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PROCESSING AND MICROBIOLOGY OF IDLI, INDIAN CEREAL-LEGUME FERMENTED FOOD

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Tiivistelmä/Referat – Abstract

Idli is a popular cereal-legume fermented food of Indian origin. It is steam cooked from fermented (lactic acid-yeast) batter of rice (cereal) and black gram (legume). Idli preparation process includes three major steps – soaking of rice and black gram, grounding and fermentation. The idli preparation process is laborious, as the whole procedure takes about 20 hours. Further, the fermented batter has a shelf life of 4-5 days at 4 ºC. Literature studies reveal less efforts has been taken to improve shelf life and nutritional quality of idli.

The overall aim of this thesis was to improve the quality of idli batter by mild heat treatment (Objective 1) and through microbial applications (Objective 2-4). First, the fermented idli batter was mild heat (MH) treated (57, 60, 63, 66 and 70 ºC) to reduce the high (10.5 log cfu/g) lactic acid bacteria and yeast counts for enhancing the shelf stability at refrigerated storage. MH treatment (at 70 ºC) induced the highest reduction (3.6 log cfu/g) without affecting the pasting profile of idli batter. During storage study (upto 10 days at 4 ºC) the microbial counts further decreased without change in pH.The second objective was to monitor the changes in physicochemical properties and B-vitamin (riboflavin, folate and vitamin B12) levels in idli batter fermentation on addition of starters - *Lactococcus lactis* N8 (SAA1) and *Saccharomyces boulardii* (YEA1). Fermentation profiles were recorded individually and in combination of starters. SAA1 and YEA1 were able to enhance or retain riboflavin and folate levels, but no change in vitamin B12 levels were observed during fermentation. Further, YEA1 individually and in combination with SAA1 significantly improved the idli batter volume, implying high gas production. The third objective was to produce nisin in idli batter by addition of SAA1 (nisin producer). The results highlighted SAA1 was capable of producing nisin in idli. However, the produced nisin was degraded by the activity of indigenous LAB and yeast in idli batter.

The final objective of this thesis was to determine the viability of probiotic *Bacillus coagulans* (BAC1) spores after cooking (steaming and microwaving) and during storage (at 4 ºC) of idli batter. Microwave cooking resulted in higher reduction of BAC1 than steam cooking. However, 5.4 log cfu/g of BAC1 spores were still viable in steamed idli from the initial added amount (8.2 log cfu/g). The BAC1 spores were not stable in idli batter suggesting spore outgrowth during storage. In summary, these results present different strategies and information for future process and product developments in idli.

Avainsanat – Nyckelord – Keywords

Idli, fermented foods, starters, yeast, LAB, nisin, riboflavin, folate, riboflavin, *Bacillus* probiotics

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PREFACE

This Masters thesis was carried out at the Division of Microbiology in the Department of Food and Environmental Sciences, University of Helsinki under the supervision of Professor Per Saris. A part of this thesis was conducted in collaboration with Division of Food Chemistry and Cereal Technology group, University of Helsinki. The entire experimental part was performed during the period of December 2012 to October 2013.

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ABBREVIATIONS

LAB	Lactic acid bacteria
RVA	Rapid viscoanalyzer
CFU	Colony forming units
RDA	Recommended daily allowance

1. INTRODUCTION

Idli is a popular cereal-legume fermented food of Indian origin. Traditionally, idli is steam cooked from the fermented batter of rice (*Oryza sativa*) and dehulled black gram (*Phaseolus mungo*) (Nisha et al. 2005). Idli is white in color, round, soft and porous in appearance with a mild sour taste (Durgadevi and Shetty 2012). In terms of nutrition, idli offers a balanced amount of carbohydrates, proteins along with essential amino acids and B-vitamins. Additionally, increased protein efficiency ratio in idli is apparent due to the combinational presence of cereal and legume (Veen et al. 1967).

The standard idli batter preparation process consists of three major steps – soaking of rice and dehulled black gram (4 – 6 hours), wet grounding and fermentation (12 – 18 hours at 30 ˚C) (Reddy et al. 1982). Consequently, the whole procedure in home scale takes about 20 hours and the idli preparation process is laborious. Additionally, household prepared idli batter lacks in consistent quality. These problems have made consumers to prefer the ready to cook idli batter from domestic markets (Nisha et al. 2005). The commercially available idli batter has received wide acceptance lately, however it, possess a very short shelf-life of 4-5 days of refrigerated storage. Presence of high amounts (10^{10}- 10^{11} cfu/g) of lactic acid bacteria and yeast, and their activity during storage could be one of the main reason spoilage of idli batter quality (Sridevi et al. 2010). Literature study highlights very less effort has been made to improve the shelf life of idli batter. However, there are several methods to improve the shelf life of foods, among existing methods mild heat treatment is a one economical treatment method. Several studies on different foods have demonstrated the efficiency of mild heat treatment on inactivation of LAB and yeast for better handling and retaining quality of foods during storage (Franz and Von Holy 1996; Tchango et al. 1997; Lamikanra et al. 2005).

Several studies on different aspects of idli fermentation has emphasized that fermentation plays a pivotal role in improving the nutritional and organoleptic properties (Reddy et al. 1982). Traditionally idli has been prepared only through natural fermentation process (Mukherjee et al. 1965).

Recently, Sridevi et al. (2010) reported usage of starters in idli batter fermentation could significantly enhance the nutritive and sensorial quality of idli. A starter culture is a microbial preparation (with one or multiple microbial strains) which is added to the food or raw material to initiate and improve the fermentation process (Leroy and de Vuyst 2004). Besides, one or more desirable property can be incorporated during food fermentation by use of an essential starter culture. For instance, Crittenden et al. (2003) used folate producing lactic acid bacterial culture to improve the folate amounts in yoghurt and fermented milk. In this way utilization of starter culture with desired character in idli fermentations could result in a product with improved nutrition and shelf life.

The food industry has been recently developing food with health benefiting properties due to increased awareness of health and wellbeing among consumers. Probiotic foods are one such health promoting food that has received wide attention among consumers lately. Probiotics are defined as 'live micro-organisms which when administered in adequate numbers confer a health benefit on the host' (FAO/WHO 2001). Numerous studies have demonstrated the positive effects of probiotics that it could contribute to human beings (Majamaa and Isolauri 1997; Salminen et al. 1998; Kalliomäki et al. 2001). Currently different varieties of probiotic foods are available. The commercially available probiotic foods most commonly use bacteria of genus *Lactobacillus* and *Bifidobacterium* (Ross et al. 2005). These strains have been mostly preferred for their good viability and stability during storage of food. However, these strains offer certain limitations in use of foods which are steamed or baked. For example, *Bifidobacterium* cannot be incorporated into bread directly as this bacteria could not resist the baking temperature (Zhang et al. 2014). Use of *Bacillus* genera of probiotics in baked and steamed food could be an alternate due to its heat stability and the survival ability at lower pH. In the USA, Isabella bakery has already launched a Muffin containing GanedanBC^{30} (probiotic *Bacillus coagulans*) (Cutting 2011). However, there are very few published studies on use of *Bacillus* probiotics in foods

The current study was initiated in order to improve the overall quality of idli batter by use of mild heat treatment, microbial processing and through addition of probiotic spores.

2. LITERATURE REVIEW

2.1 Cereal based fermented foods

Cereal based foods are the major source of human nutrition contributing 50 per cent of calories in the diet. For decades, a prominent portion of cereals has been utilized by human beings through the process of fermentation. As a result, multitude of indigenous fermented foods based on cereals originated in different parts of the world (Table 1) (Blandino et al. 2003). In general a variety of preparation and processing methods were adopted for consumption of these fermented foods. Among which, cereal fermented food prepared by supplementation of legumes is one type. Cereal-legume fermented foods could be mostly observed in regions of South and Far East Asian countries and to an extent in very few countries of South America and Africa (Mehta et al. 2012).

Table 1. Examples of cereal based fermented foods (Blandino et al. 2003; Mehta et al. 2012)

Product name	Substrate	Microorganisms	Nature of use	Regions
Boza	Wheat, millet, maize and other cereals	*Lactobacillus, Saccharomyces cerevisiae, Leuconostoc*	Thick, sweet, slightly sour beverage	Albania, Turkey, Romania and Bulgaria
Braga	Millet	Unknown	Liquid	Romania
Degue	Pearl millet	Lactic acid bacteria (LAB), yeast and moulds and coliforms		Burkina Faso
Dosa	Rice and Black gram	*Leuconostoc mesenteroides, Streptococcus faecalis, Torulopsis candida, T*. pullulans	Griddled cake for breakfast or snack food	India
Hammanatto	Wheat, soybeans	*Aspergillus oryzae, Pediococcus* and *Streptococcus*	Raisin like, soft flavoring agent for meat and fish, eaten as snack	Japan
Injera	Sorghum, maize or wheat	*Candida guilliermondi*	Bread-like staple	Ethiopia
Jamin-Bang	Maize	Yeasts, bacteria	Bread like cake	Brazil
Kenkey	Maize	*Lactobacillus fermentum, Candida, Saccharomyces, Penicillium, Aspergillus* and *Fusarium*		Ghana
Koji	Soybean, wheat, rice	*A.oryzae, A. sojae*	Alcoholic beverage	Japan
Otika	Sorghum	Unknown	Alcoholic beverage	Nigeria
Puto	Rice, sugar	*Leuconostoc mesenteroides, Streptococcus faecalis and* yeasts	Solid paste as seasoning agent, snack	Philippines
Tauco	Cereals and soybeans	*Aspergillus oryzae, Rhizopus oligosporus*	Seasoning	West Java (Indonesia)

Nutritionally, food prepared with cereal-legume combination is advantageous as it could offer an improved balance of carbohydrate (cereal) and proteins (legume) in the diet (Blandino et al. 2003). Further, the presence of legumes can also compensate the absence of certain amino acids in cereals. For example, some cereals lack in amino acids like lysine whereas legumes are rich in lysine, thus the combination counterbalances the deficiency of those aminoacids. Additionally, some cereal fermented foods contain improved B-vitamins (Nout 2009). Cereal fermentations are often progressed in the presence of LAB, yeast and molds. The microbial fermentation plays a vital role in improving of texture, flavor and shelf life of the final product, apart from nutritional enhancement (Steinkraus 1996). Traditional fermented food occupies a vital role in human nutrition, particularly in developing countries where fermented foods are consumed as staple foods. Recently, many traditional fermentation techniques have been improved by process development techniques. Thus, these process improvements could significantly improve the quality of the foods (Brandt 2014).

2.2 Idli – A traditional Indian rice cake

Idli is a popular breakfast food of Indian origin and represents itself to be one of the best examples for the cereal-legume based fermented foods. It is prepared from lactic acid fermented batter of rice (*Oryza sativa*) and dehulled black gram (*Phaseolus mungo*). Idli is round shaped steamed cake (Figure 1) which is white in color, soft and porous in texture (Reddy et al. 1982; Shetty et al. 2005). It is sour in taste with a characteristic smell, as similar to steamed sour dough bread in the western countries (Nisha et al. 2005).



Figure 1. Idli, a steamed rice cake food (ERG 2014)

In nutritional point of view, idli also serves as a good source of protein and due to its easy digestibility, it is a desirable food for infants and elderly people (Steinkraus 1996). Frequently, idli is consumed in parts of South India and Sri Lanka. On average it is consumed three or four times a week. Larger scale consumption pattern of idli can be observed in restaurants and industrial canteens in India (Nagaraju and Manohar 2000).

2.2.1 Idli preparation process and role of ingredients

The principal components of idli include rice, black gram, salt and water. In general, the idli batter preparation process (Figure 2) is progressed by a series of steps. Initially, rice and black gram are soaked in water for 5-10 hours. The soaked water is then drained and the ingredients are grounded in a wet mill, separately, by occasional addition of water. Then, the batters of rice and black gram are combined together in a separate vessel, following addition of salt. Finally, the combined batter is allowed to ferment overnight at room temperature. Next day, the fermented batter is steamed in an idli steaming vessel for 10 min and consumed (Steinkraus 1983; Soni et al. 1986; Nisha et al. 2005)

The quality of idli is often determined by the type and proportions of rice and black gram used. In this way, Kumar et al. (2005) used different cultivars (Jaya and Minilong) and type (high and low degree polished rice) of rice and examined the quality of idli obtained. The author observed differences in the fermentation profile and sensory scores of idli based on the type and cultivars of rice used. Earlier studies on idli have used varied proportions (2:1 to 4:1) of rice and black gram. However, ratios with higher portions of rice has not been preferable, as the rice flavor was dominant (Steinkraus 1996). Hence, literature studies suggest idli prepared in the ratio 2:1 (Reddy et al. 1982) and 3:1 (Sridevi et al. 2010) of rice and black gram could result idli with better quality and sensory properties.

Functionality of black gram in idli is significant as it mostly contributes to the foamy texture of the idli batter and importantly responsible for retaining the gases during fermentation (Reddy et al. 1982).

The spongy texture of steamed idli is imparted by the presence of surface active protein (globulin) and arabinogalactan (Nisha et al. 2005). Alternative attempts were also made with soybean, chick pea and green gram substituting with black gram (Steinkraus 1996). But idli with black gram was most preferred due to its organoleptic properties. Although substitutive ingredients could add more nutritive value to the food, but idli acceptability among consumers depends on the final texture and flavor. Salt is also an important ingredient that makes idli more acceptable in terms of taste (Soni and Sandhu 1989). In the past, 0.85% - 2% w/v salt has been used in the studies. Along with the main ingredients, 1.1 - 2.5 times of water are added during preparation of batter based on the requirement (Reddy et al. 1982).

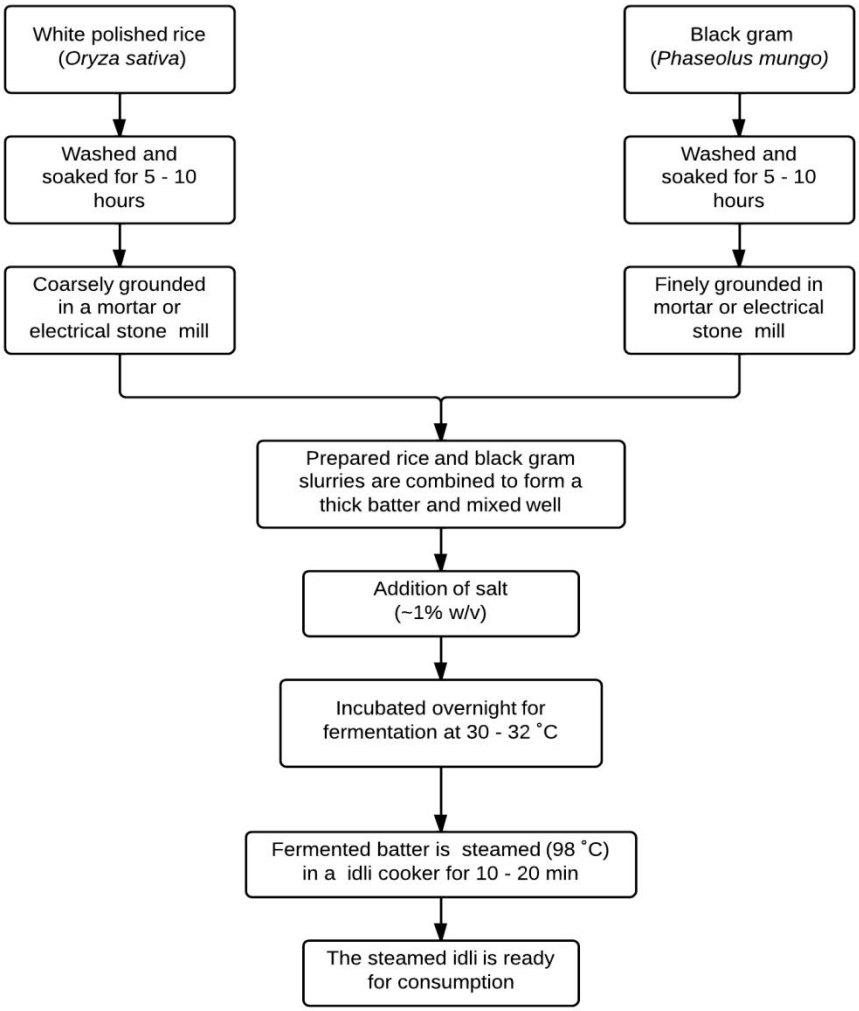

Figure 2. Steps involved in preparation of idli (Steinkraus 1983; Murthy and Rao 1997; Nisha et al. 2005)

2.2.2 Microbiology of idli

Idli remains to be widely popular in India due to its characteristic texture and flavor change occurring during fermentation (Nisha et al. 2005). Idli fermentation is naturally dominated by indigenous LAB and yeast. The microflora involved in fermentation is very often determined by the ingredients and the environment (Desikachar et al. 1960). Temperature of fermentation and seasonal variations also influences the successions of the microbial population. For instance, LAB and yeast populations were in high numbers during the winters and while in summers, yeast counts declined, possibly due to high temperature (Soni et al. 1986).

Lactic acid bacteria and the yeasts are developed during soaking of rice and black gram (Mukherjee et al. 1965; Soni et al. 1986). Both the ingredients individually contribute different species of LAB and yeast (Soni et al. 1986). So far several species of LAB and yeast have been identified that have been involved in the fermentation of idli (Table 2). In the absence of soaking, an external inoculum is essential for optimum fermentation (Reddy et al. 1982).

Along with the dominant species, prevalence of microorganisms like *Bacillus amyloliquefaciens*, *B. subtilis*, *B. polymyxa*, *Enterbacter sp*., and *Micrococcus varians* has also been reported (Soni et al. 1986). Thus the microbial community of idli fermentation is heterogeneous (Table 2). Principally the dominant organisms sequentially develop during soaking, followed by the fermentation of the ground batter. Fresh fermented idli batter contains high numbers of LAB and yeast in the range of 10^9 -10^{11} cfu/g (Mukherjee et al. 1965; Soni et al. 1986; Nisha et al. 2005; Sridevi et al. 2010; Iyer et al. 2011).

Fermentation of idli contributes two significant changes – acidification and leavening of batter. During the fermentation, microbial counts rise and the acidity of the batter increases corresponding to the decrease in pH. After fermentation for 12 – 18 hours at room temperature (30 °C), the lactic acid level in the batter ranged between 1.2 - 2.5 g, approximately with the final pH 4.5 (Mukherjee et al. 1965). Simultaneously, the batter volume increases, as a result of the release of carbon dioxide by the organisms (Steinkraus 1996).

Table 2. Different LAB and yeast species involved in idli fermentation

LAB/ yeasts	References
Lueconostoc mesenteroides	Mukherjee et al. (1965)
Streptococcus faecalis	Mukherjee et al. (1965)
Pediococcus cerevisiae	Mukherjee et al. (1965)
Lactobacillus delbrueckii	Soni et al. (1986)
Lb. fermentum	Soni et al. (1986)
Lactococcus lactis	Iyer et al. (2011)
Lb. plantarum	Iyer et al. (2011)
Saccharomyces cerevisiae	Soni et al. (1986)
Torulopsis candida	Reddy et al. (1982)
Trichosporan pullulans	Batra and Millner (1974)
Trichosporon beigelli	Soni et al. (1986)
Hansenula anomala	Soni et al. (1986)
Oosporidium margaritferium	Soni et al. (1986)
Candida robusta	Soni et al. (1986)
Kluyveromyces marxianus	Soni et al. (1986)
Candida kefyr	Soni et al. (1986)
Candida krusei	Soni et al. (1986)
Rhodotorula mucilaginosa	Soni et al. (1986)
Rhodotorula glutnis	Soni et al. (1986)

Although different organisms participate in the fermentation only certain species were capable of producing acid and gas. Accordingly, 290 bacterial strains were isolated from the idli batter at different intervals of fermentation. In the biochemical assays only 127 strains produced both acid and gas, remaining was capable of producing only acid. In the early stages of the fermentation, *Leuconostoc mesenteroides* and *Streptococcus faecalis*, low acid producers were abundant. In the later stages of the fermentation, *Pediococcus cerevisiae* prevalence was observed. The authors concluded the heterofermentative *L. mesenteroides* was dominant organism throughout fermentation and the responsible organism for leavening action. However, no activity of yeast was observed at any stage of fermentation (Mukherjee et al. 1965).

The importance of the *L. mesenteroides* in leavening was also confirmed by inoculating different pure cultures of LAB isolated from idli along with the autoclaved ingredients. In this study it was pointed both *L. mesenteroides* and *Lb. fermentum* involved in the leavening action (Ramakrishnan 1979).

Yeasts role is substantial in idli fermentation. The results of the earlier studies on function of yeast in idli fermentation were controversial. For instance, Mukherjee et al. (1965) reported only *L. mesenteroides* and other LAB were only responsible for leavening of idli batter. However, later studies evidently proved the equal participation of yeast in leavening of idli batter during fermentation (Soni et al. 1986; Sridevi et al. 2010). Diverse species of yeast have been reported from idli fermentation (Table 2), which is not surprising as they grow well at low pH.

In the biochemical assays, the predominant yeasts identified were capable of producing gas and utilizing the sugars along with the indigenous LAB. Additionally, the yeasts were capable of producing amylases which were absent in the LAB strains (Soni et al. 1986). Accordingly Venkatsubbaiah et al. (1984) hypothesized the functions of the LAB were to reduce the pH of the batter by increasing the acidity level and thereby favoring the activity of the yeast. In turn, yeast complements the growth of LAB by breaking starch through extracellular amylolytic enzymes. However, the synergism between LAB and yeasts is yet to be ascertained.

In the past in idli fermentations, the microorganisms have been enumerated through agar plate method and the organisms have been identified through the biochemical and microscopic methods (Soni et al. 1986). Commonly, for a total plate count of LAB, MRS Agar and for yeast, potato dextrose agar (Rekha and Vijayalakshmi 2011) Sabouraud dextrose agar (Sridevi et al. 2010) has been used. Additionally, for obtaining the counts of aerobic or mesophilic organisms Tryptone glucose yeast extract agar (Mukherjee et al. 1965) and nutrient agar (Rekha and Vijayalakshmi 2011) were used. However, modern molecular identification and typing approaches are yet to be handled for better identification of the microbiology involved at each stage of fermentation.

2.2.3 Nutritional profile of idli

Fermentation is an elemental part in idle as it improves the nutritional quality and organoleptic value. The preparation and fermentation process totally transforms the final quality of idli with improved flavor and texture, concurrently with enhanced digestibility. Nutritionally idli is composed of carbohydrates, proteins, vitamins and minerals (Table 3). In the literature the nutritional composition and the quality of idli differed due to the utilization of an altered proportions and variety of the rice and black gram (Rajalakshmi and Vanaja 1967; Kumar et al. 2005; Balasubramanian and Viswanathan 2007; Sridevi et al. 2010; Manickavasagan et al. 2013).

Rice is the major source of carbohydrates and additionally black gram (Rao and Belavady 1978) contributes oligosaccharides (indigestible sugars) - raffinose, stachyose and verbascose in idli. These oligosaccharides are known to cause flatulence (intestinal gas production) and often elimination of these sugars is considered to be a challenging problem (Rackis 1981). The process (soaking, fermentation and steam cooking) of idli preparation was beneficial as it reduced the oligosaccharide content up to 34 %. Besides, when steamed idli was administered to rats at 50% basal diet, the lower flatulence rate was reported (Reddy et al. 1982). As a fermentable substrate, these indigestible sugars possessed to have prebiotic properties that support the LAB proliferation as a carbon source (Chen et al. 2007; Martínez-Villaluenga and Gómez 2007).

Black gram is the vital source of protein in idli (Steinkraus 1996). Further, the protein efficiency ratio was found higher in the fermented idli than in unfermented idli mixture (Veen et al. 1967). Marked increase of amino acids were evident during fermentation regardless of the proportions of rice and black gram. Especially, methionine content increased in the range between 10 – 60% of the initial content. Nutritionally, methionine increase was advantageous as many legumes lack in sulfur containing amino acids. However, the reason for methionine increase is not yet known (Reddy et al. 1982).

Table 3. Nutrition composition of idli

Constituents	Unfermented idli	Fermented idli	References
Carbohydrate (g)	8.1	7.4	Sridevi et al. (2010)
Oligosaccharides			Reddy et al. (1980)
Sucrose (mg/g)	8.3	5.6	
Raffinose (mg/g)	ND	ND	
Stachyose (mg/g)	3.1	2.9	
Verbacose (mg/g)	15.5	12.7	
Protein (g)	13.9	14	Rajalakshmi and Vanaja (1967)
Amino acids (mg%)			Reddy et al. (1982)
Lysine	4.8	6.2	
Cystine	0.2	Trace	
Arginine	6.9	8.9	
Tryptophan	3.6	3.3	
Metheonine	1.0	1.6	
Vitamins (mg/100 g)			Ghosh and Chattopadhyay (2011)
Thiamine	0.46	0.72	
Riboflavin	0.42	0.75	
Folic acid	0.48	0.70	
Minerals			Reddy and Salunkhe (1980)
Calcium (mg/g)	0.4	0.4	
Magnesium (mg/g)	1.4	1.4	
Zinc (mg/g)	23.5	23.4	
Iron (mg/g)	46.9	46.4	
Phosphorous (mg/g)	3.7	3.7	

* The nutritional compostion varies with amount of rice and black gram used

Idli fermentation enhanced B-vitamins specially thiamine, riboflavin, folate, niacin, pyridoxine and vitamin B12 (Reddy et al. 1982). Studies have proven B-vitamin content increase two to three fold after fermentation (Balasubramanian and Viswanathan 2007). Though the rice and black gram itself contributes a certain amount of B-vitamins, further LAB and yeast participate in the fermentation process contributes to the enhancement. The indigenous strains were isolated from the fermented idli batter and tested for the vitamin production efficiency. In this perspective, few types of yeasts have been reported for the synthesis of riboflavin, thiamine and folate (Soni and Sandhu 1991) and LAB in the synthesis of vitamin B12 (Iyer et al. 2011). Recently, it was demonstrated that *L. plantarum* (isolated from idli) was capable of synthesizing 8 µg/ml of vitamin B12 and also possessed probiotic properties (Iyer et al. 2011).

On the other hand, Idli also has been a source of minerals – calcium, magnesium, zinc, iron and phosphorous. Hemalatha et al. (2007) investigated the impact of fermentation on bioaccessiblity of zinc and iron in idli batter with different proportions of rice and black gram (2:1 and 3:1). It could be proved that fermentation increased levels of zinc and iron significantly by 50 – 276 %. Conversely, steam cooking resulted in the loss (27% - 92%) of mineral contents. The bioavailability increases were attributed to the ability of microorganisms to synthesize organic acids and phytases that are involved in hydrolysis and resulted in formation of ligands of iron and zinc. Apart from the health promoting components, idli also consists of antinutritional factors such as phytic acids, saponin and trypsin inhibitors that are mainly imparted by black gram (Reddy et al. 1982). The processing methods like soaking, fermentation and steaming helped to reduce or eliminate the ANF levels in idli. During the preparations, the phytic acid levels reduced approximately 35 – 40%. Likewise, fermentation and steaming also achieved complete reduction of the chymotrypsin inhibiting activity (Reddy et al. 1982).

Use of starters in food fermentation has significantly increased the food quality in terms of final texture, nutrition and safety (Holzapfel 2002). However, until now, on a larger scale, only spontaneous fermentations have been carried out in idli fermentation. It was not possible to eliminate the indigenous microorganisms that originated during soaking of the rice and black gram (Soni et al. 1986). Thus so far in the literature studies, the role of starter cultures has been studied along with the natural microflora. In an earlier study, Soni and Sandhu (1991) added five different species of yeast along with natural flora to the idli batter. All the yeast added batters were capable of improving B-vitamin content in idli batter (Table 4). Among the added species studied, *Saccharomyces cerevisiae* performed better in the fermentations in terms of improved organoleptic, leavening and nutritional characteristics.

Table 4. Biochemical changes attained in various fermentations by additions of yeast starters (Soni and Sandhu 1991)

Parameters	**Added yeasts**											
	Control		**E1**		**E2**		**E3**		**E4**		**E5**	
	Initial	**Final**	**Initial**	**Final**	**Initial**	**Final**	**Initial**	**Final**	**Initial**	**Final**	**Initial**	**Final**
Bacteria (cfu/g)	2×10^6	8×10^{10}	9×10^5	1×10^9	1×10^6	8×10^8	2×10^5	8×10^8	6×10^5	2×10^8	1×10^5	8×10^8
Yeasts (cfu/g)	4×10^2	8×10^5	5×10^6	5×10^{10}	3×10^6	2×10^{10}	4×10^6	7×10^9	4×10^6	2×10^{10}	4×10^6	1×10^{10}
pH	6.6	4.9	6.5	5.2	6.8	5.3	6.7	5.8	6.5	5.3	6.5	5.4
Volume	200	410	200	490	200	475	200	470	200	430	200	400
Free amino acids (mg/g)	8	13	13	26	13	25	14	26	10	17	9	15
Total proteins (% w/w)	12	12.4	14	16	13.6	14.8	13.5	14.7	14	15.9	12	13.6
Thiamine (mg/100 g)	0.2	0.75	0.7	1.5	0.65	1.45	0.75	1.4	0.25	0.45	0.3	0.65
Riboflavin (mg/100 g)	0.25	0.75	0.7	1.6	0.65	1.5	0.7	1.5	0.25	0.45	0.35	0.75

Control – Uninoculated batter E1 – *Saccharomyces cerevisiae* enrichment E2 – *Torulopsis candida* enrichment E3 – *Hansenula anomala* enrichment

E4 – *Debaromyces hansenii* enrichment E5 – *Trichosporon beigelii* enrichment

2.2.4 Shelf life and safety of idli

Idli batters prepared at home scale are often consumed within two to three days at refrigerated conditions. In recent times, the ready to cook idli batter which has a shelf life of four to five days at 4 °C has started to receive wide attention from the consumers (Nisha et al. 2005). Due to the presence of high (~10^{10} cfu/g) counts of bacteria and yeast and by their activity, the quality of idli batter gets deteriorated with increase of storage time (Sridevi et al. 2010). To address this problem, steps have been taken to improve the shelf life by the canning of idli and modified atmospheric packaging (MAP) of idli batter.

In an early study, fermented idli batter was canned and processed at 125 °C for 60 min. During processing, idli formed in the can itself and the quality of idli were examined at 26 °C and 37 °C. The canned idlis possessed good organoleptic properties until 6 months at 26 °C, whereas idli stored at 37 °C, undesirable change in flavor and color was evident (Nagarathanamma and Siddappa 1965).

In another study, Durgadevi (2012) attempted to improve shelf life of idli batter by modified atmospheric packaging. Accordingly, the idli batter was packed in different plastic bags flushed with gases (oxygen, nitrogen and carbon dioxide) in different concentrations and the quality of the product was evaluated. The author concluded idli batter packed in low-density polyethylene (LDPE) with 0.012 mm thickness containing with 0% CO_2 and 7.5 to 15% O_2 increased the shelf life time by seven fold at room temperature, and maintaining a good sensory quality.

In lactic acid fermented foods, growth of pathogenic bacteria is often known to be suppressed and degrees of contaminations could be less (Adams and Nicolaides 1997). Idli is widely prepared by domestic vendors and due to improper handling procedures, there is a possibility to get idli contaminated with food spoilage organisms. However, idli batters procured from the markets were reported with prevalence of *Bacillus cereus*, *E. coli* and faecal coli forms (Roy et al. 2007)

Jama and Varadaraj (1999) studied the effect of natural fermentation on the addition of pathogens (*E. coli*, *Staphylococcus aureus* and *B. cereus*). Despite the involvement of different LAB in idli fermentation, pathogens were capable to coexist with the natural flora of idli. Simultaneously, the authors also studied the antibacterial efficacy of plantarcin LP84 (derived from *L. plantarum* isolated from idli batter) against the pathogens during the fermentations. It was concluded plantaracin added idli batter retarded the growth of these pathogens during fermentation.

2.3 Enhancement of quality of foods through processing and by microbial applications

2.3.1 Mild heat processing to reduce food spoilage

Application of heat in food processing is a commonly used method for improving the shelf life of the foods. Food industries consider thermal processing of foods to be one of the most economical ways for inactivation of the food spoilage organisms. To support this, numerous studies have spotlighted the importance of heat treatments on inactivation of spoilage and pathogenic organisms particularly in dairy products, vegetable, fruits and meat products (Doyle et al. 1987; Franz and Von Holy 1996; Li et al. 2002; Lamikanra et al. 2005). Based on the degree of heat applied in processing, the heat treatments methods are classified into pasteurization (<100 °C) and sterilization (>100 °C). The type of heat treatment method used for food processing is generally based upon the type of food and desired degree of microbial inactivation (Karel and Lund 2003).

Pasteurization or mild heat treatment is a process that inactivates a significant amount of vegetative microorganisms in food. The main objective of this process is to reduce the microbial growth in food for better safety handling and storage (Karel and Lund 2003). It is one of the minimal processing methods available that does not affect the nutritional and sensory characters of the food. In the literatures available, heat treatment of foods with in the temperature range 55 – 80 °C has been referred as pasteurization or mild heat treatment process (Malletroit et al. 1991; Franz and von Holy 1996; Kou et al. 2007).

Different temperatures (55 – 80 °C) and time combinations (15 sec – 30 min) has been used for achieving desirable reduction of spoilage organisms in foods (Rahman 2008).

The available literature studies explain that mild heat treatment has been widely applied in foods (dairy products, fruit juices, cereal and alcoholic beverages) to reduce primary spoilage LAB and yeast (Franz and von Holy 1996). However, all LAB and yeast species could not be inactivated by mild heat treatment. Franz and von Holy (1995) demonstrated different meat spoilage LAB possessed different heat resistance. For instance in the same study, when *Leuconostoc mesenteriodes* and *Lactobacillus sake* were heated at 63°C, it took 32.5 seconds and 20.2 seconds to getthe bacteria inactivated by one cycle, respectively. Similarly, Tchango et al. (1997) also demonstrated heat resistance exhibited by different spoilage yeasts (*Candida pelliculosa* and *Kloeckera apis*) vary in fruit juices. The heat resistance shown by different LAB and yeasts highlights the needs to study the effects of different temperature on reduction of the organisms in the foods for better handling and storage. Pasteurized or mild heat treated foods are often combined with other preservation methods like refrigeration or with chemical additives to enhance the shelf stability of foods. Principally heat treatments induce stress to the microbial cells and thereby increased in cellular fluidity followed by release of ions and water from cells leading to cell death (Gervais et al. 2003). Nevertheless, few studies reported certain spoilage organisms could recover from the heat induced injury during storage period of food (Wesche et al. 2009; Endo et al. 2014).

2.3.2 Incorporation of starter cultures in cereal fermented foods

Cereal fermented foods produced around the world are mostly dependent on natural fermentation process or in back slopping technique for the source of microorganism. With recent changing trends, traditional fermentation methods have been replaced by modern techniques, especially the use of starter culture as a source of inoculant in fermentation (Brandt 2014). A starter culture can be defined as a microbial preparation of a large number of cells of at least one microorganism to be added to a raw material to produce a fermented food by accelerating and steering the fermentation process (Leroy and de Vuyst 2004).

Most commonly, different groups of LAB and yeasts are the most common microorganisms in food fermentations. With existing modern techniques several strains of LAB and yeast have been isolated with one or more desirable functional properties. However, the functions of the strains have been different in the food fermentation systems. For instance, a study was performed to investigate the efficiency of *Weissella cibaria* MG1, an exopolysaccharide (EPS) producer in sour dough prepared with different flours (buckwheat, quinoa, oat, teff and wheat). It was found that the starter was suitable to synthesize good amount of EPS in sourdough prepared with buckwheat, quinoa and teff flour but not in oat and wheat (Wolter et al. 2014). Thus, it is also crucial to study the function of starter culture directly on the food system.

Selection of the appropriate strain as a starter is an essential step. According to Holzapfel (2002) a starter culture should impart considerations to improve the product quality through – rapid accelerated metabolic activities, improved and more predictable fermentation process, desirable sensory attributes and improved safety with reduced toxicological risks. Additionally, substrate of fermentation and behavior of strains in the case of addition of mixed cultures should be brought into account. Besides, functional characteristics like probiotic property, synthesis of vitamins and antagonism against pathogens could also be desirable for starters.

Currently a variety of foods are produced by the addition of starters. The most common examples include beer, wine, soy sauce, sauerkraut and Kimchi (Hansen 2002). In this way, in cereal foods, in bread and sour dough making process starters have been successfully implemented (Coda et al. 2010). However, the use of starters in many other cereal fermentations is still in the developmental phase and potential information has not been fully explored (Brandt 2014).

2.3.3 *Lactococcus lactis,* a B-vitamin and bacteriocin producer

Lactococcus lactis is a gram positive, coccal shaped homofermentative lactic acid bacteria and a non-spore forming facultative anaerobe. It is one of the most extensively studied LAB due to its wide application in the manufacture of fermented dairy foods like cheese and canned vegetables (Samaržija et al. 2001). LAB holds the status of qualified presumption of safety (QPS) and recently, the applications are being expanded in area of health improvement (as probiotic and vitamin producer) and in food preservation (as bacteriocin producer) (Kimoto et al. 1999; Capozzi et al. 2012).

Lactococcus lactis as B-vitamins enhancer

Humans cannot synthesize most vitamins and the requirements are met by intake of a balanced diet. Vitamins are naturally available to humans from different plant (vegetable, fruits and cereals) and animal (eggs, milk and meat) sources. Despite the availability of several sources, vitamin deficiencies are still persistent in populations of both developing and industrialized countries, due to malnutrition and unbalanced diet (Fenech and Ferguson 2001). To reduce the deficiencies, vitamin fortification in foods has been serving as a successful alternative way in countries like USA and Canada (LeBlanc et al. 2011) For example, In Canada folate fortification in possible foods was compulsorily amended, which contributed to decreased incidence of neural tube defects in adult female and related mortality in Canadian populations (de Wals et al. 2007).

Cereals serve to be an important source of B-vitamins in the human diet. However, like other vitamins, it is significantly lost or destroyed during food processing and cooking. Identification of LAB to synthesize B-vitamins has led to a concept of natural fortification. This could be an alternative way to address to reduce or compensate the losses of vitamins. *Lactococcus lactis* is commonly found in dairy fermented foods and also incidences have been reported to be present in cereals and associated fermented foods (Ivanova et al. 2000; Blandino et al. 2003; Iyer et al. 2011; Mukisa et al. 2012). It has also been identified that *L. lactis* has the ability to synthesize B-vitamins, particularly riboflavin and folate.

Riboflavin (B_2) and folate (B_9) are an essential source of micronutrients classified as water soluble B-vitamins. In humans, these vitamins participate in the essential cell regulation and metabolic processes. The specific function, deficiencies and recommended daily allowances (RDA) of riboflavin and folate has been presented in Table 5. Recent studies also connect the role of these B-vitamins in the prevention of cancer and cardiovascular diseases (Powers 2003; Borradale and Kimlin 2012)

Table 5. Important functions, nutritional deficiency and RDA of riboflavin and folate in humans

B vitamin	Vital function in humans	Nutritional deficiency causes	Recommended daily allowance (RDA)	References
Riboflavin (B_2)	Flavin mononucleotide and flavin adenine dinucleotide functions as cofactors and electron acceptors in important cellular metabolisms.	Ariboflavinosis, pellagra condition, brain dysfunctions, corneal opacity and anemia	1.6 mg/day	Powers (2003) EUFIC (2014)
Folate (B_9)	DNA synthesis and repair mechanism, amino acid synthesis and transport	Megaloblastic anemia, Alzheimer, cancer, cardiovascular disease, neural tube defects (NTD) in women	200 µg/day	Borradale and Kimlin (2012) EUFIC (2014)

Burgess et al. (2004) specified that *L. lactis* NZ9000 was capable of producing riboflavin and it was confirmed by the omission of the *rib* gene, which was responsible for the riboflavin pathway. Besides identification, the authors adopted two strategies to improve the riboflavin synthesis capacity of the organism by metabolic engineering and exposing it against toxic riboflavin analogue roseoflavin. As a result the metabolically engineered strain was able to synthesize more riboflavin than the strain exposed against roseoflavin. Additionally, the authors suggested identifying riboflavin producing or equivalent strains could be alternate to traditional starters for a vitamin enhancement in *in -situ* condition. Few other LAB like *L. plantarum*, *Leuconostoc mesenteroides* and *Propionibacterium freudenreichii* are reported with the ability to produce riboflavin (Capozzi et al. 2012). However, the information available in the literature is scarce on use of LAB strains in the improvement of riboflavin levels.

Naturally, several LAB strains possess the ability to produce folate and its derivatives (LeBlanc et al. 2011). Interestingly, this property has also been reported in *L. lactis*, an important industrial starter. The natural folate producing ability of *L. lactis* has been demonstrated in the study conducted by Gangadharan et al. (2010). The author isolated several LAB strains from raw milk to screen the folate producing strains. Ten LAB strains exhibited the ability to synthesize folate which was confirmed by the microbiological folate assay. The author also highlighted the highest folate producing strains - CM 28 and CM 22 (*L. lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris*). Later the author cultivated CM 28 and CM 22 in skim milk medium to enhance the folate producing capacity. However, the strains synthesized similar amount of folate (14.2 and 12.2 ng/ml) as in the folate assay medium.

Furthermore, several strains of *L. lactis* isolated from different sources had the ability to synthesize folate (Sybesma et al. 2003). In response to different growth parameters (controlled and uncontrolled conditions) and medium compositions (like NaCl concentrations) significantly improved or decreased the folate production capacity of the strains. Besides, the extracellular and intracellular concentrations of folate varied in different of *L. lactis* strains and 90 % of folate produced remained within the cell (Sybesma et al. 2003). The ability of strain to synthesize B-vitamins is a very specific or strain dependent trait and production capacity varies depending to the strain (Capozzi et al. 2012). Some strains even consume vitamin like folate (Kariluoto et al. 2006) thus highlighting the specificity of the vitamin synthesizing ability.

L. lactis as nisin producer

Bacteriocins are class of natural antimicrobial compounds that is ribosomally synthesized by bacteria (O'Sullivan et al. 2002). Many LAB-derived bacteriocins efficiently eliminate the food-borne pathogens such as *Listeria monocytogenes* and *Staphylococcus aureus* and also prevents food spoilage. In food systems bacteriocins can be used in three ways – as purified or semi purified antimicrobial additives, as bacteriocin based ingredients from fermented foods and by employment of bacteriocin producing LAB in food fermentations (Sobrino-López and Martín-Belloso 2008).

Nisin is a lantibiotic (antimicrobial compounds synthesized by LAB are known as lantibiotic) produced by *L. lactis* subsp. *lactis* strains, which is grouped under class-Ia bacteriocin (Delves-Broughton et al. 1996). Several studies have been documented on presence of nisin producing *L. lactis* in milk, vegetable, cereal, fish, meat and closely related fermented foods (Rodriguez et al. 1995; Choi et al. 2000; Noonpakdee et al. 2003; Beasley and Saris 2004; Settanni et al. 2005; Tuncer and Ozden 2010). Nisin gained an important role due to its strong antagonistic activity against a broad spectrum of Gram-positive and Gram-negative bacteria. It is the only bacteriocin that has been accepted as a food additive (E234) in Europe and also regarded with GRAS status by the Food and Drug Administration (FDA) in USA. The use of nisin producing *L. lactis* as starter culture in food systems, particularly in dairy fermented foods, has been extensively studied (O'Sullivan et al. 2002; Sobrino-López and Martín-Belloso 2008) but its potential role in other food fermentations are still under exploratory stage.

Foods associated with rice are frequently reported with presence of pathogenic *Bacillus* species like *B. cereus* (Agata et al. 2002; Roy et al. 2007; Ankolekar et al. 2009). The harmful emetic toxins and enterotoxins from these species are regarded to cause foodborne diarrheal diseases in humans (Wang et al. 2013). Nisin being a food preservative and its antagonistic activity has also been demonstrated against different *Bacillus* species. Kato et al. (2001) investigated the role of nisin producing *L. lactis* IFO12007 as starter culture in rice miso fermentation and its effectiveness to inhibit the spiked *Bacillus subtilis*. The results of the study indicated, the nisin producing strain completely inhibited the activity of *B. subtilis* during fermentation by exhibiting a nisin activity of 6400 IU/g in rice.

In situ nisin synthesis efficiency of the starter culture and the efficacy of this lantibiotic in the food system can be highly influenced by several food related factors like food structure, microbial diversity and other physicochemical factors from the substrate. For example, Rosenquist and Hansen (1998) studied the effectiveness of nisin (from nisin producing LAB and as purified additive) against *Bacillus* spores in sour dough fermentation. Nisin had no effect on the *Bacillus* counts. However, the nisin activity was observed in the agar plates. The ineffectiveness of nisin in the sour dough was attributed to pH of the bread crumb, poor solubility and uneven distribution of nisin in the bread.

Furthermore, it has been reported nisin can also be produced in mixed culture conditions. Liu et al. (2006) synthesized nisin from *L. lactis* in combination with *Saccharomyces cerevisiae*. Additionally, Shimizu et al. (1999) also highlighted the ability of *L. lactis* to synthesize nisin in mixed culture system along with *Kluyveromyces marxianus.*

On the other hand, Tolonen et al. (2004) also pointed out the possibility to synthesize nisin with *L. lactis* N8 in sauerkraut (fermented cabbage) fermentations in presence of mixed microflora. In this way, *L. lactis* N8 produced 1400 IU/ml of nisin in sauerkraut fermentation and even after 13 days the nisin presence was noticeable. Contrastingly, Breidt et al. (1995) observed nisin produced by *L. lactis* NCK400 in sauerkraut fermentation was degraded by natural microflora. In the same study, the authors found similar trends of depletion of added purified nisin in sauerkraut fermentation. However, the reasons for depletion of nisin during fermentations were not determined.

In the literature studies, nisin producing *L. lactis* have been preliminarily screened using different nisin sensitive indicator strains, among them *Micrococcus luteus* has been widely used (Rogers and Montville 1991; Pongtharangkul and Demirci 2004; Beasley and Saris 2004). Similarly nisin has been quantified and detected using various methods based upon agar diffusion and immunochemical methods. Nevertheless, these methods are considered to be unreliable due to cross-chemical interactions and interference of other substances during the assay. To avoid those kinds of complications, Hakovirta et al. (2006) developed a sensitive bioassay for detection and quantification of nisin. The authors constructed a nisin indicator strain (*L. lactis* LAC275) which emits bioluminescence, when grown in presence of nisin.

2.3.4 Use of *Saccharomyces boulardii* as a functional starter in food fermentations

Saccharomyces boulardii is non-pathogenic yeast that has been used as a probiotic supplement in more than 100 countries (Cascio et al. 2013). Clinically, it has been in use as biotherapeutic agent particularly in treating several types of diarrheal and bowel diseases.

Earlier studies highlighted that *S. boulardii* and *S. cerevisiae* (Baker's yeast) shared similarities (McFarland 2010). However, recent studies focused on metabolomics and molecular approaches precisely explained that *S. boulardii* uniquely differed from *S. cerevisiae* both genetically and in metabolomical aspect (McCullough et al. 1998; Mitterdorfer et al. 2002; Edwards-Ingram et al. 2007). Moreover, *S. boulardii* has been a clinical research subject for a long time but its studies related to application as food additive is very limited.

In the literature, uses of *S. boulardii* in cereal-legume fermentations were reported to be nutritionally beneficial. Sindhu and Khetarpaul (2004) attempted to develop a barley based probiotic fermented food and evaluated the effects of *L. casei* and *L. plantarum* individually and in combination with *S. boulardii* on the nutritional profile and acceptability of the product. During the fermentations, *S. boulardii* proliferated (9 log cfu/g) well with *L. casei* and *L. plantarum*, approximately with a final pH 4.2. In a similar study the fermentations carried out with *S. boulardii* reduced the antinutrients like phytic acid and trypsin inhibiting activity by improving the starch and protein digestibility (Sindhu and Khetarpaul 2003).

Various types of yeasts have been shown to synthesize B-vitamins like thiamine, riboflavin, folate, and pyridoxine (Soni and Sandhu 1991; Hjortmo et al. 2005; Batifoulier et al. 2005; Kariluoto et al. 2006). Additionally, the literature studies have recommended for use of these organisms in the food matrix for potential enhancement of B-vitamins (LeBlanc et al. 2011; Capozzi et al. 2012). *S. cerevisiae*, due to its wide applications in bread fermentations, has been in research focus for its B-vitamins producing efficiency and the role in B-vitamin enhancement during fermentation (Kariluoto et al. 2004; Hjortmo et al. 2005; Kariluoto et al. 2006; Katina et al. 2007; Hjortmo et al. 2008). *S. boulardii* was recently reported to synthesize folate and could enhance B-vitamins during fermentation of soymilk. Rekha and Vijayalakshmi (2010) analyzed the changes in vitamin B complex of soy milk, fermented by *S. boulardii* along with other probiotic LAB. The soy milk fermented with *S. boulardii* was capable of improving riboflavin and niacin content from the initial contents. Enhancement of riboflavin and niacin content were also evident in soymilk fermented with combinational cultures. Despite the increase, thiamine contents of the soymilk decreased during fermentations (Table 6).

Table 6. Changes in B-vitamin content (on a wet weight basis) in fermented and unfermented soymilk with different LAB along with yeast incubated for 24 h at 37 °C. (mg/100 ml) (Rekha and Vijayalakshmi 2010)

Cultures	Thiamin	Riboflavin	Niacin
Soymilk (control)	2.17	0.36	0.10
Sb + La	2.10	0.57	0.83
Sb + Lb	2.30	0.67	0.78
Sb + Lc	2.60	0.63	0.98
Sb + Lp	1.60	0.63	0.73
Sb + Lh	1.50	0.43	0.77
Sb + Lf	1.70	0.50	0.97
Sb	1.60	12.84	0.73

Sb – *Saccharomyces boulardii*, La - *Lactobacillus acidophilus*, Lb - *Lactobacillus bulgaricus*, Lc - *Lactobacillus casei* , Lp - *Lactobacillus plantarum*, Lh – *Lactobacillus helveticus*, Lf – *Lactobacillus fermentum*.

Similarly, *S. boulardii* ability to synthesize folate was accounted by Hjortmo et al. (2005). The authors presented a study with an overview of total contents and types of folates synthesized by *S. cerevisiae*, its related species and wild species of yeast isolated from different sources. The results explained that several species of yeast cells were capable of synthesizing folate and specifically *S. boulardii* cells contained folate of about 6.5 mg/100 g of dry matter. However, majority of *S. cerevisiae* strains contained higher folate than *S. boulardii*.

LAB probiotics are often used in dairy foods. Alternatively, studies have also been attempted to employ yeast probiotics in dairy foods. Lourens-Hattingh and Viljoen (2001) evaluated the growth and survival of *S. boulardii* in different types of milk yoghurts. *S. boulardii* was found to grow well in the sweetened and fruit yoghurt but in the plain yoghurt and ultra-heat treated (UHT) yoghurt the counts remained to be stable. The increase of counts in the sweetened and fruit flavored yoghurt were attributed to the utilization of fermentable sugars (sucrose and fructose) and in these yoghurts formation of carbon dioxide and alcohol was observed. This pattern was not found in plain and UHT yoghurts. Finally, the authors suggested *S. boulardii* as a promising probiotic in plain milk and yoghurt.

Likewise, Karaolis et al. (2013) has recommended *S. boulardii* as a potential probiotic organism in goat yoghurt that was capable of surviving until the 3rd week of storage. Moreover, the study suggested the consumption of yeast probiotic yoghurts could be an optional way for treating individuals suffering from antibiotic-associated diarrhea.

A study conducted by Krasowska et al. (2009) reported that filtrate obtained from *S. boulardii* grown culture medium could potentially diminish the virulence factors of *Candida albicans* (a fungus responsible for opportunistic infections in humans). A similar effect was also seen with live *S. boulardii.* For the action of the filtrate of *S. boulardii* against *Candida albicans,* the authors proposed for the presence of active antagonistic compound in the filtrate. Additionally, the use of *S. boulardii* on fermentation of rice bran positively altered the functionality of the bioactive compounds. Further, the extracts of the fermented rice bran reduced the growth of human B lymphomas (type of blood cancer cells) on comparison with the unfermented rice bran (Ryan et al. 2011).

2.3.5 Probiotics and their technological characteristics

Probiotics are defined as 'live micro-organisms which when administered in adequate numbers confer a health benefit on the host' (FAO/WHO 2001). Consumption of probiotics offers unique health benefits to human beings (Figure 3) and food consisting with probiotics underlies in the classification of functional foods (Prado et al. 2008). Mostly these live cultures are incorporated in fermented dairy foods and non-dairy foods based on cereals, fruits and vegetables. Additionally, probiotics also available commercially as supplements in the form of tablets, capsules and freeze dried preparations (Rivera-Espinoza and Gallardo-Navarro 2010).

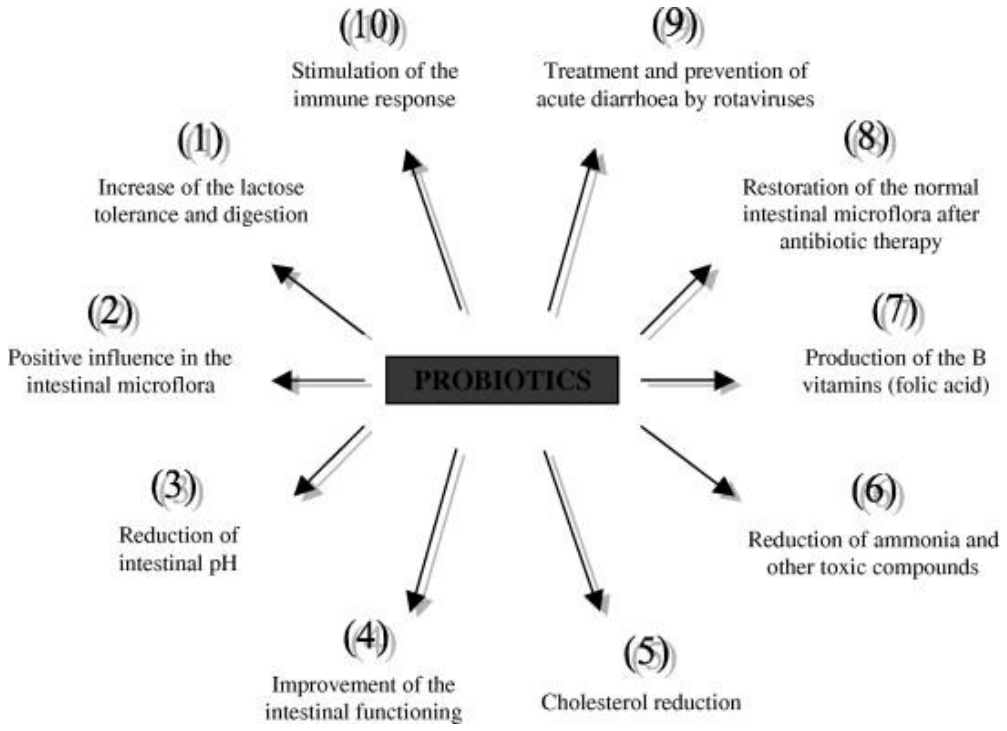

Figure 3.Probiotic beneficial effects on human health (Prado et al. 2008).

The commercially available probiotic foods most commonly use bacteria of genus *Lactobacillus* and *Bifidobacterium* (Ross et al. 2005). A number of studies have demonstrated the efficacy and stability of these organisms in the dairy and nondairy in refrigerated storage. Recent increasing consumer demands and diversification of food choices, has also led to an attempt to incorporate probiotics in non-traditional food matrices like fresh foods, cereal baked foods, chocolates and beverages (Luckow et al. 2006; Aragon-Alegro et al. 2007; Possemiers et al. 2010). However, sometimes *Lactobacillus* and *Bifidobacterium* has been reported with low viability and stability in the non-traditional food.

The efficacy and survival ability of many probiotic organisms in food matrices are greatly influenced by the processing and storage conditions. For example, Zhang et al. (2014) evaluated the viability of probiotic *Bifidobacterium lactis* Bb12 during bread baking. During the baking process, the viable counts reduced drastically and exposure to high lethal temperature (baking conditions) has been regarded as the reason for viability reduction. In another study, Rodgers (2007) investigated the survival ability of *L. acidophilus* and *Bifidobacterium animalis* ssp. *lactis* in fresh food service products (sushi, smoothies, burger and condiments). The author observed a 4 log reduction of the added organisms in burger and the condiments and suggested the probiotic

strains used were not suitable for these kinds of foods. Besides, temperatures higher than 60˚C also inhibited the viability of the probiotic strain. Thus, these studies highlight the importance of selecting an appropriate probiotic organism considering the specific food preparation and processing methods.

2.3.6 *Bacillus* probiotics

Bacillus probiotics are one among the most extensively studied organisms following *Lactobacillus* and *Bifidobacterium* (Duc Le et al. 2004). The spores of different *Bacillus* species like *Bacillus subtilis, B. clausii, B. cereus, B. coagulans* and *B. licheniformis* are used as probiotics and available as commercial supplements (Cutting 2011). Apart from human consumption, *B. licheniformis* and *B. subtilis* are being commercially used as probiotics for aquaculture in countries like USA and Vietnam (Gatesoupe 1999). For human consumption *Bacillus* probiotics have been sold as a medicinal supplement in Italy for more than 50 years in the brand name 'Enterogermina' (Cutting 2011) and recently in Finland it is available as 'Gastronel+'.

Bacillus species are also widely known for their pathogenic nature. Frequently strains related to *B. cereus* from different food sources have been responsible for producing harmful emetic and enterotoxins that causes food poisonings (Drobniewski 1993). Additionally, numerous diarrheal outbreaks due to different *Bacillus* species have been well documented (Salkinoja-Salonen et al. 1999). These reports raise the concerns about the use of *Bacillus* probiotics in humans. However, it should be taken into account that the pathogenic nature of *Bacillus* species is totally strain specific. For instance, *B. subtilis* is the major organism involved in the fermentation of traditional Japanese food, natto (Morinaga et al 2006). Besides, several studies have been highlighting the safety of *Bacillus* probiotics. Sorokulova et al. (2008) evaluated the safety of the two commercially available *Bacillus* probiotic strains (*B. subtilis* and *B. licheniformis*). *In vivo* studies on different animal models were carried out to examine the acute and chronic toxic effects of these *Bacillus* probiotics.

It was noted, these strains had no toxic effects on the tested animals and the authors recognized these strains to be safe for human consumption. The safety of other species (*B. cogulans*, *B. indicus* and *B. subtilis*) for consumption has also been investigated and concluded to be safe for humans use (Hong et al. 2008; Endres et al. 2009).

The *Bacillus* probiotics possess certain interesting technological characters that makes them advantageous over other available probiotics. The *Bacillus* spores can survive high temperature, pressure and harsh processing condition. and the spores are shelf stable for a longer time at both room temperature and refrigerated conditions (Cutting 2011). These competitive abilities make *Bacillus* probiotics a desirable alternative probiotic additive in foods. Erdem et al. (2014) first attempted to develop a synbiotic (presence of probiotic and prebiotic together) dark chocolate with *Bacillus indicus* HU36, maltodextrin and lemon fiber. During processing and storage, *Bacillus indicus* HU36 exhibited a high survival rate (5 log cfu/g) and its presence did not affect the sensory characteristics of the formulated chocolate.

Permpoonpattana et al. (2012) studied the efficacy of *Bacillus subtilis* spores as an ingredient in the whole-meal biscuits baking process (235 °C for 8 minutes). The spores were capable of surviving the baking process with a one log reduction from initial concentration. The results of these attempts lead the possibilities for development of novel probiotic foods. Recently, *B. coagulans* strain GanedenBC^{30} received self-affirmed GRAS (Generally Regarded as Safe) status in the USA (Endres et al. 2009). *Bacillus coagulans* is a spore forming bacteria which was previously reported as *Lactobacillus sporogenes*, according to Bergeys manual until 1974. Later classification techniques associated with taxonomic characteristics, 16S RNA ribotyping and fatty acid profiles undoubtedly explained the reasons for rectification as *Bacillus coagulans*.
Recently, a probiotic food "Active Muffin" with GanedenBC^{30} has been released by Isabella's Health Bakery in USA (Cutting 2011).

3. EXPERIMENTAL RESEARCH

3.1 Aims and overview of the study

The overall aim of the study was to improve the quality of idli batter by mild heat processing and through microbiological applications. Four individual experiments were done to reach the overall aim of the study which were:

1. to study the effect of mild heat treatment on LAB and yeast counts in idli batter after treatment and during storage at 4 °C
2. to evaluate the changes in physicochemical properties and B-vitamins levels (riboflavin, folate and vitamin B12) of idli batter on addition of microbial starters - *Lactococcus lactis* N8 (SAA1) and *Saccharomyces boulardii* (YEA1)
3. to investigate the ability of *Lactococcus lactis* N8 (SAA1) as starter culture to produce nisin in the idli batter during fermentation
4. to determine the viability of the probiotic *Bacillus coagulans* spores during idli cooking and in the idli batter during storage at 4 °C.

An overview of the experimental study with objectives is presented in Figure 4.

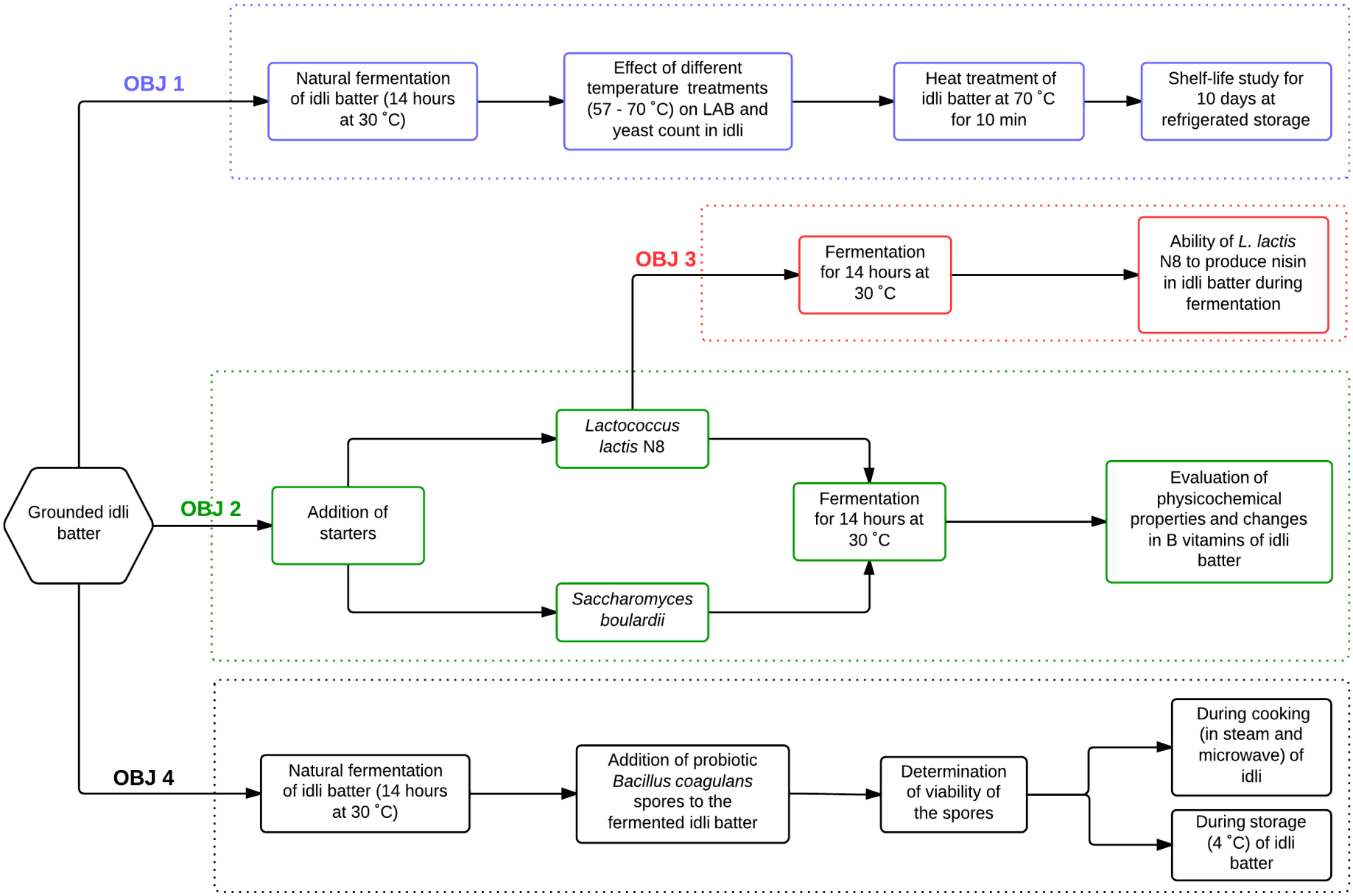

Figure 4. An overview of the experimental study

4. MATERIALS AND METHODS

4.1 Preparation of idli batter

Parboiled rice (*Oryza sativa*) and dehulled black gram (*Phaseolus mungo*) were procured from domestic market (Helsinki, Finland) for the experiments. Rice and dehulled black gram were taken in 3:1 ratio separately and thoroughly washed in potable water, soaked in sterile water for 6 hours at 28 ± 2°C. The ingredients were finely and coarsely ground separately, with periodic additions of water in an electrical wet grinder (Sowbaghya, India) presented in Figure 5. Ground batters were blended together with 0.85% (w/v) salt and allowed to ferment for 14 hours at 28 °C. For all the experiments, the same procedure was followed in preparation of idli batter.



Figure 5. Electrical wet grinder used to ground the idli batter

4.2 Effect of mild heat treatment on idli batter

4.2.1 Mild heat treatments

Fermented idli batter was subjected to different temperature treatments to analyze its effects on LAB and yeast population. For the treatments, 35 g of freshly fermented idli batter was taken in sterile 50 ml BD Falcon tube (BD Biosciences, USA) in duplicates.

The tubes were capped and treated at 57, 60, 63, 66 and 70 °C in a water bath (Type 1012, GFL, Germany) for 10 min. After each treatment, the temperature inside the sample was measured and then cooled for 10 min. The experiments were repeated in two batches with freshly fermented idli batter and the unheated fermented idli batter was considered as control.

Microbiological analysis

Influence of the mild-heat treatments on LAB and yeasts Colony Forming Units (CFU) were determined by Total-Plate Count method (TPC), using spread plate technique. The replicates of thermally treated and untreated samples (0.1 g) were taken in Eppendorf tubes and homogenized with 0.9 ml sodium chloride solution (0.85%) for 2 min in the shaker at an optimal speed. Thereafter, the samples were serially diluted to tenfold and using a sterile bent glass rod, desired dilution (0.1 ml) was spread plated on de Man, Ragosa, Sharpe (MRS; Oxoid Ltd., UK) and yeast peptone dextrose (YPD, Appendix 1) agar plates for LAB and yeast count, respectively. The plates were incubated aerobically for 24 to 48 hours at 30 °C and the CFU of LAB and yeast were enumerated.

4.2.2 Pasting properties of idli batter with respect to temperature

The pasting profile of idli batter with respect to two different holding (95 and 70 °C) temperatures were studied with Rapid Visco Analyzer (RVA), (model RVA-4, Newport Scientific, Australia) connected to a computer controlled by Thermocline windows software

(TCW, Version 2.2). Idli batter (14% moisture) and distilled water was weighed together, in the aluminum canister, and the stirrer was then introduced into the canister and vigorously paddled for proper mixing. Canister with stirrer was then inserted into RVA and centered onto the canister assembly point. Prior to initiation of the RVA analysis, the desired programs to be performed were preset using the Thermocline software. The RVA analysis program included three phases - heating phase from 50 °C, holding phase (95 and 70 °C) and a cooling phase in 50 °C**.** Finally, the RVA test was initiated and the changes in viscosity of idli batter with respect to different temperature phases were recorded.

4.2.3 Shelf life study with idli batter treated at 70 °C

Idli batter samples (35 g) were taken in sterile 50 ml BD Falcon tube and treated at 70 °C in the water bath for 10 min. Following treatment the samples were cooled to room temperature for 10 min and stored at 4 °C for 10 days. The stored batter samples were evaluated at two days interval until the tenth day. During the storage study, duplicates of treated and control samples were plated as previously described in two days interval until the tenth day.

Changes in microbiological counts and pH of the batter

The changes in the LAB and yeast counts of idli batter were enumerated from 0-10th day of storage at 4 °C on MRS and YPD agar, respectively. All the counts were represented in log cfu/g.

The pH changes in heated (70 °C) and unheated (control) idli batter were measured directly using the digital pH meter (model 420A+, Thermo Orion, USA) on each sampling day (Steinkraus et al. 1996).

4.3 Role of starters in idli fermentation

Starter cultures and growing conditions

In this study, *Lactococcus lactis* N8 (SAA1) and *Saccharomyces boulardii* (YEA1) were used as LAB and yeast starter culture. SAA1, a nisin producer was originally obtained from Valio Ltd. (Graeffe et al. 1991) and was cultured in M17 (Oxoid Ltd., UK) agar or broth supplemented with 0.5% (w/v) glucose (M17G) at 30 °C for 11 hours. YEA1 was procured from pharmacy (Helsinki, Finland) as probiotic supplement (Precosa®, Biocodex). A capsule from it was broken and cultured in YPD broth at 30 °C for 12 hours. Thereafter, the stock cultures were maintained in 0.87% of glycerol at -20 °C and the working stock cultures at 4 °C.

Supplementation of starters to idli batter

To the freshly grounded idli batter, the starter cultures were added. For this, 150 ml of fresh cultures of SAA1 (9.8 log cfu/ml) and YEA1 (9.5 log cfu/ml) were prepared in M17G and YPD broth, respectively. From the prepared fresh cultures, the starters were taken in Falcon tube and centrifuged (5804 R, Eppendorf, Germany) at 7000 rpm for 10 min at 4 °C.

The supernatant was discarded without disturbing the pellet formed and it was resuspended in distilled water. The resuspended pellet solution with starters was added to idli batter individually and in combination. Detailed description is presented in Figure 6. Naturally fermented idli batter (without starter) was used as control. The idli batter samples were fermented at 28 ± 2°C for 14 hours. For the analysis the samples were considered as follows

- unfermented idli batter (for vitamin analysis)
- naturally fermented idli batter (control)
- SAA1 fermented idli batter
- YEA1 fermented idli batter
- SAA1 and YEA1 (in combination) fermented idli batter

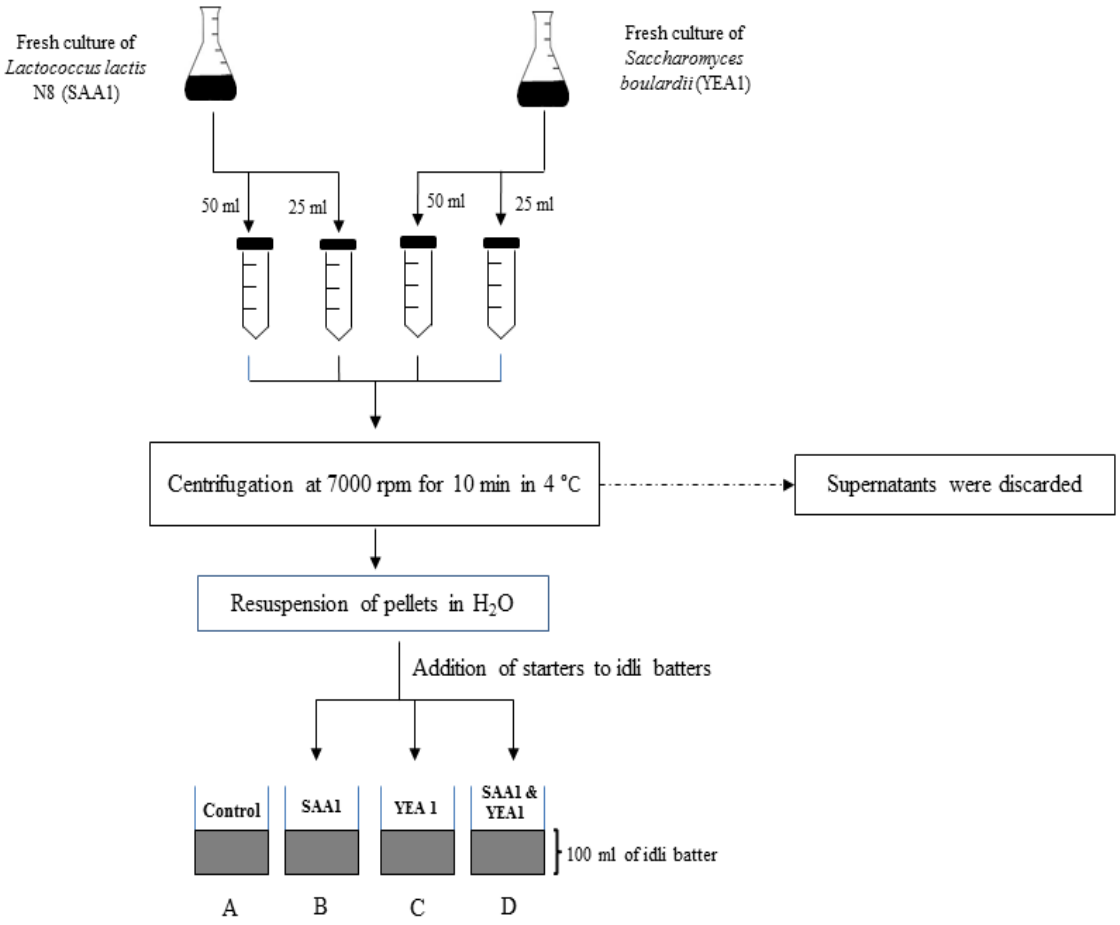

Figure 6. Schematic representation on addition of starters to idli batter (100 ml).

4.3.1 Evaluation of pH and changes in batter volume

The pH of the different idli batter samples was measured directly on the pH meter (model 420A+, Thermo Orion, USA) before and after fermentation.

For the measurement of the change in batter volume, 100 ml of the idli batter was taken into 250 ml beaker. After 14 hours of fermentation, the change in the batter volume from each sample was measured in 'ml'.

4.3.2 Microbiological analysis

The microbial counts in the batter before and after fermentation of the samples were enumerated on MRS, M17G agar for LAB and YPD agar for yeast count. The plating technique was carried out as mentioned previously. The total plate counts were expressed as 'log counts'.

4.4 B-vitamin analysis

Prepared idli batter samples were analyzed for B-vitamins – riboflavin, folate and B12 at Division of Food Chemistry, University of Helsinki. For the analysis, 150 g of naturally fermented batter, batter fermented with starters and unfermented batter were freeze dried. All the analyses were performed in subdued light as B-vitamins were sensitive to light.

4.4.1 Determination of vitamin B12 content by microbiological assay

Vitamin B12 content in idli batter was determined by microbiological assay (MBA). In this assay *Lactobacillus delbrueckii* ATCC 7830 was used as the test organism.

Preparation of calibrant

Two calibrants were used in this assay. Stock solution 1 was prepared with cyanocobalamin (Supelco, USA) (200 µg/mL) in 25% ethanol in Milli-Q water. At 361 nm absorbance, the concentration of the stock solution 1 was confirmed spectrophotometrically with 25% ethanol as blank. The stock solution 2 was prepared by diluting the stock solution 1 in 1:100 with ethanol and prepared stock solutions were stored in dark at 4 °C.

Sample extraction

Extraction buffer (8.3 mmol/L of sodium hydroxide and 20.7 mmol/L of acetic acid) of pH 4.5 was prepared and the part of the buffer was taken separately which was adjusted to pH 6.2. Further, this buffer was used for the final dilution of the extracted sample.

The sample (0.5-1.0 g) was weighed in the 30 ml plastic tube in duplicate. For extraction, 10 ml of extraction buffer (pH 4.5) was added to the samples and blank buffer. In the tubes, 1% sodium cyanide solution was then added and vortexed. Thereafter, the tubes were placed in 100°C water bath for 30 min and cooled on ice. The tubes were centrifuged (Hemle, Germany) at 8000 rpm, 10 min at room temperature and supernatants were transferred onto new 30 ml plastic tubes. With extraction buffer (pH 6.2) the residual tubes were vortexed again to ensure maximum extraction. The pH of the supernatant was adjusted to 6.2 with 3% sodium hydroxide. Finally the extracts were filtered through a filter paper (∅90 mm, VWR, Belgium) into a volumetric flask (25 ml) and then the volume was made up to the mark with pH 6.2 buffer.

Sample dilutions for assay

A calibrant solution (0.1 ng/ml) was prepared by diluting stock solution 2 in 1:200 and 1:100 concentration with buffer (pH 6.2). Two dilutions of the each sample were prepared in duplicate with buffer pH 6.2 in volumetric flasks and same dilutions were also prepared for the blank.

Assay medium and culture inoculation

The assay medium (Merck, Germany) was prepared of pH 6.2 and filtered through 0.2 µm sterile syringe filter (Pall, USA).The calibrant (0 – 80 µl) at eight levels were added onto the wells of 96-well microtiter plate, with four wells for each dilution and buffer pH 6.2 was added to obtain a final volume of 100 µl in each well. Diluted sample extracts of 100 µl were added into the remaining wells, with four well for each extract. The cryopreserved *Lactobacillus delbruckii* ATCC 7830 was thawed and 100 µl of it was added to the assay medium. 200 µl of the inoculated medium were added into each well.

The plates were incubated for 19 hours at 35 °C. After incubation the optical density of the wells were measured with a microplate (Multiskan Ex, Labsystem, Finland) reader at 595 nm. A calibration curve was constructed and results were calculated with appropriate blank corrections and dilution factors.

4.4.2 Determination of riboflavin content by Ultra High Perfomance Liquid Chromatography (UHPLC) method

Riboflavin standard

Riboflavin (Sigma-Aldrich, Germany) was used as the external standard. A stock solution was prepared with the riboflavin standard and diluted acetic acid (0.02 M). From this, the working standards were prepared by diluting in diluted acetic acid (0.02 M).

Sample preparation

The samples preparation involved three steps – extraction, enzyme treatment and filtration. First for extraction, about 0.5 g of each sample was weighed in the extraction tube and 15 ml hydrochloric acid solution (0.1 M) was added. The pH of the prepared solution was adjusted to 2.0 with diluted acetic acid and the extraction tubes were placed in a water bath at 100 °C for 1 hour.

After cooling to room temperature, the pH of the sample extract was adjusted to 4.5 with sodium acetate (0.1 M), the optimal pH for enzyme to act. Following, Taka-Diastase (Pfaltz & Bauer, USA) with β-amylase (Sigma-Aldrich, Germany) was added to the extract. The sample mixture was then incubated at 37 °C for 24 hours for enzymatic dephosphorylation reaction. The sample mixture was filtered through Whatman filter paper and transferred to the 25 ml volumetric flask and the volume was made up with diluted acetic acid (0.02M). Finally, the extracts were filtered and injected by syringe onto the UHPLC vials using a membrane filter (0.2 µm, Pall, USA).

Determination of riboflavin

The sample extracts were analyzed with UHPLC system (Waters, USA). The extracts (10 µl) were injected with an auto sampler under following parameters. Mobile phase A: Methanol (HPLC grade) 30%, Mobile phase B: Ammonium acetate in H_20 (filtered with 0.2 µm membrane) 70%, Flow rate: 0.2 ml/min, Column: BEH C18 column (2.1 mm x 100 mm; 1.7 µm), Detector: Fluorometric detector. Finally the total amount of riboflavin was quantitatively determined with an external calibration curve.

4.4.3 Determination of folate

Total folate contents in idli were analyzed by MBA with *Lactobacillus rhamnosus* ATCC7469 as an indicator organism (Kariluoto et al. 2004). The assay was performed at Division of Food Chemistry by Dr. Susanna Kariluoto.

4.5 Ability of SAA1 to produce nisin in idli batter

Addition of SAA1

SAA1 was added as pellet (9.7 log cfu/ml) to idli batter as a starter culture in different concentrations – 0.5 ml, 5 ml and 50 ml to analyze its efficiency to produce nisin in the idli batter system. After fermentation for 14 hours, the batter samples were analyzed for the presence of nisin by plate test with *Micrococcus luteus* nisin sensitive indicator organism (grown in Luria Bertani (LB) agar plate, Appendix 3) and by nisin fluorescence assay.

4.5.1 Plate test with *M. luteus*

For this assay, nisin (Sigma, 2.5 % pure) was taken as the standard (10,000 IU) and added to 0.1% Tween 80 acidified to pH 2.5. From the prepared stock, diluting in acidified Tween 80 - 10, 25, 50 and 100 IU/ml standards were prepared. The idli batter fermented with SAA1 in two different concentrations were taken (0.1 g) into Eppendorf tubes.

The samples were homogenized in 0.9 ml of acidified Tween 80 (0.1%) by vortexing it for 2 min. Following mixing, the samples were centrifuged (7000 rpm for 10 min) and the supernatants were collected. Then the supernatants of samples were boiled in 100 °C waterbath for 10 min (to inactivate the vegetative microbial cells) and cooled in ice for 5 min.

Different dilutions of samples prepared were added (3 µl) to the LB plate with *M. luteus*. The plates were incubated at 37 °C for 15 hours. The inhibition zones in the plate represent the presence of nisin, but in the case of the batter samples it may also indicate the presence of other *M. luteus* inhibitory substances.

Nisin fluorescence assay

The samples were prepared as for plate test with *M. luteus* assay. Desired dilutions were prepared and the assay was performed as described by Hakovirta et al. (2006).

4.5.2 Antagonistic activity of naturally originating idli microflora against SAA1

From the plates (MRS and M17G plates) with colonies originated from naturally fermented idli batter (NAT isolates), 30 colonies were randomly picked and a pure culture was prepared in M17G agar and incubated at 30 °C for 16 hours. NAT isolates were co-streaked in "+" form against a streak of SAA1 on M17G agar. The plates were incubated at 30 °C for 16 hours. Also the colonies that exhibited inhibitory zones in SAA1 survival test were streaked against NAT colonies.

4.5.3 Survival ability of SAA1 in idli batter during fermentation

Thirty colonies (ISO 1 – 30) originated from SAA1 fermented idli batter were isolated and pure cultures were made. The isolates were streaked onto the indicator lawn of *M. luteus* and incubated at 37 °C for 16 hours. Colonies exhibiting zone of inhibition were potentially considered to be SAA1.

4.5.4 Nisin fluorescence assay

The ability of the ISO strains isolated from the idli batter (SAA1 fermented), having inhibitory activity against *M. luteus*, to synthesize nisin was confirmed with nisin fluorescence assay. Indicator strain *Lactococcus lactis* LAC275 (Hakovirta et al. 2006) was used in this assay obtained from Per Saris culture collection. The strain LAC275 was cultured overnight in M17G broth supplemented with chloramphenicol (5 µg/ml). Approximately before 6-8 hours of this assay, a fresh culture of LAC275 was prepared. A LAC275 solution was prepared, the pre-cultured cells were diluted 1:100 in M17G broth with chloramphenicol (5 µg/ml) and Tween 80 (10% w/v).

Nisin standards (2.5% pure, Sigma) were prepared in Tween 80 (0.1%) acidified to pH 2.5 with HCl dissolved in distilled water. The nisin standards (10 to 70 pg/ml) were prepared with Tween 80 (0.1%, acidified). Then, it was added to LAC275 solution and this solution was added onto the microtitre wells, five wells for each standard concentration with final volume of 225 µl in each well.

The isolates that exhibited inhibitory zones were selected and grown in M17G broth for 12 hours at 30 °C. For the assay, one ml of fresh culture was centrifuged at 7000 rpm for 10 min at room temperature. The supernatant was transferred to new eppendorf tube and was placed in a waterbath (100 °C for 10 min). Following, it was cooled and different dilutions were made with Tween 80 (0.1%). Then diluted samples (175 µl) were loaded to six wells for each dilution of the sample. To the wells with diluted samples, the LAC275 suspension solution (50 µl) was then added with the final volume of 225 µl in each well. The blank was prepared with LAC275 solution and Tween 80 (0.1%) without nisin.

The microtitre plates with cells were grown at 30 °C for 18 hours without aeration. Prior to fluorescence measurement, 175 µl of supernatants from the wells were removed from the incubated plate. The presence of nisin was determined by the fluorescence in the Fluroscan Ascent 374 flurometer connected to a computer by using Ascent software (version 1.2, Labsystems, Helsinki, Finland).

The fluorescence was measured at excitation and emission filters of 373 nm 538 nm, respectively. The fluorescence was expressed in relative fluorescence units (RFU). The presence and absence of nisin in each sample were recorded.

4.5.5 Fate of added nisin in idli batter during fermentation

To determine the fate of nisin in idli batter during fermentation pure nisin (1000 IU/ml) was added to it. The batter samples were taken and checked for the presence of nisin by *Micrococcus luteus* plate assay. For the assay 0.1 g of idli batter was homogenized in 0.9 ml of Tween 80 and centrifuged (7000 rpm for 10 min). The supernatant was transferred to a new eppendorf tube and boiled at 100 °C for 10 min.

The tubes were cooled on ice and desired dilutions were made. Diluted samples were added onto the plate and the plates were incubated at 37 °C for 18 hours. After incubation, based on the inhibition zones formed, presence of nisin was confirmed. Idli batter without nisin was taken as control. Finally the samples were also subjected to Nisin fluorescence assay.

4.5.6 Screening of naturally nisin producing isolates

The NAT isolates were also subjected to the nisin fluorescence assay and *M. luteus* plate assay to determine their ability to produce nisin**.**

4.6 Stability of probiotic *Bacillus coagulans* spores in idli batter

Addition of spores

Commercial preparation of *Bacillus coagulans* spores (BAC1) was used for the viability study. The probiotic spores (8 log cfu/g) were diluted in 0.85% saline (5 ml) and then resuspended into 25g of freshly fermented idli batter.

After addition, the viability of the spores in idli batter was assessed at two steps - after cooking (steam and microwave cooking) and during storage of the batter at 4 °C.

4.6.1 Viability after cooking

The BAC1 spores added idli batter (25 g) was steam cooked (98 °C for 20 min) in idli mold and microwave cooked (model M1770,UPO, Finland) for three min at medium-high mode in a microwavable bowl. Cooked idli by both the methods were cooled at room temperature for five min, taken in stomacher bags, diluted in 0.85% saline (75 ml) and placed in stomacher for two min. From obtained suspensions tenfold dilution was made and spread plated on Tryptone glucose yeast (TGY, Appendix 2) agar. The plates were incubated at 37 °C for 48 hours and enumerated.

4.6.2 Viability during storage

The BAC1 spores added idli batter was stored at 4 °C. Viability of the spores was evaluated at 2nd and 12th hour of storage. For evaluation the idli batter was steamed in the idli mold at 98 °C for 20 min. The cooked and cooled idli was stomached in 0.85% saline (75 ml) and plated in TGY agar. After the incubation at 37 °C for 48 hours, the plates were enumerated.

4.7 Statistical analysis

The results presented were obtained from two independent experiments and each sample was measured in duplicate (n = 4). All the values were expressed as mean ± standard deviations. The microbiological cell counts were initially obtained as colony forming units (CFU) and converted into log units. Analysis of variance and significant differences for the mean values of samples were determined by one-way ANOVA and Tukeys test. Data was processed through Microsoft excel (Microsoft, USA) and Statgraphics Centurion XVI (Version16.1).

5. RESULTS

5.1 Effect of temperature treatment on LAB and yeast population in idli batter

This study was performed in order to evaluate if mild temperature treatment could improve the shelf life of idli batter by reducing the LAB and yeast counts. Temperature treatments reduced the indigenous LAB and yeast counts present in all the mild heat treated idli batter samples. The reductions in the counts were observed to be uniform and the changes in counts are presented in Table 7. Initially, the populations of total LAB and yeast in idli batter were 10.9 log cfu/g. With increase in the temperature, the counts gradually and significantly ($p<0.05$) reduced until 60 °C and reductions observed during treatment at 63 and 66 °C were not significantly ($p<0.05$) different. Thus, among the treatments, the lowest reduction (2.6 log units) in LAB and yeast counts was found at 57 °C and the highest inactivation (3.6 log units) was achieved at 70 °C.

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Table 7. Effect of temperature on LAB and yeasts counts in idli batter**.** Values are presented as mean log cfu (n=4). Different superscripts in a column signifies statistically significant difference ($p<0.05$).

Treatment temperature (°C)	LAB count (log cfu/g)	Yeast count (log cfu/g)
Untreated batter (control)	10.93 ± 0.05^{a}	10.90 ± 0.05^{f}
57	8.17 ± 0.05^{b}	8.30 ± 0.28^{g}
60	7.92 ± 0.06^{c}	7.88 ± 0.06^{h}
63	7.80 ± 0.07^{cd}	7.86 ± 0.05^{h}
66	7.74 ± 0.10^{d}	7.73 ± 0.02^{h}
70	7.26 ± 0.12^{e}	7.15 ± 0.10^{i}

5.1.1 Shelf-life study with idli batter treated at 70°C

Changes in microbiological counts

The treatment at 70 °C on fermented idli batter helped to reduce the higher populations of LAB and yeast colony counts. The changes in the LAB and yeast counts of untreated and treated idli batter at refrigerated storage are presented in Figure 7 and Figure 8. As in the preliminary experiments of temperature treatments at 70 °C, almost 3.6 log unit counts were reduced from

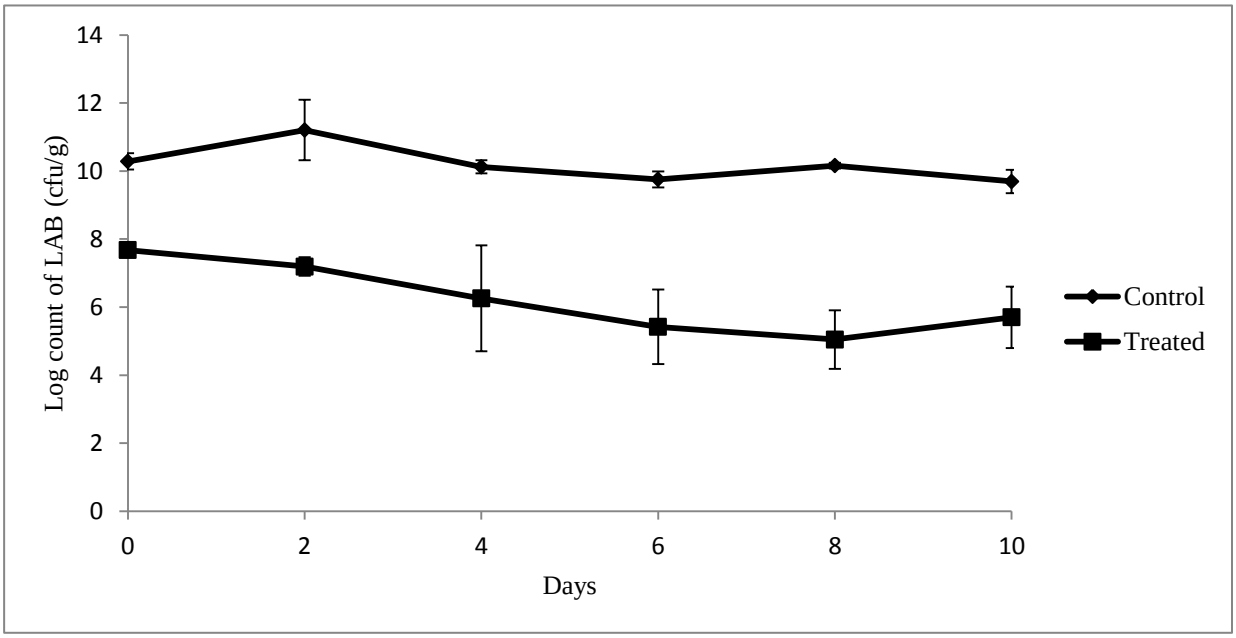

Figure 7. Changes in LAB counts of idli batter treated at 70°C and untreated idli batter during storage at 4°C for 10 days (n=4).

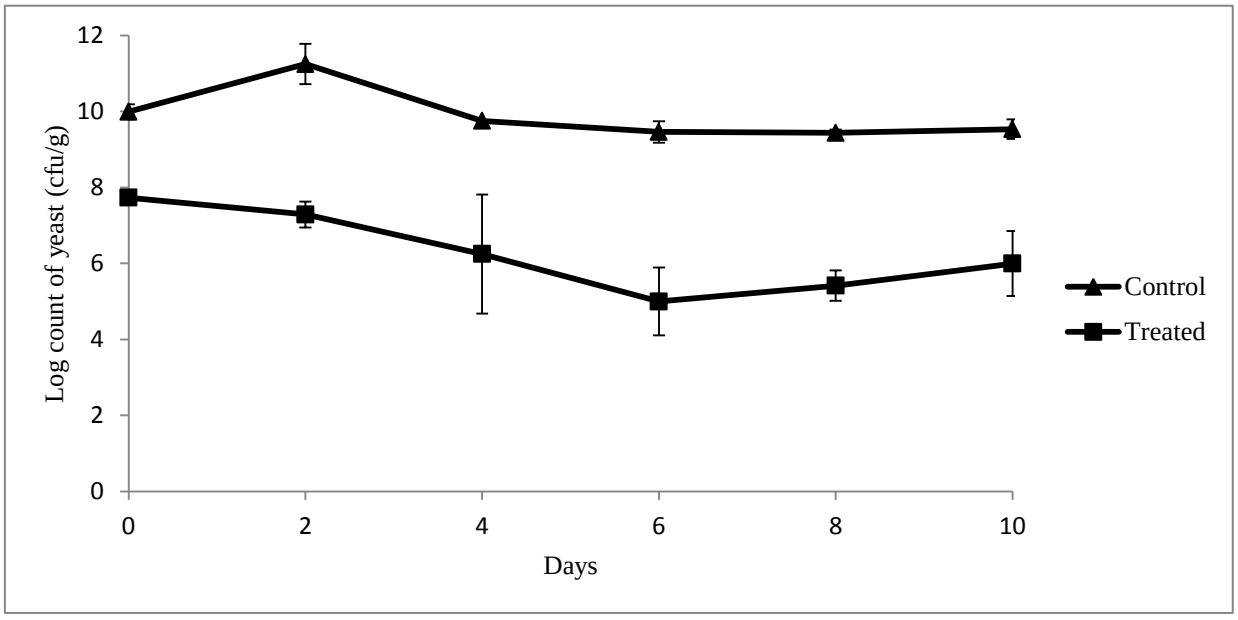

Figure 8. Changes in yeast counts of idli batter treated at 70°C and untreated batter during storage at 4°C for 10 days (n=4)

the initial LAB and yeast population of 10.5 log units. During the storage period in the untreated idli batter the LAB and yeast counts gradually started to decrease significantly with slight fluctuations. After 10 days of storage, 0.5 log units decrease was observed. The same pattern of reduction was also observed in the populations of the heat treated idli batter. Here, the significant reduction was observed from the 4th day, but from thereafter a gradual reduction in the mean counts was seen until 10th day but the reduction was not statistically significant ($p<0.05$) . During the storage period, on the whole the counts reduced in the range of 1.4-2.6 log units (from the 0th day of storage). However, the counts in the untreated batter, still remained to be higher until 10th day.

5.1.2 Viscosity profile of idli batter

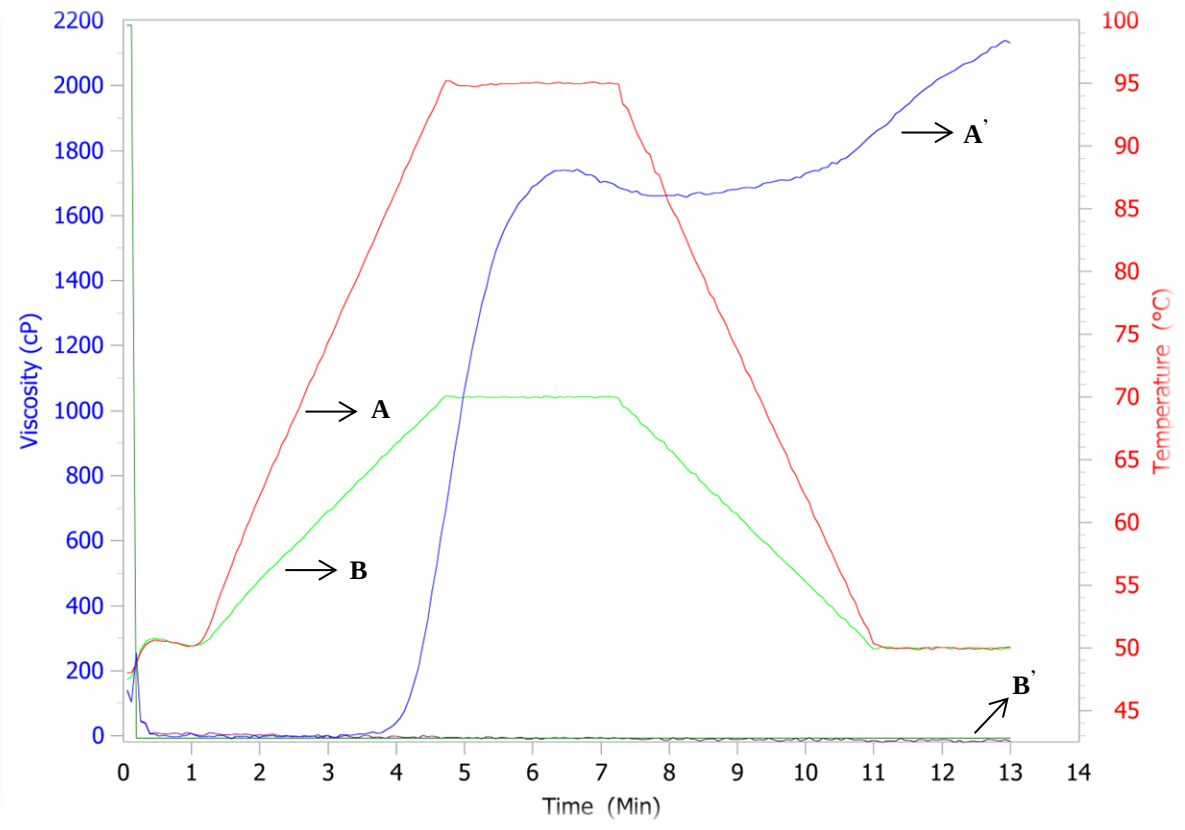

Figure 9. Viscosity vs temperature profile of idli batter. Curve A and B represent temperature profiles of idli batter with holding at 95 and 70 °C respectively. Curve A' and B' represent the viscosity profile of idli batter at the corresponding temperatures.

The changes in the idli batter with respect to temperatures were studied through RVA measurement. The viscosity profile curves obtained with two different temperature holdings generated by RVA is illustrated as in Figure 9. Both measurement profiles were initiated at 50 °C and the temperature holding phase lasted for four min. The first profile had the holding temperature (A) 95 °C, exhibited a sharp change in viscosity (A') at 86.5 °C. On further cooling, it reached the final viscosity 2133 centipoise (cP). The second profile with holding temperature 70 °C, did not exhibit any change in viscosity on heating and cooling until end of 13th min. From the obtained measurements, the temperature treatment at 70 °C for extended shelf life apparently had no effect on idli batter viscosity.

5.1.3 Change in pH during storage

During the storage period, no change in pH was observed in both mild heat treated and untreated (control) idli batter samples. On the 0th day pH of idli batter was 4.3 ± 0.09 and the values recorded during other storage days were statistically similar to 0th day.

5.2 Effect of starters in idli batter fermentation

5.2.1 Change in pH

Freshly grounded (unfermented) idli batter had an intial pH of 5.91 ± 0.10. After 14 hours of fermentation, the pH of all (control and starter added) idli batter samples reduced to 4.35 ± 0.14. However, the pH value obtained in the starter added idli batter was statistically ($p<0.05$) similar to the natural fermented idli batter.

5.2.2 Change in idli batter volume

In all the idli batter samples there was change in volume at the end fermentation (Figure 10).

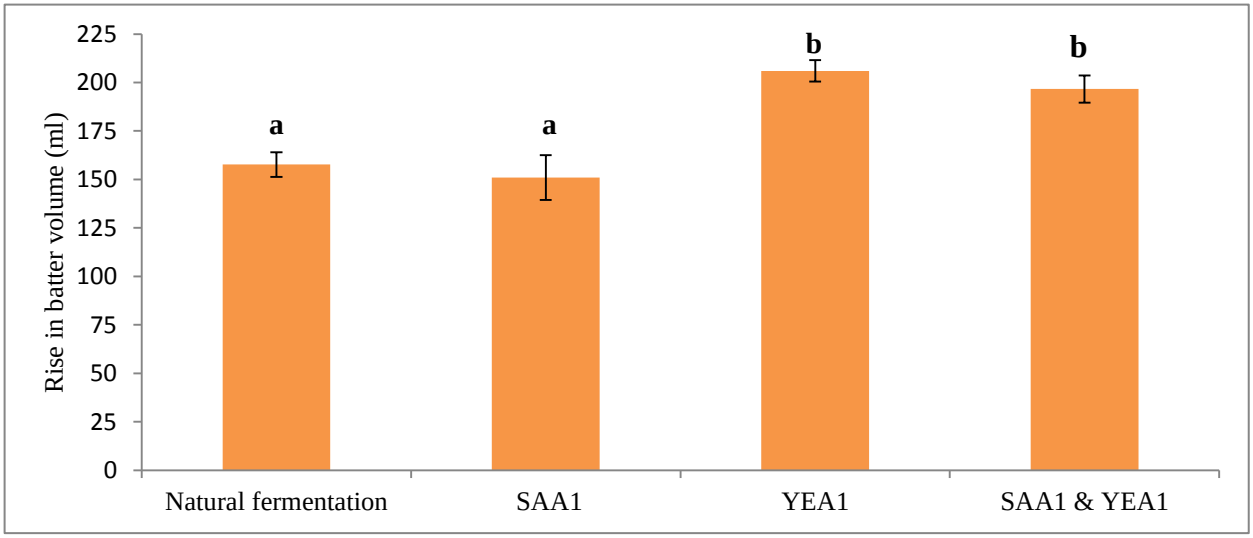

Figure 10. Rise in idli batter volume fermented with LAB and yeast starter (individually and in combination). Different alphabets indicate significant differences ($p<0.05$) between the samples ($n=4$).

Among the fermented idli batter samples, higher volume increase was observed in the YEA1 individually and combination with SAA1, although the volume of these samples were not statistically significant ($p<0.05$). Further, statistically similar lower volume increase was observed in the naturally fermented and SAA1 added idli batter. In comparison, the YEA1 in combination and individually was capable of improving the batter volume significantly implying higher gas production.

5.2.3 Microbiological profile of the idli batter

The final population of the yeast and LAB ranged between 9.7 – 9.9 log cfu/g. Among the samples, 2.8 log unit increase was observed only in the naturally fermented idli batter. In the YEA1 fermented idli batter, a rise of 1 log unit from the initial count (8.6 log) was observed. While in the idli batter with starters, the initial and final populations were virtually the same with minor fluctuations (Table 8).

Table 8. Microbiological profile of idli batters fermented with different starters (n=4)

Idli batter fermented with starters	Cell counts before fermentation (log cfu/g)			Cell counts after fermentation (log cfu/g)		
	LAB count		Yeast count	LAB count		Yeast count
	MRS	M17G	YPD	MRS	M17G	YPD
Natural fermentation	7.08 ± 0.61	7.21 ± 0.56	7.15 ± 0.39	9.88 ± 0.05	9.92 ± 0.05	9.89 ± 0.08
SAA1	10.2 ± 0.15	10.2 ± 0.15	10.08 ± 0.02	9.82 ± 0.85	9.99 ± 0.06	9.94 ± 0.07
YEA1	8.67 ± 0.13	8.72 ± 0.17	8.61 ± 0.07	9.79 ± 0.17	9.71 ± 0.28	9.76 ± 0.17
SAA1 & YEA1	9.44 ± 0.34	9.14 ± 0.57	9.61 ± 0.06	9.86 ± 0.18	9.79 ± 0.16	9.86 ± 0.15

5.2.4 Effect of starters on the B-vitamins

Influence on riboflavin content

Riboflavin significantly ($p<0.05$) increased in the idli batter by addition of starter (Figure 11). YEA1 individually and in combination with SAA1 efficiently improved the riboflavin in the range between 53 – 62% from the initial content of 0.5410 µg/g. Natural fermentation in presence of endogenous organisms did not significantly ($p<0.05$) affect the initial amounts. Consequently, upon addition of SAA1, a 22% increase of riboflavin content was exhibited. However, the increase was found to be similar as in the naturally fermented idli batter.

Influence on folate content

Figure 12 shows the change in folate content analyzed by microbial assay in presence and absence of starters. Fermentation process affected the initial folate amounts in the idli batter.

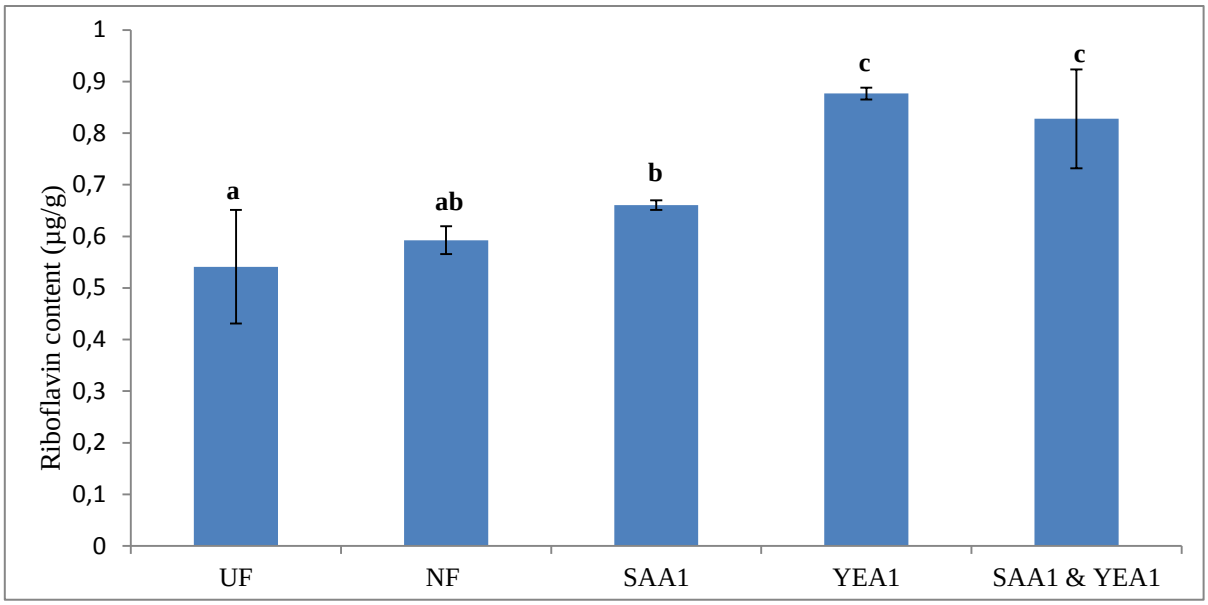

Figure 11. Change in riboflavin content before and after fermentation by different starters (individually and in combination). Different alphabets indicate significant differences ($p<0.05$) between the samples (n=4).

UF – unfermented idli batter, NF – naturally fermented idli batter

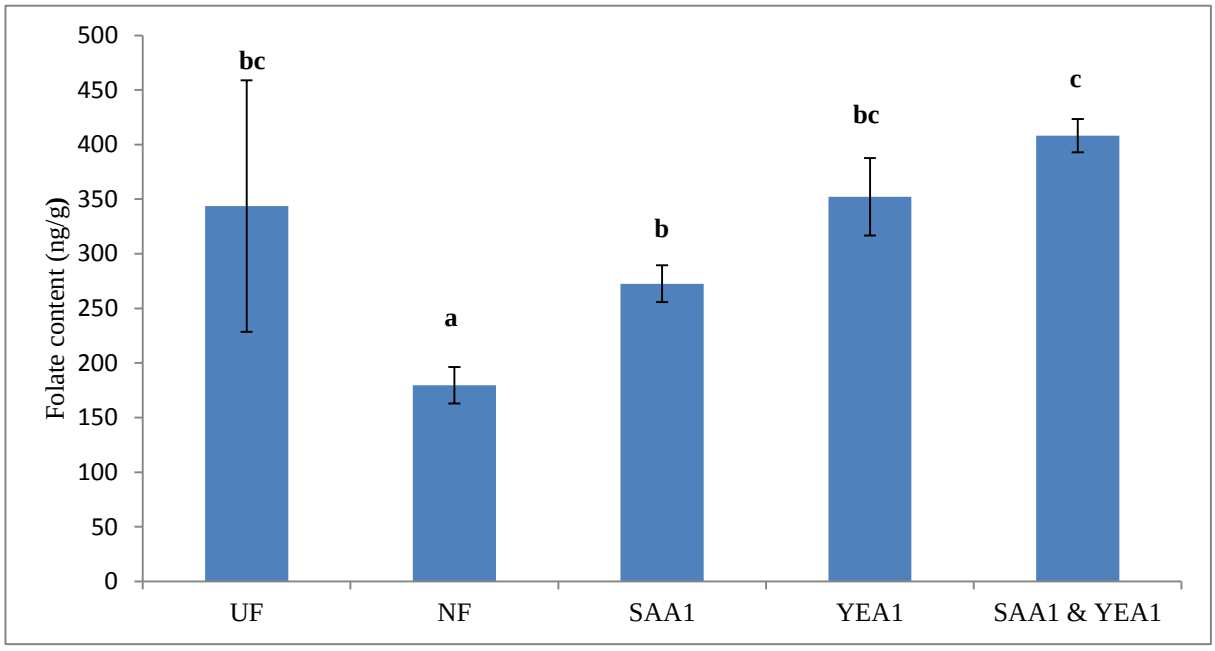

Figure 12. Change in folate content in idli batter before and after fermentation by different starters (individually and in combination). Different alphabets signify the statistical differences ($p<0.05$) between the samples (n=4)

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UF – unfermented idli batter, NF – naturally fermented idli batter

Accordingly, natural fermentation process decreased the folate significantly from 345.5 ng/g to 179 ng/g. However, increased trend on folate amounts were found in idli fermentation progressed in presence of starters.The combinational starter fermented idli batter had the highest amount of folate (408 ng/g) whereas the SAA1 fermented batter contained only 272.8 ng/g. Nevertheless, the folates amounts present in combinational starter fermented idli were statistically ($p<0.05$) similar to YEA1 fermented idli (352 ng/g). A substantial loss of 52 % of folate content occurred through natural fermentation, which was not observed in the starter fermented idli batters. On the other hand, SAA1 and YEA1 strains were neither able to synthesize folate nor prevented the indigenous organisms in idli to utilize folates. Vividly, in presence of starters the folate loss has been either reduced or prevented.

Influence on vitamin B12 content

Addition of starters had no significant effect on vitamin B12 content in the idli batter. According to the MBA assay performed, the unfermented idli batter contained 1.35 ng/g of vitamin B12 on average. Further, it is also evident that SAA1 and YEA1 did not synthesize or could not induce any change in vitamin B12 content in idli during fermentation.

5.3 SAA1 ability to produce nisin in idli batter

In the initial experiments, *Micrococcus luteus* plate assay was used to confirm the presence of antibacterial activity (putative nisin) in the idli batter. Idli batter added with SAA1 in three concentrations (0.5, 5 and 50 ml) did not exhibit any inhibition zones on the plates signifying either degradation of synthesized nisin during fermentation or that SAA1 was not capable of producing nisin in the idli fermentation system. In the study, it was assumed that due to low nisin concentration in idli batter, it was not detectable in *M. luteus* assay, hence I used the sensitive nisin fluorescence assay. Here nisin was detectable in the 50 ml of SAA1 added idli batter but in low amount and the concentration was approximately 1 µg/g.

5.3.1 Antagonisitic activity of natural flora on SAA1

It was suspected that the natural flora in idli might hinder the activity of SAA1. Accordingly, 30 colonies were isolated from the natural fermented idli batter (from M17 plates) and streaked against SAA1. No streaks exhibited inhibitions zones. Thus it potentially suggested SAA1 was able to coexist with the indigenous LAB in the idli batter during fermentation.

5.3.2 Survival ability of SAA1 in idli batter

The survival ability of SAA1 during fermentation was checked by isolating 30 colonies from the SAA1 fermented idli batter. Among the isolated colonies eight colonies exhibited clear inhibitions zones on *M. luteus* plates (Table 9). These strains were further confirmed as nisin producers by nisin fluorescence assay.

Table 9. Nisin production profile of the isolates originated from SAA1 fermented idli batter

Isolates	ISO1	ISO2	ISO3	ISO4	ISO5	ISO6	ISO7	ISO8
***M. luteus* assay**	+	+	+	+	+	+	+	+
Nisin fluorescence assay	+	+	+	+	+	+	+	+

5.3.3 Fate of pure nisin in the idli batter during fermentation

Presence of low amount of nisin signified that nisin could be degraded in idli batter. In this aspect, the fate of pure nisin (2.5% pure) in idli batter was studied upon fermentation in periodic time intervals. Presence of nisin was detected on the *M. luteus* plates until 6th hour of fermentation. This vividly indicated the degradation of nisin and at 6th hour of fermentation, only 20 IU/g of nisin was detectable in the idli batter. The degradation was dramatic, as the concentration of nisin reached (6th hour) 20 IU/g from initial concentration of 1000 IU/g. However, the nisin presence was still detectable after 24 hours of fermentation through nisin fluorescence assay. The final concentration nisin after 24 hours of fermentation was 1 IU/g.

5.3.4 Isolation of naturally nisin producing isolates

The 30 isolates (NAT) from the naturally fermented batter were subjected to *M. luteus* plate assay, out of which 13 isolates showed inhibition zones on the plate (Table 10). Finally through nisin fluorescence assay, 12 isolates were shown to be nisin producers. NAT1 isolate was not nisin positive suggesting the presence of other potential bacteriocin producers in idli batter.

Table 10. Ability of the isolates (naturally fermented batter) to produce nisin

Isolates	**NAT1**	**NAT2**	**NAT3**	**NAT4**	**NAT5**	**NAT6**	**NAT7**	**NAT8**	**NAT9**	**NAT10**	**NAT11**	**NAT12**	**NAT13**
***M. luteus* assay**	+	+	+	+	+	+	+	+	+	+	+	+	+
Nisin fluorescence assay	-	+	+	+	+	+	+	+	+	+	+	+	+

5.4 Viability of BAC1 spores in idli batter during cooking and storage

The probiotic BAC1 spores showed better stability during cooking than during storage at 4 ˚C. The obtained result was judged by number of colonies obtained from the analyzed prepared idli samples. BAC1 viability counts were obtained from steam and microwave cooking samples were 5.41 ± 0.22 log cfu/g and 4.68 ± 0.10 log cfu/g, respectively. Cell reduction was observed to be higher (3.5 log) in microwave cooking than steam cooking (2.7 log) from the initial viable count (8.20 log cfu/g) of BAC1.

The probiotic spores could not be detected after 12 hours after presence in idli batter, as no colonies were detected from the cooked idli samples. Stability of probiotic BAC1 spores in idli batter during storage was tested by adding the BAC1 spores to fermented idli batter and by obtaining viable counts from the steam cooked idli at different time points (2nd and 12th hour after addition). After two hours of addition only 4.80 log ± 0.32 cfu/g could be detected from initial count (8.2 log cfu/g) and at 12th hour no counts could be obtained. Further, its clear BAC1 spores were not stable in idli batter.

6. DISCUSSIONS

6.1 Impact of mild heat treatment on idli batter

Idli is a LAB and yeast fermented food. It is one the most important staple food in Indian subcontinent. For a long time idli was being prepared at home scale, although the preparation procedure of idli batter is laborious (Nisha et al. 2005). Due to this reason the commercially packed idli batter started to receive attention among consumers. However, the commercial idli batter has a low shelf life of four to five days at refrigerated storage (Sridevi et al. 2010). The fermented idli batter consists of high number (~10 log cfu/g) LAB and yeast counts. Upon storage with high microbial counts at refrigerated conditions (4 °C) the activity of the organisms spoils the quality of the batter leading to hard textured idli.

In our study it was attempted to reduce the counts of LAB and yeast in the idli batter by application of mild heat treatment. Our preliminary experiments focused to heat treat the naturally fermented idli batter within the temperature range of 57 °C – 70 °C. The temperatures were chosen above 55 °C on the basis of previous studies that LAB (Franz and Holy 1995) and yeast (Tchango et al 1997) could be inactivated by treatment above 55 °C. Besides, maximum temperature for treatment was chosen to be 70 °C in consideration with the pasting temperature (i.e. viscosity) of the batter. It has been reported that when a starch slurry is treated above its pasting temperature, the viscosity of the starch slurry is affected due to the formation of viscous mass and this process is not reversible on cooling to room temperature (Blevins and Protzman 1976). The idli batter is also a viscous paste made out of rice and black gram consisting of approximately 75 % starch (amylose and amylopectin) (Durgadevi 2012). Adequate information on the pasting temperature of idli batter was not available. As a result the pasting profile of the idli batter was studied using RVA. The pasting profile of idli batter (14% moisture) was studied with two holding temperatures - higher temperature (95 °C) and milder temperature (70 °C). The obtained profiles elucidated the pasting temperature of idli batter was 86.5 °C and therefore no change of viscosity in idli batter at 70 °C was observed.

Existing literatures report pasting temperatures of different rice and black gram cultivars were between 71.5 – 79 °C (Singh et al. 2006) and 75.8 – 80.3 °C (Kaur and Singh 2007), respectively. Contrarily, my study reports the pasting profile of idli batter (prepared with rice and black gram in 3:1 ratio) is higher than temperatures reported individually for different rice and black gram cultivars. The combinational presence of parboiled rice and black gram together could have affected the pasting temperature. Sharma et al. (2013) suggested stone ground batter prepared with parboiled rice exhibits higher pasting temperature due to the presence of pregelatinized starch. In addition, the information on the cultivars of rice and black gram used in this study could not be retrieved, as the raw materials were procured from domestic market (Helsinki, Finland).

The results of different mild heat treatments (57 – 70 °C for 10 min) indicated the possibility to reduce the counts of LAB and yeast in idli batter. Similar reductions of LAB and yeast counts due to mild heat treatments have been previously demonstrated in different food matrices (meat products, fruits and fermented beverages)(Franz and Von Holy 1996; Tchango et al. 1997; Rahman et al. 2011). During the treatments, apparent reductions (2.6 log cfu/g) of LAB and yeast were evident already at 57 °C. With increase in temperature, the counts of the organisms declined steadily and highest reduction was observed at 70 °C (3.6 log cfu/g). These reductions highlights the presence of heat sensitive LAB and yeast in idli batter and that intensity of heat applied has a strong influence on the count reduction. Similarly, Tchango et al. (1997) and Franz and von Holy (1995) have also proved the efficiency of heat intensity applied in reduction of the spoilage LAB and yeast in fruit juices and Vienna sausages.

Exposure of LAB and yeast to mild heat treatment or pasteurization temperatures could damage the vital cellular components of the organisms thereby causing severe injury or stress. The injured cells might die or recover, which are totally strain dependent properties. Additionally, Leistner (2000) suggested that the simultaneous exposure of organisms in different stressful environments could have a deleterious effect on the metabolic activities of the microorganism leading to cell death. In this line, the acidic environment of idli batter in combination with heat treatment could be one such stressful environment that might have also potentially reduced the counts of the yeast.

Tchango et al. (1997) recorded the similar trend in the decline (4 log cfu/ml of reduction) of spoilage yeast (*Candida pelliculosa* and *Koleckera apes*) counts in pineapple juice (pH 3.95) at different intensity of mild heat treatments. After each mild heat treatment of idli batter, the internal temperature of the batter was analyzed. A notable difference was observed between temperature applied and the internal temperature of idli batter (results not shown). For instance, idli batter treated at 57 °C for 10 min had an internal temperature of 49 °C, an 8 degree difference could be seen. This difference in temperatures might be due to thermal resistance offered by idli batter and the Falcon tube used. Mattea et al. (1986) also suggested that porous food with high moisture content can also affect the heat conduction. Accordingly, idli batter is a porous viscous liquid bubbles formed during fermentation with approximately 65% moisture and this is likely to have affected the heat conduction. This temperature difference could be identified as a hurdle in idli batter processing through mild heat treatment. In future, it is beneficial to consider this hurdle during processing of idli batter as suggested by Leistner (2002).

Previous studies have demonstrated heat treatment of food and storage at refrigerated storage has been a successful strategy to improve the shelf life of foods (Vicente et al. 2002; Cortés et al. 2008). A similar strategy of heat treatment and storage at refrigerated storage was followed in this study. However, upon storage (four days) of unheated and heated idli batter at room temperature, both the idli batters were observed to be spoilt simultaneously (results not shown).
During the 10 days storage of unheated idli batter, the LAB and yeast counts remained to be stable, until the 10th day. Certain microorganisms that optimally grow at 20 – 30 °C are capable of surviving even at refrigeration temperatures (4 – 6 °C) but at lower rates. The microbial activities at lower temperatures potentially spoil the quality of chilled foods (Russell 2002). A similar observation could be made with idli batter on storage at 4 °C. However, in the heat treated idli batter the counts of LAB and yeast reduced during the storage period. Mild heat treatment at 70 °C could have injured the LAB and yeast cells and reduced their survival ability at 4 °C. This trend was also observed by Gardiner et al. (2000) where spray dried *Lactobacillus salivarius* UCC 118 cells reduced during storage at 5 °C. The authors suggested extensive heat damage could have affected the survival ability.

Following 10 days of storage reduction of LAB and yeast counts were evident in both unheated and heated idli batters. This decrease could be explained by the process of autolysis which is an irreversible cellular process that occurs at the end of the stationary growth phase of the microorganism. The LAB and yeast counts decreased through autolysis process was also observable during storage of wine and cheese. It has been reported autolysis process varies with organisms (Alexandre and Guilloux-Benatier 2006). Heat treatment in combination with sodium chloride could additionally speed up the autolysis of cells in the food matrices (Crow et al. 1995).

6.2 Effect of starters on idli batter fermentation

Mostly idli fermentation has been carried out through natural fermentation. Recently, Sridevi et al. (2010) suggested incorporation of starters during fermentation resulted in better quality of idli batter than natural fermentation. In my study *Lactococcus lactis* N8 (SAA1) and *Saccharomyces boulardii* (YEA1) were used as starter cultures and changes in physicochemical properties, B-vitamins (riboflavin, folate and vitamin B12) and microbiological counts were observed.

Idli fermentation imparts two significant changes – acidification and leavening of batter. Based on these parameters the success of idli fermentation could be accessed (Agrawal et al. 2000). In our study pH reduction and rise in batter volume was observed in all idli batter samples fermented for 14 hours and these results were in agreement with previous studies on idli fermentation (Venkatsubbaiah et al. 1984; Balasubramanian and Viswanathan 2007; Rekha and Vijayalakshmi 2011; Ghosh and Chattopadhyay 2011). The reduction in pH and raise in batter volume signifies the increased activity of the microorganisms by utilizing the substrate (Reddy et al. 1982). Reduction in pH could be attributed by an increase in lactic acid content through the due activity of LAB during fermentation (Sridevi et al. 2010). Besides, the starter culture addition did not affect the pH of the batter which is beneficial, as too sour idli is not preferred for consumption.

On the other hand, increase in idli batter volume is due to the metabolism of heterofermentative *Leuconostoc mesenteroides* (Mukherjee et al. 1965) and different yeasts involved in the natural fermentation (Soni et al. 1986). The highest increase in batter volume was observed in the batter fermented with YEA1 individually and in combination with SAA1. However SAA1 added batter did not affect the leavening process rather improve the volume. Its apparent YEA1 played significant role in improving the batter. A similar observation was made by Soni and Sandhu (1991) on addition of different pure cultures of yeast during idli fermentation. *S. boulardii* ability to produce carbon dioxide in fruit yoghurt when used as probiotic culture has been reported and is undesirable (Lourens-Hattingh and Viljoen 2001). However, the leavening characteristic induced by YEA1 in idli fermentation is advantageous as it resulted in soft and porous idli. Usage of probiotic yeast in idli fermentation cannot impart any probiotic effect based on living cells, as the idli is steamed cooked resulting in inactivation of the yeast cells. Nevertheless, (Krasowska et al. 2009) suggested filtrate of *S. boulardii* grown culture also has the potential ability to inhibit virulent *Candida albicans*.

Due to the process of soaking of rice and blackgram, a natural microflora (LAB and yeast) develops around 10^6 -10^7 cfu/g (Mukherjee et al. 1965). LAB and yeast cell counts obtained in our study were approximately similar to previous counts reported by Iyer and Ananthanarayan (2008). In starter added idli batter increased initial counts could be observed, as a result of starter addition. Though the final counts of the starter added batter possessed similar final counts, but the reduction of pH and rise in batter highlights the progression activity of the organisms. In this study, high counts (~9.5 log cfu/g) of YEA1 and SAA1 were used in the experiments. It is worth noting that freshly grounded idli batter consists of 7 log cfu/g of LAB and yeast counts (indigenous organism). Besides, the ability of SAA1 and YEA1 to co-exist with the indigenous organisms was unknown. Thus, preliminarily high counts were used for the study.

In general, the results of the vitamin analysis of fermented (starter and natural) and unfermented idli batter revealed the riboflavin and folate contents were significantly affected by the activity of LAB and yeast. An initial level of riboflavin and folate is contributed by the cereal matrix (rice and dehulled black gram) (Reddy et al. 1982; Soni and Sandhu 1991).

In my study it was observed natural fermentation could not induce significant changes in the riboflavin content but drastically reduced the folate content. Contrastingly, Ghosh and Chattopadhyay (2011) reported natural idli fermentation could enhance folate levels. It is worth noting early studies have adopted chemical methods for determination of folates and currently chemical methods are rarely employed for folate analysis (Arcot and Shrestha 2005).
Besides, MBA assay on vitamin B12 showed for presence of low amounts of vitamin B12. The natural fermentation and starter aided fermentation could not induce any change in vitamin B12 and additionally the amounts obtained in this assay might be contributed by the cereal matrix itself.

Addition of SAA1 and YEA1 individually enhanced the riboflavin content (22% and 61%) during idli fermentation. Previously Burgess et al. (2004) demonstrated the riboflavin producing trait in *L. lactis* NZ900 and similarly, *S. boulardii* has been reported to improve the riboflavin levels during fermentation of soy milk (Rekha and Vijayalakshmi 2010). In corroboration with previous reports on the starters efficiency to enhance riboflavin levels, my study results proposed SAA1 and YEA1 are potential riboflavin enhancers. Besides, the same trend of riboflavin enhancement was evident in idli batter fermented in combination of SAA1 and YEA1. This observation signifies combinational presence of starters does not necessarily have any negative effect on the riboflavin enhancement during the fermentation process.

Individual and combinational starter fermented idli batter had higher folate levels than the naturally fermented idli batter. These results suggested two possibilities - either the added starters might have interrupted or influenced the metabolic pattern of natural microflora in consumption of folate or the added starters have the ability to synthesize folate. The first possibility was suggested based on the previous report that the introduction of LAB or yeast to a mixed culture system, can significantly influence the metabolic pattern of the other organisms. For example, *S. cerevisiae* wine fermentation pattern was found to be different during sequential fermentation with non-saccharomyces wine yeasts (*Hanseniaspora uvarum, Torulaspora delbrueckii* and *Kluyveromyces thermotolerans*) than in individual wine fermentation (Ciani et al. 2006). Another example that could be pointed out is the sequential growth of *Leuconostoc* along with *Lactococcus,* reduced the acetaldehyde synthesizing capacity of *Lactococcus*

(Tamime and Marshall 1997). In this line, a similar effect could have happened in our study on addition of the starters in idli batter along with the natural microflora. The second possibility was drawn based on the reports of the ability of *L. lactis* (Burgess et al. 2004) and *S. boulardii* (Hjortmo et al. 2005) to synthesize folate.

Folate producing LAB (Sybesma et al. 2003) and yeast (Hjortmo et al. 2005) possessed different levels of intracellular and extracellular folate. In my study the added starter cultures could have also contained intracellular folates thereby increasing the folate levels even without an increase of *S. boulardii* cells during fermentation. The folate sample preparation process might have enabled the release of intracellular folate into the food matrix as a result of cell lysis. However, the presence of starters could efficiently compensate the folate losses occurring in natural fermentation Similar folate losses during baking was compensated in yeast fermented bread (Kariluoto et al. 2004).

The maximum amount of riboflavin (0.88 µg/g) and folate content (408 ng/g) was found in YEA1 and in mixed starter added idli batter. Naturally fermented idli batter had less riboflavin (0.60 µg/g) and folate (179.5 ng/g) content in comparison with the starter fermented batter. The RDA of riboflavin and folate is 1.6 mg/day and 200 µg/day, respectively (EUFIC 2014). On average, people consume around 2-3 idli/day (approximately 100 g). By consumption of starter fermented idli batter people could meet approximately 5% and 20% of riboflavin and folate, respectively. But with natural fermentation only 3.7% and 9% of needed riboflavin and folate content is obtained. Thus, bioprocessing of idli batter with presence of YEA1 and SAA1 starters would increase the nutritional value and the quality.

6.3 Production of nisin by application of *Lactococcus lactis* N8 in idli batter

Commercially available idli batter has been encountered with presence of food pathogens like *B. cereus* and *E. coli* (Roy et al. 2007). Additionally, Jama and Varadaraj (1999) reported survival sof food pathogens (*B. cereus*, *E. coli* and *Staphylococcus aureus*) was evident in natural fermentation and low pH did not inhibit the activity of the pathogens. These reports raise concerns for the microbial safety of idli batter.

Recently, natural antimicrobial compounds are widely applied for maintaining the microbiological safety of the foods. Nisin (produced by *L. lactis*) is one such natural antimicrobial that has been widely used in food due to its antagonistic activity against a wide range of Gram positive and negative bacteria (Cleveland et al. 2001).

In this study nisin producing *L. lactis* N8 was added to idli batter along with the natural microflora and its ability to synthesize nisin in idli batter was investigated. Initially different concentrations of SAA1 fermented idli batter revealed the presence of low levels of nisin only in the idli batter fermented with 50 ml of SAA1 (9. log cfu/g). This result signifies either SAA1 growth could have been affected by natural micro flora or the produced nisin may have been degraded by the activity of natural microflora.

Investigations on the antagonistic activity of 30 isolates (from natural fermented idli batter) against SAA1 showed all the isolates grew well without exhibiting any inhibition zones on the plates. Besides, the eight colonies isolated from SAA1 fermented idli batter also exhibited potential nisin activity in both *M. luteus* plate assay and in nisin fluorescence assay. These results in corroboration strongly indicated the natural microflora in the idli batter could not have inhibited the growth and survival of SAA1. Similarly, Shimizu et al. (1999) also reported *L. lactis* growth was not affected in the mixed culture system. Additionally, Tolonen et al. (2004) and Breidt et al. (1995) demonstrated *L. lactis* can produce high amounts of nisin along with natural microflora, which is contradictory with the results obtained in this study.

It was suspected nisin was degraded during natural idli batter fermentation. In this line, the fate of pure nisin in idli batter during natural fermentation was examined. A strong degradation of nisin was observed from the 3rd hour of fermentation. A similar result was also reported by Tolonen et al. (2004) and Breidt et al. (1995). Accordingly, Tolonen et al. (2004) observed nisin production by *L. lactis* N8 during sauerkraut fermentation during initial phases, however, the nisin levels came down in the later periods during storage. Likewise, Breidt et al. (1995) reported nisin activity was detectable on the first and no nisin was observed in the fifth day. The decrease in nisin concentration could be due to proteolysis of bacteriocins (Parente and Hill 1992; Daba et

al. 1993; de Vuyst and Vandamme 1994). Additionally, addition of purified nisin (1000 IU/g) in idli batter before fermentation also resulted in idli with undesirable quality (results not shown).

During the experiments, the 30 isolates from the natural fermented batter were initially screened for nisin producing ability with *M. luteus* plate assay. Out of these, 13 strains exhibited inhibition zones. Futher, nisin fluorescence assay showed 12 strains were capable of producing nisin. The corresponding results agreed with Iyer et al. (2011) report for presence of *L. lactis* in idli batter. Thus, these results suggests supplementation of *Lactoccoccus lactis* along with natural flora is not a suitable strategy to produce nisin in idli batter. However, addition of starter culture by excluding natural flora could be one option to synthesize nisin in idli batter. Additionally, addition of purified nisin after idli fermentation can be another option to incorporate, if the degradation of nisin can be hindered.

6.4 Survival ability of probiotic *Bacillus coagulans* spores in idli batter

Most commonly used probiotic species cannot be incorporated directly in foods like bread, as these strains are heat sensitive strains. Recent studies showed *Bacillus* species have been used in food due to its increased resistance to heat and acidic conditions (Cutting 2011). In my study *Bacillus coagulans* spores were directly added to idli batter and their viability after cooking and during batter storage at 4 °C was observed.

Two cooking methods were used in this study – steam and microwave cooking. Both the cooking methods reduced the added BAC1 spore counts, but the highest reduction (3.5 log) was observed in microwave cooking. Permpoonpattana et al. (2012) also observed one log reduction of *B. subtilis* spores in viability, during biscuit baking (235 °C for 8 min) Besides, Celandroni et al. (2004) pointed microwave radiations could effectively inactivate *Bacillus* spores. The authors also demonstrated with an exposure of *B. subtilis* spores to microwave radiation for 4 min that it reduced viable spore counts 2 log and similar results could be observed in this study. However the reported reduction was higher in my study, which might be due to higher power microwave cooking.

On the other hand, BAC1 spores were not stable in idli batter during storage at 4 °C. The viability already reduced after 2nd hour of addition and no viable counts were detected after 12 hours of storage. This observation signifies acidic conditions in the idli batter possibly could have stimulated BAC1 spores to germinate and further steam cooking of idli could have inactivated the germinated BAC1 cells. Thus, BAC1 can be added to idli batter before steaming to achieve the necessary probiotic effect. Nevertheless, idli batter is not a suitable probiotic vehicle for BAC1 spores, as it loses its viability.

7. CONCLUSIONS

In this study four standalone experiments were performed to enhance the nutritional and shelf life quality of idli batter by mild heat treatment and microbial applications.

The first objective of the study was to reduce the LAB and yeast counts in idli batter by mild heat treatment. The results of this study highlighted mild heat treatment at 70 ˚C can induce a strong reduction (3.6 log units) of LAB and yeast counts without affecting the viscosity of idli batter. During the storage study, there was no change in pH level, but a further drop of microbial count was evident. Reduced populations of LAB and yeast can ensure for stable and consistent quality of idli batter until 10 days. Further descriptive sensory analysis of mild heat treated idli on different storage days could help in estimating the shelf life of mild heat treated idli batter.

The second objective of the current study was to examine the changes of physicochemical properties and B-vitamins (riboflavin, folate and vitamin B12) levels on addition of starter culture (SAA1 and YEA1) in idli batter. Among the chosen starters, YEA1 in combination and individually significantly improved the idli batter volume in comparison with natural fermentation. Technologically, this character is beneficial as it results in soft and porous idli. The added starters were not capable to improve the vitamin B12 content; however, they (individually and in combination) were able to enhance the riboflavin and retained folate content in idli batter. Though SAA1 and YEA1 were capable of increasing folate and riboflavin levels, further investigation at *in vitro* conditions is essential to prove their potential ability to synthesize

riboflavin and folate. On the other hand, the natural fermentation process did not enhance vitamin B12 and riboflavin contents instead depleted folate levels in idli batter. These results highlight the importance of using starters for idli fermentation. It also provides a possibility to enhance the B-vitamin levels and sensory quality of idli in an economical way.

The third objective of the study was to produce nisin in idli batter by addition of SAA1. The results of the study indicated the produced nisin was degraded by the action of natural microflora despite the survival of SAA1. As low amounts of nisin were detected, application of SAA1 along with natural microflora is considered as an ineffective strategy. However, addition of SAA1 excluding the natural microflora, might be one efficient way to synthesize nisin in idli batter.

The final objective was to use determine the viability of BAC1 spores during cooking and storage (refrigerated conditions). During storage at 4 ˚C BAC1 spores was not stable. During steam and microwave cooking also loss of BAC1 spores was evident. However, the loss of BAC1 spores was lesser in steam cooking. This result illustrates a practical possibility of addition of freeze dried BAC1 spores directly into idli batter while serving fresh foods in restaurants. In future by increasing the BAC1 inoculum level, the required levels of probiotic cells can be achieved compensating for the cooking loss.

In summary, the whole study presents different possibilities to improve the nutritional and shelf life of idli batter. Further, the results of the entire study provide preliminary informations for future process developments of idli.

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APPENDICES

Appendix 1

Yeast peptone dextrose (YPD) agar

Ingredients	**Amount of ingredients (g/L)**
Yeast extract	10
Peptone	20
Dextrose	20
Agar	20

Appendix 2

Tryptone glucose yeast extract (TGY) agar

Ingredients	**Amount of ingredients (g/L)**
Tryptone	5
Glucose	1
Yeast extract	2.5
Agar	20

Appendix 3

Luria Bertani (LB) agar

Ingredients	Amount of ingredients (g/L)
Tryptone	10
Yeast extract	5
Sodium chloride	10
Agar	20