic cultures however the problem in these cultures particularly *L.acidophilus* is slow growth the addition of CPP enhance the growth of these cultures by 2 times ,thus this is of great significance in probiotic industry. *S.thermophilus* and *L. bulgaricus* are constituents of yoghurt culture, while *L. bulgaricus* is a fast growing culture *S.thermophilus* is comparatively slow growing. From the result obtained it is very clear that addition of CPP can enhance the growth of *S.thermophilus* equivalent to that of *L.bulgaricus* thus the findings may have significance in yoghurt industry.

The observation with respect to influence of CPP on the development of acidity as an indicator of growth of lactic culture is further being confirmed by determination of total viable count in control and CPP added samples at 0.6% and 1.0% level. The preliminary investigation indicated that the total viable count increased with additional CPP in case of *S.thermophilus* at the best incubation period of 8hrs. Similarly the increase in total viable count is also observed in samples with added CPP in the case of *Bifido. bifidum* while not much difference was noticed with respect to *L.bulgaricus* fermented milk with or without CPP. Addition of CPP increased the total viable count during the fermentation of milk by all the lactic cultures. The influence of CPP is highly significant in the case of *L.acidophilus* compared to other cultures; this may be of great importance in probiotic industry.

The total viable counts were estimated during incubation of milk with different cultures. The best values with distinct colonies are presented in table 4.

Table 4: Effect of CPP on viable cell count

Cultures	**Optimum Incubation Time(hours)**	**Viable Assay (CFU/ml)**		
		control	**0.6% of CPP**	**1%of CPP**
L.acidophilus	8	12.934($86X10^{11}$)[a]	14.045 ($111x10^{12}$) [b]	15.812($65x10^{14}$) [c]
L.bulgaricus	10	11.770($59x10^{10}$)[a]	12.886($77x10^{11}$) [b]	13.732($54x10^{12}$) [c]
Bifido.bifidum	8	11.959($91x10^{10}$) [a]	12.968($93x10^{11}$) [b]	13.939($87x10^{12}$) [c]
S.thermophilus	16	12.913($82x10^{11}$) [a]	13.924($84x10^{12}$) [b]	15.004($101x10^{13}$) [c]

The total viable count is expressed in log values and the actual counts are given in parentheses.

The addition of CPP increased the total viable count in all the cultured milks. However the increase in total viable count was highly significant in CPP added milk especially in *L. acidophilus* which is very slow growing probiotic. This may be of importance in probiotic industry.

CONCLUSION

Addition of CPP to milk enhanced the growth of all the lactic cultures significantly. The influence of CPP for enhancement of growth of lactic cultures was highly significant particularly in slow growing cultures like *L.acidophilus, B.bifidum* and *S.thermophilus*. The setting time for desirable maximum acidity level of 1% was achieved for *L.acidophilus* within7.5hrs in milk with CPP against 14 hrs in control similarly in case of *S.thermophilus* the time required for this acidity was 10 hrs and 5 hrs. And in the case of *B.bifidum* 5.5 hrs for CPP added and 12 hrs for control sample .The total viable counts was more in fermented milk with added CPP was much higher to the extent of 10^{-14} dilution level compare to the control which shown counts only to the dilution level 0f 10^{-11}. These findings are very useful in yoghurt and probiotic and dairy product industry. Further the added CPP can also enhance the nutraceutical value of the products because of bioactivity of CPP in enhancing the calcium absorption.

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How to Cite this Article: Parisa Behbahani, M.Jayashankara, G.S.Bhat"Influence Of Caseinophosphopeptides On Performance Of Lactic Cultures In Fermented Milk" Science Journal of Microbiology, Volume 2013, Article ID sjmb-264, 7 Pages, 2012. doi: 10.7237/sjmb/264