Middle East Journal of Applied Sciences, 4(4): 924-930, 2014
ISSN 2077-4605

Isolation, Identification and Selection of Lactic Acid Bacterial Cultures for Production of Riboflavin and Folate

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ABSTRACT

Folate (vitamin B_{11}), an important B-group vitamin, participates in many metabolic pathways such as DNA and RNA biosynthesis and amino acid inter-conversions. Mammalian cells cannot synthesize folate; therefore, an exogenous supply of this vitamin is necessary to prevent nutritional deficiency. Riboflavin (vitamin B_2) is an essential component of basic cellular metabolism since it is the precursor of the coenzymes flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD).

Twenty samples of Laban Rayeb (Natural sour milk), raw milk and soft & Ras cheeses were collected from different locations in Egypt. MRS and M17 agar media (Oxoid) were used to isolate *Lactobacilli* and *Lactococci* respectively. Typical colonies were picked and further purified in three successive passage on MRS agar and M17 agar. Cultures were routinely maintained on appropriate solid media at 40 º C. Lactic acid bacteria organisms were identified to *Lactobacillus plantarum , Lactococcus lactis , Streptococcus thermophilus and Lactobacillus rhamnosus* . Results reveals the presence of relatively high number of lactobacilli in the Egyptian milk and dairy products, 27% of the identified cultures were *Lactobacillus plantarum.* Twenty three percent of identified strains were *Lac. lactis* subsp. *lactis*. Two *Lactococcus lactis* strains, 2 *Lactobacillus plantarum,2 Lactobacillus rhamnosus* and 2 *Streptococcus thermophilus* strains, were tested for their ability to produce folate and riboflavin in Elliker broth . Medium was inoculated by 5% of cultures and incubation at 37 ºC /27 hr . It was observed that *Lac.lactis* (Srain 2) and *S.thermophilus*(Srain 2) yielded a higher riboflavin compared to *Lb. plantarum and Lb.rhamnosus*. Further *Lac.lactis* yielded a maximum riboflavin content of 13.57 µg/ ml on 48 hr in Elliker medium. However, *S.thermophilus* yielded a maximum riboflavin content of 10.39µg/ml.Also, *Lac.lactis*(Srain 2) and *Lb. plantarum* (Srain 1)yielded a higher folate compared to *S.thermophilus and Lb.rhamnosus*.

Key words: Lactic acid bacteria, Riboflavin, Folate, *Lactobacillus, Lactococcus,Streptococcus*

Introduction

Lactic acid bacteria (LAB) are industrially important microbes that are used all over the world in a large variety of industrial food fermentations. Their contribution in these fermentation processes primarily consists of the formation of lactic acid from the available carbon source resulting in a rapid acidification of the unprocessed food material, which is a critical parameter in the preservation of these products. However, besides their lactic acid forming capacity, LAB also have the ability to contribute to other product characteristics such as flavour, texture and nutrition. Next to their most important application, which is undoubtedly in the dairy industry, LAB are also applied at an industrial scale in the fermentation of other raw food materials such as meat and vegetables(Capozzi *et al.,*2012) .

Over the past few years a number of new food ingredients labelled as being nutraceuticals have been launched on the food and pharmaceutical market. These include components that have a proven beneficial effect on human health, such as low-calorie sugars and B vitamins. Lactic acid bacteria, in particular *Lac. lactis,* have been demonstrated to be ideal cell factories for the production of these important nutraceuticals. The term ‘nutraceuticals’, launched by Stephen DeFelici in the 1980s, defines a wide range of foods and food components with a claimed medical or health benefit(Pszczola,1992) . Over the past five to ten years a large number of new food ingredients labelled as being nutraceuticals have been launched on the food and pharmaceutical market. The beneficial action of these components ranges from the supply of essential minerals or vitamins to protection against several infectious diseases. In the majority of cases, especially in the control of or protection against disease, the health claims are not supported by sound scientific data and we are merely dealing with, rather shaky, circumstantial evidence (LeBlanc *et al.* 2011). Lactic acid bacteria (LAB), in particular *Lac. lactis* are demonstrated to be ideal cell factories for the production of several important nutraceuticals. Not only do LAB have great potential for *in situ* production in fermented foods, but they also have huge potential in metabolic engineering strategies. Several examples are presented here where the production of a certain nutraceutical has been enhanced or newly induced through overexpression and/or disruption of relevant metabolic genes (Sybesma *et al.*2003) . Some vitamins such as folate (B11) are mainly found in products from plant origin. As de

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novo synthesis of folate does not occur in animals, the natural folate found in meat, eggs or dairy products originates from plant sources. Other B vitamins such as cobalamin (vitamin B12) are naturally found in animal foods products including fish, poultry, milk and milk products, eggs and meat, and are not present in plants. Riboflavin (B2) originates from organ meat, nuts,cheese, eggs, milk and lean meat, but good quantities are also found in green leafy vegetables, fish, legumes, whole grains and yoghurt. These three B vitamins are essential in the human diet. Specific population groups in Western society, such as the elderly, adolescents and other socially isolated groups, run a high risk of insufficient daily intake of these vitamins. Many food-grade bacteria produce excess of B vitamins such as riboflavin (vitamin B2), folate (vitamin B11) and cyanocobalamine (vitamin B12). This unique property of bacteria offers the possibility to fortify raw food materials such as soya, milk, meat and vegetables with B vitamins by natural means (LeBlanc *et al.* 2011). Although most vitamins are present in a variety of foods, human vitamin deficiencies still occur in many countries, mainly because of malnutrition not only as a result of insufficient food intake but also because of unbalanced diets.Even though most lactic acid bacteria (LAB) are auxotrophic for several vitamins, it is now known that certain strains have the capability to synthesize water-soluble vitamins such as those included in the B-group (folates, riboflavin and vitamin B12 amongst others).

Many industrially important LAB such as *Lac. lactis* and *S.thermophilus* have the ability to synthesize folate (Papastoyiannidis *et al.* 2006). This explains why some fermented dairy products, including yogurt, contain higher amounts of folate compared with nonfermented milks. It was shown that folate concentration in yogurt may be increased to values above 200 $\mu g\ l^{-1}$ (Wouters *et al.* 2002). However, the ability of microbial cultures to produce or utilize folate varies considerably being a strain-dependent trait. Most authors claim that *S. thermophilus* normally produce folates whereas *Lb. delbrueckii* subsp. *bulgaricus* is a folate consumer, so the selection of adequate combination of strains is essential to develop fermented foods with increased vitamin concentrations. Not only *S. thermophilus* and *Lac. lactis* have the ability to produce folates, but also other LAB like *Lb.acidophilus* and *Lb. plantarum* have been reported to produce folate in CDM (LeBlanc *et al.* 2010) as have *Leuconostoc lactis and Bifidobacterium longum*. Also, *Lb.reuteri* JCM1112, a well-known producer of vitamin B_{12}, can produce high quantities of folates, so this LAB could potentially increase folate levels in milk (Santos *et al.* 2008). Another example of LAB producing folates is the combination of *S. thermophilus* and *Bifidobacterium animalis* that increased the levels of this vitamin sixfold (Crittenden *et al.* 2003). Riboflavin (vitamin B_2) plays an essential role in cellular metabolism, being the precursor of the coenzymes flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD) both acting as hydrogen carriers in biological redox reactions involving enzymes such as nicotinamide adenine dinucleotide (NADH) dehydrogenase. Normal adults need to consume between 0·9 and 1·6 mg of this vitamin on a daily basis as the human body cannot adequately store riboflavin (Institute of Medicine 1998). Although dairy products contain riboflavin, they are not considered a good source of this essential vitamin. Considering that milk contains *c.* 1·2 mg of riboflavin per litre, an average adult person and a pregnant woman would need to consume, respectively, 1 and 1·6 l of milk per day to meet their daily requirement.

The aim of the present work was to isolate and identify the lactic acid bacteria from different dairy products and to select LAB that are able to produce vitamins riboflavib and folate.

Materials and Methods

Bacterial Strains

Isolation of Lactic acid bacteria:

Twenty samples of Laban Rayeb (Natural sour milk), raw milk and soft & Ras cheeses were collected from different locations in Egypt. MRS and M17 agar media (Oxoid) were used to isolate *Lactobacilli* and *Lactococci* respectively. Typical colonies were picked and further purified in three successive passage on MRS agar and M17 agar. Cultures were routinely maintained on appropriate solid media at 40 ^{o} C.

Identification of LAB Strains:

Lactic acid bacteria organisms were identified according to the criteria described by Hardi(1986), Kandler & Weiss (1986) and Mundt (1986).

Source of commercial strains:

Lactobacillus rhamnosus was obtained from Northern Regional Research Laboratory (NRRL). *Lactobacillus plantarum* was provided by Agriculture College Ain-Shams Uni. (MIRCEN). *Streptococcus thermophilus and Lactococcus lactis* were obtained from Chr. Hansen's Lab., Denmark.

Media

Elliker medium broth (Elliker et al 1956) was used to produce vitamins by lactic acid bacteria. The lactic acid bacteria were cultivated in Elliker broth and incubated at 35 ^{o}C for 24hr.

Screening of vitamin production

Active cultures of LAB were tested by using Elliker broth. All cultivation media were seeded with 5 % (by volume) inoculums and incubated at 35 C ^{0} for 48 hr.

HPLC analysis

The HPLC system was a HP 1100 chromatograph (Agilent Technologies, Palo Alto, CA, USA) equipped with an auto-sampler, quaternary pump and a diode array detector were used. The quantitation was integrated by Chemstation chromatographic software interfaced to a personal computer.The chromatographic column was C18 Zorbax XDB (250 mm x 4.6 mm, 5 µm film thicknesses). The column was kept at room temperature at a flow rate of 0.8 ml/min with a total run time of 12 min. Separation of vitamins was carried out by gradient elution with methanol (A) and 1% TFA containing water (B). the elute composition was initially 8 % A + 92 % B, held for 2 min, and changed linearly to 92 % A + 8 % B in the next 4 min and held for 6 min. Detection wave length for detection of cyanocobalamin was set at 254 nm. The retention time of cyanocobalamin was about 7.059 min.

Results and Discussion

Thirty lactic acid bacteria isolates were classified into the genera *Leuconostoc, Streptococcus, Lactococcus* and *Lactobacillus* based on their morphology and biochemical characters .Table (1) shows the percentage distribution of different genera of LAB . Of the cultures, 50 % belonged to the genus *Lactobacillus*. Also, Table (1) reveals the presence of relatively high number of lactobacilli in the Egyptian milk and dairy products, four strains were *Streptococcus*, *Leuconostoc* were two in numbers. Nine strains belonged to *Lactococcus* . The results of the identification are presented in Table (2). Twenyt seven percentages of the identified cultures were *Lactobacillus plantarum.* Twenty three percent of identified strains were *Lac. lactis* subsp. *lactis*.

Production of folate by different lactic acid bacteria

A variety of lactic acid bacteria were screened for their ability to produce folate.Two strains of *Lactococcus lactis*, 2 *Streptococcus thermophilus* and *2 Lactobacillus plantarum and 2 Lactobacillus rhamnosus*, all produced folate. Large differences in folate production for different strains. From Table (3), folate was produced by *Streptococcus thermophilus* strain at level 0.831-1.455 µg/ml in Elliker medium. *Lactococcus lactis* also produced folate ,the data are shown in Table (4) . Strain (2) produced 2.495 µg/ml in Ellliker , but strain (1) produced only 1,455µ g/ml in Elliker. From Table (5) results revealed that *Lactobacillus plantarum* produced folate by two strains at range 2.22 µg/ml to1.95µg/ml in Elliker. Also, in Table (6) the data cleared that *Lactobacillus rhamnosus* produced variable quantities from folate by two strains. Folate, an important B-group vitamin, participates in many metabolic pathways such as DNA and RNA biosynthesis and amino acid inter-conversions. Mammalian cells cannot synthesize folate; therefore, an exogenous supply of this vitamin is necessary to prevent nutritional deficiency (LeBlanc *et al.* 2007). Numerous researchers have reported that lactic acid bacteria, such as *Lac.lactis* and *S. thermophilus* have the ability to synthesize folate **(**Sybesma *et al.*2003 , Crittenden *et al.,*2003 , Laiño *et al.,*2012). Sybesma *et al.*2003), found that Several species and strains from the lactic acid bacterial genera *Lactococcus*, *Lactobacillus*, *Streptococcus*, and *Leuconostoc* were screened for folate production. LeBlanc *et al.* (2007) and Patel *et al.* (2013) reported that among LAB, many *Lactobacillus* spp. and *lactococcus* spp. including *Lb. Plantarum, Lb. bulgaricus, Lac. lactis,S.thermophilus* and *Enterococcus* spp. have the ability to produce folate. The lactic acid bacteria *Lac. lactis* MG1363 and *S. thermophilus* B119 were further analyzed for folate production under different growth conditions. *Lac. lactis*, *S. thermophilus*, and *Leuconostoc* spp. Produced folate in the range of 5 to 291 µg/liter. *Lactobacillus* strains, with the exception of *Lb. plantarum*, did not produce folate . On the other hand, Rossi (2011) found that some lactobacilli (*Lb. gasseri, Lb. salivarius, Lb.acidophilus* and *Lb. johnsonii*) used as both starter cultures and probiotics, cannot synthesize folate because they lack few specific genes involved in folate biosynthesis. Goswami (2012) evaluated production of folic acid and biotin from *Lb. helveticus* MTCC 5463, a probiotic strain and *Lb. rhamnosus* MTCC 5462, a normal lactobacilli. Both the strains increased contents of folic acid in fermented milks either used singly and in combination; however, biotin concentration increased

only with probiotic strain. Several *Lb.plantarum* strains able to produce high amounts of folate were recently identified and folate production conditions optimized (Sybesma *et al.* 2003 and Nor *et al.* 2010). *Lac. lactis* and *S. thermophilus* strains have the ability to synthesize folate (Crittenden *et al.* 2003; Burgess *et al.* 2009; LeBlanc *et al.* 2010) which may further explains why some fermented dairy products, including yogurt, contain higher amounts of folate than non-fermented milks .

Table 1. The percentage distribution of different genera of LAB.

Genera	No	%
Lactobacillus	15	50 %
Streptococcus	4	13 %
Lactococcus	9	30 %
Leuconostoc	2	7 %

Table 2. Identification of 30 LAB isolates

Species	No	%
Lactobacillus plantarum	8	27 %
Lactobacillus bulgaricus	6	20 %
Lactobacillus rhamnosus	1	3 %
Lactococcus lactis subsp lactis	7	23 %
Lactococcus lactis subsp diacetylactis	2	7 %)
Streptococcus thermophilus	4	13 %
Leuconostoc mesentroides	2	7 %

Table 3. Production of Folate µg/ml by *S. thermophilus*

Type	Folate µg/ml
Strain 1 Local Strain	1.455906822
Strain 2 commercially strain	0.831946755

Table 4. Production of Folate µg/ml by *Lactococcus lactis*

Type	Folate µg/ml
Strain 1 Local Strain	1.455906822
Strain 2 commercially strain	2.495840266

Table 5. Production of Folate µg/ml by *Lactobacillus plantarum*

Type	Folate µg/ml
Strain 1 Local Strain	2.221297837
Strain 2 commercially strain	1.955074875

Table 6. Production of Folate µg/ml by *Lactobacillus rhamnosus*

Type	Folate µg/ml
Strain 1 Local Strain	1.913477537
Strain 2 commercially strain	1.081530782

Additionally, Laiño *et al.* (2012 **)** reported that *Lb. delbrueckii subsp. bulgaricus* and *S. thermophilus* strains were isolated from artisanal Argentinean yogurts and were grown in folate-free culture medium (FACM) and nonfat milk after which intracellular and extracellular folate production were evaluated. From the initial 92 isolated LAB strains, 4 *Lb. delbrueckii subsp. bulgaricus* and 32 *S. thermophilus* were able to grow in the absence of folate. *Lb. delbrueckii subsp. bulgaricus* CRL 863 and *S. thermophilus* CRL 415 and CRL 803 produced the highest extracellular folate levels (from 22.3 to 135 µg/L) in FACM. In nonfat milk, these strains were able to increase the initial folate concentrations by almost 190%. This is the first report where native strains of *Lb. delbrueckii subsp. bulgaricus* were shown to produce natural folate. Laiño *et al.*(2013) used the LAB strains identified in the previous study in developing novel fermented products bio-enriched in natural folates that could in turn be used as an alternative to fortification with the controversial synthetic chemical folic acid.

Production of Riboflavin by different lactic acid bacteria

Riboflavin or vitamin B2 is a dietary necessity for humans as, unlike many plants, fungi and bacteria, they are unable to synthesise the vitamin. Two sources of riboflavin are available to humans: adietary source and riboflavin produced by microflora of the large intestine (Hill, 1997). Some microorganisms (mainly bacteria and fungi), rather than producing riboflavin, are capable of riboflavin overproduction. In bacteria this trait can be achieved either by metabolic engineering (Perkins *et al.* 1999) or by exposure to purine analogues and/or the toxic riboflavin analogue roseoflavin (Burgess *et al.* 2006). In *Lac. lactis* both of these approaches have been used with success (Burgess *et al.* 2006). The toxic analogue approach has also been successfully employed for *Lb. plantarum, Leuconostoc mesenteroides* and *Propionibacterium freudenreichii* (Burgess *et al.* 2006) and a fermented dairy product made with the vitamin B2- overproducing *P. freudenreichii* strain has been reported

to counteract riboflavin deficiency in an animal model (LeBlanc *et al.* 2005; LeBlanc *et al.* 2006). From Table (7) ,riboflavin was produced by *Streptococcus thermophilus* strain (2) at level 10.39µg/ml, But strain 1 gave 7.59µg/ml in Elliker medium . *Lactococcus lactis* also produced riboflavin , the data are shown in Table (8) strain (2) produced 13.57 µg/ml and strain (1) produced 8.73µ g/ml in Elliker medium. From Table (9), *Lactobacillus plantarum* produced riboflavin at range 7.026 µg/ml to 7.08 µg/mlin Elliker medium . Also, in Table (10), the data cleared that *Lactobacillus rhamnosus* produced low quantity from riboflavin by two strains.

Such strategies do not attempt to generate alternative production strains, but rather replacing riboflavin consuming strains used in traditional food fermentation processes with riboflavin-producing counterparts, thereby increasing riboflavin bioavailability in the food product and introducing an added health benefit. The elevated levels of the vitamin which would be produced in such foods would not have any negative health implications as no upper limit of intake has been set for riboflavin due to a lack of evidence on adverse effect in humans (Flynn *et al.* 2003). Grain products contain only low amounts of riboflavin as much of the vitamin is lost due to processing. However, chemical fortification practices make certain breads and cereals very good sources of riboflavin (Powers 2003). Recently, spontaneous roseoflavin-resistant derivatives of *Lb. plantarum* previously identified from natural sourdoughs were used during a standard bread-making or pastamaking procedures (Capozzi *et al.* 2011). In both cases, the approach resulted in a considerable increase of vitamin B2 content (about a twofold and threefold increase in pasta and bread, respectively), thus representing a convenient and efficient food-grade biotechnological strategy for the production of vitamin B2-enriched bread and pasta (Capozzi *et al.* 2012), even though the stability of the mutants phenotype **.** Juarez del Valle *et al.* (2014),selected riboflavin-producing strains of LAB from 179 strains, only 42 were able to grow after the fourth passage in a riboflavin free-medium.. Only five strains (*Lb. fermentum* CRL 220 and CRL 345, *Lb. plantarum* CRL 725, *S. thermophilus* CRL 417 and *Lb. paracasei subsp. paracasei* CRL76) were selected due to their high riboflavin producing capabilities in the B2-free medium, and were then used to inoculate soymilk. The criteria of selection were the highest producers of extracellular riboflavin and optimal growth in riboflavin-free culture medium. Selected strains showed an extracellular concentration of riboflavin above 190 ng/mL reaching high values of 260 ng/mL. The levels of riboflavin produced by these strains were higher than those described by Capozzi *et al.* (2011) in LAB isolated from sourdough.

Table 7. Production of Riboflavin µg/ml by *Sreptococcus thermophilus*

Type	Riboflavin µg/ml
Strain 1 Local Strain	7.592593
Strain 2commercially strain	10.39216

Table 8. Production of Riboflavin µg/ml by *Lactococcus lactis*

Type	Riboflavin µg/ml
Strain 1 Local Strain	8.736383
Strain 2commercially strain	13.57298

Table 9. Production of Riboflavin µg/ml by *Lactobacillus plantarum*

Type	Riboflavin µg/ml
Strain 1 Local Strain	7.08061
Strain 2commercially strain	7.026144

Table 10. Production of Riboflavin µg/ml by *Lactobacillus rhamnosus*

Type	Riboflavin µg/ml
Strain 1 Local Strain	7.875817
Strain 2commercially strain	6.786492

Conclusion

Lactic acid bacteria served as inimitable source for developing novel products and applications, especially those that can satisfy the increasing consumer's demands for natural products and health benefits. The identification and application of LAB and related strains delivering health-promoting compounds is very promising field and furthermore, their ability to enrich the food matrix or human body with the aid of producing vitamins and biocatalysts further enhance the scope of utilizing them for medicinal and health applications. It is expected that the food industry will exploit novel and efficient vitamin-producing strains to produce fermented products. Such products are expected to provide economic benefits to food manufacturers since increased 'natural' vitamin concentrations would be an important value-added trait without increasing production costs. The increase of B-group vitamin concentrations in fermented/ functional foods is possible through judicious selection of microbial species and cultivation conditions. In addition, the use of microencapsulation technique to increase vitamin production by LAB strains will be applied in the further research.

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