LWT - Food Science and Technology 86 (2017) 277–284

Contents lists available at ScienceDirect

LWT - Food Science and Technology

journal homepage: www.elsevier.com/locate/lwt

Ability of lactobacilli isolated from traditional cereal-based fermented food to produce folate in culture media under different growth conditions

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ARTICLE INFO

Article history:
Received 27 April 2017
Received in revised form
11 July 2017
Accepted 1 August 2017
Available online 3 August 2017

Keywords:
Folate
Fermented products
Lactic acid bacteria
Vitamin fortification

ABSTRACT

Folates are essential vitamins (B9). Lactic acid bacteria (LAB) can be used to increase the folate levels in foods during fermentation. Here, 151 LAB strains isolated from fermented food were screened for folate production. Folates were extracted by heat treatment coupled with enzymatic extraction. Total, extra- and intracellular folate were quantified by microbiological assay. Most *Lactobacillus plantarum* and *Lactobacillus fermentum* strains synthesized folate, *Pediococcus pentosaceus* strains consumed folate, and *Pediococcus acidilactici* strains either consumed or produced. Seven strains were further examined for their ability to synthesize or consume folate during growth in rich folate medium (MRS) and folate-free medium (FACM). For most strains, the differences observed in MRS were attenuated in FACM. *L. fermentum* 8.2 and *L. plantarum* 6.2 produced the highest levels of folates in MRS (97 and 93 ng·mL^{-1}) and FACM (29 and 44 ng·mL^{-1}). In most cases, production reached a maximum from 9 or 11 h to 24 h. Incubations in FACM with mixed LAB cultures showed that folate production is not additive. We demonstrated how biosynthesis of folate by LAB depends on species, strains, and is highly influenced by time and medium composition. Characterized LAB strains can be further explored for novel folate bio-enriched functional food.

1. Introduction

Folate (vitamin B9) refers to tetrahydrofolate molecule (THF) and its numerous derivatives. Folic acid is the synthetic form of folate and it is commonly used for food fortification and nutritional supplements. It is formed by a pteridine ring linked to *p*-amino-benzoic acid (*p*-ABA) and L-glutamic acid, two precursors for folate synthesis (Hanson, Gage, & Shachar-Hill, 2000). Folate is essential to the human body as it is involved in DNA replication, cell division (Duthie, Narayanan, Brand, Pirie, & Grant, 2002), methylation, biosynthesis of nucleotides and amino acid metabolism (Cossins & Chen, 1997; Hanson et al., 2000). In addition, it has an effect in the prevention of neural tube defects (Wald & Sneddon, 1991) as well as against colorectal and pancreas cancer (Giovannucci et al., 1998; Lin, An, Wang, & Liu, 2013; Van Guelpen et al., 2006).

Humans cannot synthesize folate. Therefore, an exogenous intake of this vitamin is essential. Leafy greens (spinach), legumes (lentils, chick peas, beans) nuts, fruits (citrus), liver, certain cheeses and fermented dairy products are good sources of folate (Eitenmiller & Landen, 1999). However, folate deficiency still occurs worldwide, leading to severe diseases such as megaloblastic anaemia and congenital malformation (McLean, de Benoist, & Allen, 2008; McNulty & Scott, 2008). For those reasons, many countries have adopted national fortification programs (Youngblood et al., 2013). Though, folic acid fortifications may have possible side effects as to mask the symptoms of a vitamin B12 deficiency or promote growth of pre-neoplastic lesions (Cuskelly, Mooney, & Young, 2007; Kim, 2007). A natural enrichment of folate in foods via fermentation represents a possible alternative to synthetic enrichment. The folate content of food can be highly increased by fermentation using selected folate producing lactic acid bacteria (LAB) (Kariluoto et al., 2014; LeBlanc, de Giori, Smid, Hugenholtz, & Sesma, 2007). In this context, most of the work

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http://dx.doi.org/10.1016/j.lwt.2017.08.007
0023-6438/

has been done on dairy products, which are highly consumed in developed countries (LeBlanc et al., 2011; Saubade, Hemery, Guyot, & Humblot, 2016). Nevertheless, fermented cereal foods are also extensively consumed in developing countries, and may thus meaningfully contribute in increasing dietary intake of folate. Their fermentation is dominated by LAB (Humblot & Guyot, 2009; Lei & Jakobsen, 2004). Cereal-based fermented porridges made from pearl millet are commonly consumed by young children in different countries of Africa (Lei & Jakobsen, 2004; Tou et al., 2006). A recent publication of our group shows that the folate content of a fermented porridge made from pearl millet and called *ben-saalga* in Burkina Faso, varied from 0 to 3.3 $\mu g \cdot 100\ g^{-1}$ on a fresh weight basis, which is rather low (Saubade, Humblot, Hemery, & Guyot, 2017). Nevertheless, genes *folK* and *folP* encoding enzymes involved in folate biosynthesis have been detected by PCR in 80% of these food samples (Saubade et al., 2017) and in 96% of a collection of 151 LAB isolated from *ben-saalga* (Turpin, Humblot, & Guyot, 2011). A gap is therefore present between the genetic potential identified in the actors of *ben-saalga* fermentation and the nutritional reality of the final fermented product.

The aim of the present study was to investigate the actual ability of LAB previously isolated by our group from *ben-saalga* (Turpin et al., 2011) to produce or consume folate when grown in medium containing folates and their precursors. To better investigate the conditions for the synthesis of folate by these LAB strains, a selection of the most producing and consuming ones was studied more deeply during growth in folate-free medium, which only contains one of the precursors required for folate synthesis (*p*-ABA). Growth and folate concentration were measured along time. In addition, differentiation between intra- and extracellular folate was determined. Such strains can be potentially explored for the development of folate bio-enriched fermented food products for functional application in future.

2. Materials and methods

2.1. Identification of folate producing and folate consuming LAB

A total of 151 LAB strains belonging to four species (*Lactobacillus fermentum, Lactobacillus plantarum* and its subspecies *Lactobacillus paraplantarum, Pediococcus acidilactici* and *Pediococcus pentosaceus*), previously isolated from *ben-saalga* (Turpin et al., 2011), were used in this study. The strain *L. plantarum* WCFS1 was also included as a positive control for folate quantification (Santos et al., 2007; Wegkamp, de Vos, & Smid, 2009). All strains were stored in de Man Rogosa and Sharp medium (MRS, Difco, Sparks, USA) at −80 °C in glycerol (40%) before use.

For identification of folate producing and folate consuming LAB, each strain was plated on MRS agar and incubated for 48 h at 30 °C. Then, an isolated colony from each strain was inoculated into 4.5 ml of MRS broth and incubated for 16 h at 30 °C. Samples were then flushed with nitrogen and kept at −20 °C until total folate extraction and quantification as described below (2.3 and 2.5). A sample of each batch of MRS broth was also stored the same way, to quantify folate initially present in the broth.

2.2. Kinetics of growth and distribution of folate for strains grown on folate-free medium (FACM)

From each species of the collection, one folate producer and one folate consumer strain were selected, with the exception of *P. pentosaceus* species where no folate producer was identified. The strain *L. plantarum* WCFS1 was included as a positive control.

The ability of those strains (n = 7) to consume or produce folate was confirmed by measuring total folate after incubation in a folate containing medium (MRS) for 24 h at 37 °C. Results were compared to those obtained by the same seven strains grown in a folate-free medium, which contained 2 $mg \cdot L$ of *p*-ABA that is a precursor required for folate synthesis (Folic Acid Casei Medium, FACM, Difco), incubated in the same conditions.

Extracellular and intracellular folates were extracted during growth in FACM medium. Briefly, overnight MRS pre-culture were washed twice with sterile saline solution (0.9% w/v NaCl, Sigma-Aldrich), and then re-suspended in this solution in the same volume. Each strain was inoculated individually in FACM medium at 1% (v/v) and incubated without stirring at 37 °C for 24 h. At different time points, samples were taken for pH and colony forming units (CFU) determination. CFU were determined by plating serial dilutions of the samples on MRS agar (incubated at 30 °C for 48 h). Plate counting was performed in duplicate and average of three different incubations was expressed as $Log10\ CFU \cdot mL^{-1}$. Samples were also kept at − 20 °C until analysis of intracellular and extracellular folate as described below (2.4 and 2.5). Three independent experiments were performed.

2.3. Extraction of total folate from bacterial cultures

Folate extraction procedure was adapted from Kariluoto and Piironen (2009). Samples were diluted twice in extraction buffer (pH 7.85; 50 mM CHES/50 mM HEPES, 10 mM 2-mercaptoethanol, sodium ascorbate 2% (w/v), Sigma-Aldrich), the tubes were flushed with nitrogen and heated to 100 °C for 10 min. They were vortexed twice during thermal extraction and then cooled on ice. The pH was adjusted to 4.7 with acetic acid (10% w/v) and 1 mL of a γ-glutamylhydrolase solution (a conjugase prepared from desiccated hog kidney, 5 $mg \cdot mL^{-1}$ in mQ water), was added to each tube. The tubes were flushed with nitrogen and incubated in a thermostatically controlled water bath for 3 h at 37 °C in the dark, under continuous agitation (Memmert, 100 rpm). The pH was readjusted to 6.1 and the volume to 50 mL with sodium ascorbate at 0.5% (Sigma-Aldrich). Centrifugation was performed at 20,000 g for 5 min at 4 °C. The same extraction procedure was applied to samples of non-inoculated MRS broth and to blank samples (extraction buffer plus conjugase), used as controls. After centrifugation, the supernatants were stored at −20 °C, protected from light, for a maximum of two weeks before folate analysis (2.5).

2.4. Extraction of intracellular and extracellular folate from bacterial cultures

The extraction of folate was performed as described above, with some modifications. Briefly, a centrifugation step (12000×*g*, 10 min, and 20 °C) was performed before the extraction, to separate the supernatant (extracellular folate) from the pellet (intracellular folate) (Sybesma et al., 2003). The supernatant was then diluted 1:1 (v/v) in extraction buffer, while the pellet was re-suspended in 1 ml of sodium ascorbate (0.5% w/v) and 0.5 g of zirconia and silica beads of 0.1 mm (BioSpec Products, Zirconia/Silica) were added to the suspension. The cells were disrupted at 30 Hz for 3 min at 20 °C in a vibration mill (TissueLyser II Retsch®, QIAGEN®; IKA, Wilmington, NC). The cell extract was centrifuged (10,000 g, 5 min, 20 °C) and the supernatant was re-suspended in 1:1 (v/v) of the extraction buffer.

Folates were then extracted by thermal treatment and deconjugation, as described before (2.3). Extracts were stored at −20 °C, for a maximum of two weeks before folate quantification (2.5).

2.5. Folate quantification

The quantification of extracted folate (total as well as extra- or