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Occurrence and natural enhancement of folate in oats and barley

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ACADEMIC DISSERTATION

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ABSTRACT

Folate, one of the B vitamins, has a well-established role in preventing neural tube defects (NTDs) in the developing foetus and megaloblastic anaemia. Folate intake is below recommended levels, especially in countries where mandatory folic acid fortification is not practiced. In these countries, interest to innovate ways for the natural enhancement of folate is high. Wholegrain cereals provide a high proportion of natural folate. Oats and barley have once again attracted attention as cereals with health potential due to their high beta-glucan contents. However, knowledge on the folate content in oats and barley and their milling fractions is limited. In turn, several microbes are potential folate producers in aqueous processes. Folate content in cereal-based products could be further improved by utilising folate-rich milling fractions or by using the ability of microbes to synthesise folate.

In this thesis, oats and barley were studied as sources of folate. The total folate was determined in five oat and barley cultivars over three years with a microbiological method. Different fractions were produced from oats by oat processing and from barley by scarification and industrial milling. The folate vitamer distribution in fractions was examined with the ultra-high performance liquid chromatography (UHPLC) method. Furthermore, bacteria isolated from cereal products, food-grade yeasts and lactic acid bacteria (LAB) were studied for their folate-production in rich medium and in aqueous processes of oat and barley bran and flour. In addition, the profile of the produced folate vitamers was studied in rich medium and in oat flour and barley bran matrices.

The validated UHPLC method proved to be fast and sensitive for determining seven folate vitamers in cereal and microbe samples. New data was obtained on the folate content and its variation in oat and barley cultivars. The total folate content in barley grains was slightly higher at 770 ng/g dm, than the folate content in oats (690 ng/g dm) when determined shortly after harvest. These contents were higher than had been previously found in wheat. In addition, the variation among the cultivars in each year was moderate. This study also showed that oat and barley grains might lose folate during storage. Dry-fractionation of oats and barley yielded fractions with high folate content. Among the oat fractions, the highest folate content was found in its by-products. The folate content in the residual flour from oat flaking was 2.5-fold that of native oat grain. In barley grain 40–60% of the folate was lost during industrial dehulling and pearling processes. The total folate content in oat and barley fractions demonstrated that folate was localised in the outer layers and germ. The main folate vitamers in the oat and barley fractions were 5-CH_3-H_4folate, 5-HCO-H_4folate and 5,10-CH^+-H_4folate.

A few endogenous bacteria isolated from oat bran produced folate in rich medium more than *Saccharomyces cerevisiae,* which is known as a good folate producer. In cereal matrices, several food-grade yeasts produced a significant amount of folate with glucose addition, but folate production by LAB was low. Folate content in the oat flour matrix fermented with *Pseudomonas* sp. for 24 h and stored for 2 weeks in the cold was 9-fold that of the control sample. Bacteria and yeasts accumulated the most 5-CH_3-H_4folate followed by H_4folate and 5,10-CH^+-H_4folate.

The results in this thesis show that oats and barley are good sources of folate. Introducing folate-rich milling fractions into cereal products would increase the folate intake of consumers. Further, food-grade yeasts and bacteria have potential for folate enhancement in aqueous cereal processing. Particularly, the folate production by some cereal-based endogenous bacteria offers possibilities for natural folate enrichment in beta-glucan-rich oat and barley matrices.

PREFACE

This academic journey has been long and winding, but extremely rich and fruitful to me. During this voyage, I have also enjoyed maternity leave periods to focus on my family. My research in Food Sciences has taken me from the field of sterols, through lipid oxidation, antioxidants and phenolic compounds and finally into the challenging world of folates, first as a researcher in the EU-project Healthgrain and then in the Folafibre project.

The practical part of this study was carried out at the Department of Food and Environmental Sciences, at the Division of Food Chemistry, University of Helsinki. It was part of the project "Folafibre — aqueous processing of oats and barley: *In situ* enhancement of folate and associated bioactivity compounds while maintaining soluble dietary fibre physiologically active". The project was funded by the Academy of Finland and partially by the August Johannes and Aino Tiura Foundation. Their financial support is gratefully acknowledged.

I owe my sincerest appreciation and gratitude to my supervisors Professor Vieno Piironen and Docent Susanna Kariluoto. Vieno, you gave me the possibility to work on this topic and to build it up into my dissertation. You trusted, encouraged and gave me patient guidance during the writing period with incredible professionalism. Susu, you taught me how to take care of sensitive folates. Your expertise in folates and gentle supervision with encouraging words gave me motivation towards my goal. Susu, it has been easy to work with you as well as share the joys and sorrows of our lives.

I greatly appreciate Professor Elżbieta Gujska and Professor Cornelia Witthöft for pre-examination of this dissertation. Thank you for critically evaluating my thesis and for the constructive comments and suggestions you provided.

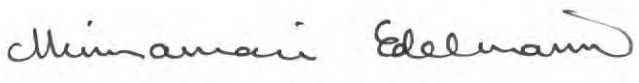
My colleagues in the Folafibre research team are greatly appreciated. I deeply thank Professor Laura Nyström for valuable comments, discussions and supportive words during the preparation of the manuscripts and the thesis. I sincerely appreciate the expertise in cereal technology provided by Dr Tuula Sontag-Strohm and Professor Hannu Salovaara. Thank you for the conversations regarding oats and barley and for the nice lectures and lab works in cereal chemistry and technology. I owe my special thanks to Docent Matti Korhola. Thank you Matti for patiently answering my funny questions about microbes and for providing very helpful comments and suggestions. In addition, I wish to thank Dr Mirkka Herranen for competently culturing the microbes used in this study. I thank Dr Reetta Kivelä for her knowledge on β-glucan and Outi Brinck for her skillful assistance with analyses of the numerous porridge samples. Furthermore, I am thankful to Docent Anna-Maija Lampi for practical advice in food chemistry, inspirational comments and her positive and joyful character.

I wish to express my warm thanks to my present and former colleagues in Viikki D-building. During these years I have had the pleasure of working with many fantastic colleagues and students in an encouraging and easy-going atmosphere. I thank Professor Marina Heinonen and Docent Velimatti Ollilainen for inspiration, support and expertise in food chemistry. I want to thank Dr Tanja Nurmi, Dr Mari Lehtonen, MSc Tuuli Koivumäki and Dr Petri Kylli for all their support and friendship. Tanja, you were a good example to me as a doctoral student. Thank you for the moments in the lab and for all conversations about our lives full of kids and family. Mari, I thank you for your positive energy and good advice as I finalized this thesis. Tuuli and Petri, you have been treasures, always ready to assist with computer issues and you gave invaluable advice in lab works. Miikka Olin, thank you for being my personal technical assistant in UHPLC and in other technical problems. I have enjoyed working with the current doctoral students: Tuuli, Annelie Damerau, Göker Gürbüz, Marjo Pulkkinen, Bei Wang and Bhawani Chamlagain. Special thanks to Bhawani, for working together in vitamin B12 research.

I cannot thank my friends outside the lab enough for their loving support. You have offered me happy moments and long-lasting friendship through all stages of life and enthusiastically cheered me on as a “senior-student”.

I owe my dearest thanks to my family. I thank my parents, May and Kari, for all their support, encouragement and especially for taking care of our children and house-keeping. Mum and Dad, thank you for giving me the basis of what I am today. Further, I wish to thank my dear sister, Kasimiira, for loving me. Finally, my dear husband and our four lovely children deserve heartfelt thanks. Otso, Maaria, Pyry and Kaisla, you have had a “cool” attitude towards my work. Special thanks to Maaria, for the grain figure. Hopefully, I have shown you that learning is not limited by age, but rather is an ongoing process. Mika, thank you for loving, caring and believing in me throughout this journey. Thank you for sharing life with me.

Nurmijärvi, February 2014



Minnamari Edelmann

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred to in the text by Roman numerals:

I Edelmann M, Kariluoto S, Nyström L, Piironen V. 2012. Folate in oats and its milling fractions. Food Chem 135: 1938–1947.

II Edelmann M, Kariluoto S, Nyström L, Piironen V. 2013. Folate in barley grain and fractions. J Cereal Sci 58: 37–44.

III Kariluoto S, Edelmann, M, Herranen M, Lampi A-M, Shmelev A, Salovaara H, Korhola M, Piironen V. 2010. Production of folate by bacteria isolated from oat bran. Int J Food Microbiol 143: 41–47.

IV Kariluoto S, Edelmann M, Nyström L, Kivelä R, Herranen M, Korhola M, Sontag-Strohm T, Salovaara H, Piironen V. 2014. *In situ* enrichment of folate by microorganisms in beta-glucan rich oat and barley matrices. Int J Food Microbiol. Accepted. DOI: 10.1016/j.ijfoodmicro.2014.01.018.

Contribution of the author to papers I to IV:

I, II Minnamari Edelmann planned the study together with the other authors and was responsible for folate analyses. She had the main responsibility for interpreting the results and she acted as the corresponding author of the paper.

III, IV Minnamari Edelmann planned the study together with the other authors and she was responsible for folate analyses. She had the main responsibility for interpreting the results and participated in the preparation of the manuscript.

ABBREVIATIONS

AOAC	Association of Official Analytical Chemists
ATP	Adenosine triphosphate
BHMT	Betaine-homocysteine methyltransferase
CHES	2-(N-cyclohexylamino) ethanesulfonic acid
DAD	Diode array detector
DFE	Dietary folate equivalent
DHF	Dihydrofolate
DHFR	Dihydrofolate reductase
DHP	Dihydroptreoate
DHN-P	Dihydroneopterin monophosphate
DHPPP (HMDHP-PP)	6-hydroxymethyl-7,8-dihydropterin pyrophosphate
DM	Dry matter
DNA	Deoxyribonucleic acid
EHFC	Enterohepatic folate cycle
FBP	Folate binding protein
FLR	Fluorescence
FPGS	Folypolyglutamate synthase
FR	Folate receptor
FW	Fresh weight
GCH	GTP cyclohydrolase
GCPG	Glutamate carboxypeptidase
GTP	Guanosine triphosphate
HEPES	N-(2-hydroxyethyl)piprazine-N'-2-ethanesulfonic acid
HK	Hog kidney conjugase
HMDHP	6-hydroxymethyldihydropterin
HPLC	High performance liquid chromatography
IUPAC	International Union of Pure and Applied Chemistry
LAB	Lactic acid bacteria
LC	Liquid chromatographic
LC-MS	Liquid chromatographic-mass spectrometry
LOD	Limit of detection
LOQ	Limit of quantification
MA	Microbiological assay

MCE	Mercaptoethanol
MES	2-(*N*-morpholino)ethanesulfonic acid
MRP	Multidrug resistance-associated protein
MS	Methionine synthase
MTHFR	Methylenetetrahydrofolate reductase
NADPH	Nicotinamide adenine dinucleotide phosphate
NTD	Neural tube defect
OD	Optical density
PAB	Propionicacid bacteria
*p*ABA	*para*-aminobenzoic acid
PCFT	Proton-coupled folate transporter
PDA	Photodiode array
r	Pearson's correlation coefficient
RFC	Reduced folate carrier
RSD	Relative standard deviation
SAM	S-adenosylmehionine
SIDA	Stable isotope dilution assay
SPAE	Solid phase affinity extraction
SPE-SAX	Solid-phase extraction with strong anion exchange
UHPLC	Ultra-high performance liquid chromatography
UV	Ultraviolet
YPD	Yeast extract, peptone, dextrose

H_4folate	Tetrahydrofolate
5-CH_3-H_4folate	5-methyltetrahydrofolate
5-HCO-H_4folate	5-formyltetrahydrofolate
10-HCO-PGA	10-formylfolic acid
10-HCO-H_2folate	10-formyldihydrofolate
5,10-CH_2-H_4folate	5,10-methylenetetrahydrofolate
5,10-CH^+-H_4folate	5,10-methenyltetrahydrofolate
PGA	Folic acid

CONTENTS

1 INTRODUCTION

Folate is an umbrella term referring to the different forms of the water-soluble B vitamin that have the same biological activity as folic acid. Folate is essential for all organisms and provides one-carbon groups for nucleotide biosynthesis, amino acid metabolism and deoxyribonucleic acid (DNA) methylation (Hanson and Gregory 2011). Folate metabolism in the cells includes several forms of folate, vitamers, which usually exist as polyglutamates with varying one-carbon substitution and oxidation states. Folic acid is the synthetic oxidised form containing only one glutamate residue. It is more stable than reduced folates and is used in supplements and fortified foods.

Folate is currently one of the most actively studied vitamins. This is mainly due to its well-established role in preventing neural tube defects (NTDs) in the developing foetus, which is considered as one of the most important nutritional discoveries of the last 50 years (Katan et al. 2009). NTDs are congenital malformations of the brain and spinal cord caused by failure of neural tube closure between 21 and 28 days following conception (Blencowe et al. 2010). Thus, sufficient folate is essential during early pregnancy when the embryo is rapidly growing and the folate requirement for DNA synthesis and methylation reactions is intense. Insufficient or suboptimal intake of folate is classically associated with megaloblastic anaemia. In recent years, research has focused on the role of folate in many other symptoms. Normal folate metabolism decreases the concentration of blood homocysteine. A high level of homocysteine in the blood is regarded as a risk factor for coronary heart disease and stroke (Cui et al. 2010). Furthermore, folate deficiency is thought to influence the risk for several types of cancers, such as colorectal, pancreatic and breast cancers, thorough disturbances in DNA synthesis and repair (Mason 2011). Folate may also have a role in cognitive functions as in Alzheimer's disease (Smith 2008). It has also been suggested that low maternal folate status in early pregnancy may link to behavioural problems in childhood (Schlotz et al. 2010).

To maintain normal blood levels of folate, dietary intake should be 150–200 µg/day, but an intake of 300 µg/day maintains the folate concentration in the blood above, and the homocysteine concentration below the accepted cut-off values. Thus, the recommended intake of folate is set to 300 µg/day (NNR 2012). Women of reproductive age should have 400 µg/day of folate, which is an adequate supply to reduce the risk of NTDs (De Benoist 2008; NNR 2012). Today in Europe, however, folate intake is inadequate. In Finland, for instance, the average folate intake of men was 270±149 and that of women 234±98 µg/day in 2012 (Helldán et al. 2013). In Sweden, in the years 2010–2011, less than 20% of the adult females reached the recommended intake, and the average folate intake of men and women was 266±95 and 253±114 µg/day, respectively (Riksmaten – vuxna 2010–11).

The relationship between apparent folate deficiency and NTD occurrence was hypothesised as early as 1965. After a number of studies suggested that folic acid might reduce the risk of NTDs, the United States decided to implement mandatory folic acid fortification of enriched cereal grain products in 1998. This resulted in a 19–32% reduction in the prevalence of NTDs

in 10 years (Crider et al. 2011). Currently, the gap between recommended and actual folate intake has led to mandatory folic acid fortification of wheat flour in more than 60 countries around the world, except in Europe. On the other hand, very high intake has been questioned, as it could mask vitamin B12 deficiency or promote the growth of pre-neoplastic lesions (Jägerstad 2012).

Natural folate enhancement has gained special attention in countries where mandatory folate fortification is not practiced. Alhough folate content is relatively high in such foods as liver, green vegetables, legumes, peas, nuts and oranges, cereal products have a high daily contribution to folate intake. The importance of cereal products as a source of folate was demonstrated by a Finnish study, which recently reported that bread and other cereal products accounted for as much as 29% and 33% of the total dietary folate intake for women and men, respectively (Helldán et al. 2013). The levels of folate vary among cereal species as well as among cultivars of the same species (Andersson et al. 2008; Nyström et al. 2008; Piironen et al. 2008; Shewry et al. 2008). The folate content and its variation in wheat has been studied the most, yet there is only scattered information on the folate content in oats and barley.

Oats and barley are important cereal grains for Finnish agriculture. In 2012, their production was 1073 and 1581 million kg, respectively (TIKE 2013). Oats and barley have been mostly used as feed and barley for the brewing industry. The lack of or minimal amounts (in barley) of gluten protein restricts their use in baking. Nevertheless, oats and barley have once again attracted much attention as cereals with health potential. They contain a high amount of beta-glucan, a dietary fibre, when compared to wheat (Sullivan et al. 2013). The regular consumption of beta-glucan has been shown to reduce the content of total and low-density lipoprotein cholesterol in serum, which has led to approval of the health claims for beta-glucan (EFSA 2009). Bioactive compounds, such as folate, are unevenly distributed in cereal grain. Dry-fractionation has been used successfully, mostly for wheat, to achieve tissues, such as bran, aleurone and germ rich in bioactive compounds including folate. However, information on the folate content in oat and barley milling fractions is still missing. Even though bread making solely from oat and barley wholegrain flours is not meaningful, introducing their folate-rich fractions into baking and other cereal products could provide for natural enhancement.

In addition to using folate-rich fractions of oat and barley grain to increase the folate content in cereal products, natural folate enhancement by microbes may also have potential. Microbes are known to produce beneficial bioactive compounds, such as folate (Rossi et al. 2011). Folate biosynthesis has been studied mainly in lactic acid bacteria (LAB) and bifidobacteria, and it seems to depend strongly on species, strain, growth time and cultivation conditions. Traditional sourdough fermentation increased folate content in rye bread (Kariluoto et al. 2004), and through the selection of the yeast strain and optimisation of the cultivation procedure, folate enhancement was transferred to white wheat bread (Hjortmo et al. 2008c). However, little is known about the capability of endogenous bacteria to produce or consume folate. Oats and barley are consumed traditionally as wholegrain products. Recently, they

have also been used in snacks, biscuits and prebiotic drinks and they still have potential for novel food applications. Aqueous processes could be tailored to combine the enhancement of bioactive compounds such as folate by microbes with the beneficial effects of dietary fibre, especially beta-glucan. Heat-treated oat mash was found to be a suitable medium for fermentation by LAB and yeast strains, and the resulting ferments had high and stable beta-glucan content (Angelov et al. 2005). Further, barley flour and barley malt flour proved to be potential substrates for LAB fermentation (Rathore et al. 2012).

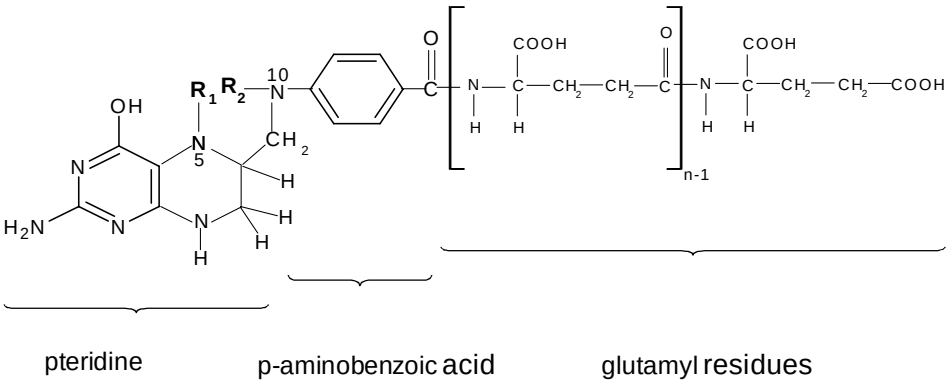
In this thesis, the literature review gives an overview of the structural features, metabolism and analytical methods of folate. The occurrence of folate and its different forms in cereal grains and grain fractions is reviewed. In addition, an overview of folate production by microbes is given. The experimental part of this thesis is a summary of the data published in the attached papers **I–IV**. Total folate content and variation in oat and barley cultivars were studied first and the ultra-high performance liquid chromatography (UHPLC) method was validated. The localisation of folate in the grain was examined by determining folate content and folate vitamer distribution in different milling fractions of oat and barley. Folate production by selected cereal-associated bacteria and food-grade yeasts and bacteria was studied under different cultivation conditions and finally in the aqueous processing of oat and barley. The significance of the results is discussed, concluding remarks are made and suggestions for further research are offered.

2 REVIEW OF THE LITERATURE

2.1 Folate chemistry and metabolism

2.1.1 Structure

Folate is a term that is used to represent the different forms of the water-soluble B vitamin that have similar biological activity to folic acid. The basic structure of folate consists of a pteridine ring (2-amino-4-hydroxy-methylpterin) joined to *para*-aminobenzoate (*p*-aminobenzoate) through a methylene bridge. In addition, one or more L-glutamic acid residues are conjugated to *p*-aminobenzoate with a γ-peptide linkage (Figure 1).



R_1	R_2	Vitamer
H	H	Tetrahydrofolate, H_4folate
CH_3	H	5-methyltetrahydrofolate (5-CH_3-H_4folate)
HCO	H	5-formyltetrahydrofolate (5-HCO-H_4folate)
H	HCO	10-formyltetrahydrofolate (10-HCO-H_4folate)
	–CH_2– (bridge)	5,10-methylenetetrahydrofolate (5,10-CH_2-H_4folate)
	–CH= (bridge)	5,10-methenyltetrahydrofolate (5,10-CH^+-H_4folate)

Figure 1. The structure of polyglutamyl tetrahydrofolates.

The folate pool of a cell or a food source is a mixture of related molecules, called folates or folate vitamers, which differ in their oxidation state, in the attached one-carbon (C1) unit and in the length of the glutamate tail (Blancquaert et al. 2010). In theory, over 150 folate forms exist, but less than 50 have been found in animals and plants.

One-carbon units at various levels of oxidation (formyl –CH=O; methyl –CH_3; methylene -CH_2-; methenyl –CH=; formimino –CH=NH) can be substituted at the N5, N10 or both positions of the pteridine ring. These C1-substituted folates are enzymatically interconvertible and serve as C1 donors for various metabolic reactions that are crucial for cellular functionality. The list of naturally occurring units is shown in the structural formula (Figure 1). The pteridine ring of folate can exist as the tetrahydro, dihydro or fully oxidised form.

According to the recommendations of the International Union of Pure and Applied Chemistry (IUPAC), if the pteridine ring system is fully reduced at the 5, 6, 7 and 8 positions, a vitamer is called a tetrahydrofolate (5,6,7,8-tetrahydropteroyglutamic acid) and is abbreviated as H_4folate. When the pteridine ring is fully oxidised, the monoglutamate form of the vitamer is folic acid (pteroylglutamic acid, PGA). Partial reduction of the pteridine moiety at the 7,8-positions leads to dihydrofolate (DHF; H_2folate) (Blancquaert et al. 2010).

In addition, up to 6–9 glutamate residues are typically attached to the first glutamate via a γ-peptide linkage. The length of the glutamate chain differs from one cell type to another or even within different organelles. However, the penta- and hexaglutamate forms are predominant in most eukaryotic cells (Tibbets and Appling 2010). The polyglutamyl tail is important for the physiological roles of folate. The folylpolyglutamates are more effective substrates for most of the enzymes involved in C1 metabolism, whereas folate transporters prefer monoglutamyl forms. The glutamate chain enhances folate stability, because the tail promotes enzyme binding. Bound folates are far more stable than free folates against oxidative cleavage. Chain elongation increases the anionic nature of folate and decreases its affinity for membrane carriers. As a result, the folylpolyglutamates are retained effectively within cells and subcellular compartments (Hanson and Gregory 2011; Ravanel and Rébeillé 2012). Important features of folate also include its diasteromery. That is, naturally occurring fully reduced folates have two chiral centres: one is at the α-carbon of the L-glutamate and the other at C6 of the pteridine ring. The biologically active forms of H_4folate, 5-CH_3-H_4folate and 5-HCO-H_4folate, are [6S, αS] diastereoisomers, whereas the active forms of 10-HCO-H_4folate, 5,10-CH_2-H_4folate and 5,10-CH^+-H_4folate are [6R, αS] diastereoisomers (Gregory 2008).

2.1.2 Folate metabolism

The essential processes in folate metabolism include conversion of dietary folylpolyglutamates to monoglutamates, intestinal absorption, receptor- and carrier-mediated transport across cell membranes, cellular metabolism and excretion.

Absorption

Dietary folates predominantly exist in the reduced polyglutamate forms. Prior to absorption, they have to be hydrolysed to corresponding monoglutamates in order to cross over cell membranes and be transported. The hydrolysis occurs primarily in the proximal part of the small intestine, the jejunum, via glutamate carboxypeptidase II (EC 3.4.17.21) (GCPII), which is anchored to the intestinal apical brush border (Figure 2) (Bailey and Caudill 2012).

Monoglutamylated folates are absorbed across the brush border membrane of the enterocyte by a saturable pH-sensitive transport system. The high-affinity proton-coupled folate transporter (PCFT) is a more recently identified transport protein, which predominantly transports oxidised and reduced monoglutamated folates at acidic pH. Its optimal transport activity is achieved at pH 5.5, which explains its role as the major folate transporter in the

apical brush border at pH 5.8–6.0 (Zhao et al. 2011). When pharmacological doses (>10 µM or > 200 µg) of folic acid are consumed, absorption takes place by a nonsaturable diffusion-like process and most of the diffused folic acid appears unchanged in the portal circulation (Shane 2008). Natural folate monoglutamates and moderate levels of folic acid are metabolised to 5-CH_3-H_4folate inside the enterocyte. Natural folate monoglutamates are first converted to H_4folate and then via 5,10-CH_2-H_4folate to 5-CH_3-H_4folate. Folic acid is first reduced to H_2folate and then to H_4folate.

Another potential source of folate may be folate produced by colon bacteria. Recently, it has been proposed that colonic absorption may contribute to total folate absorption and folate availability (Auftreiter et al. 2009).

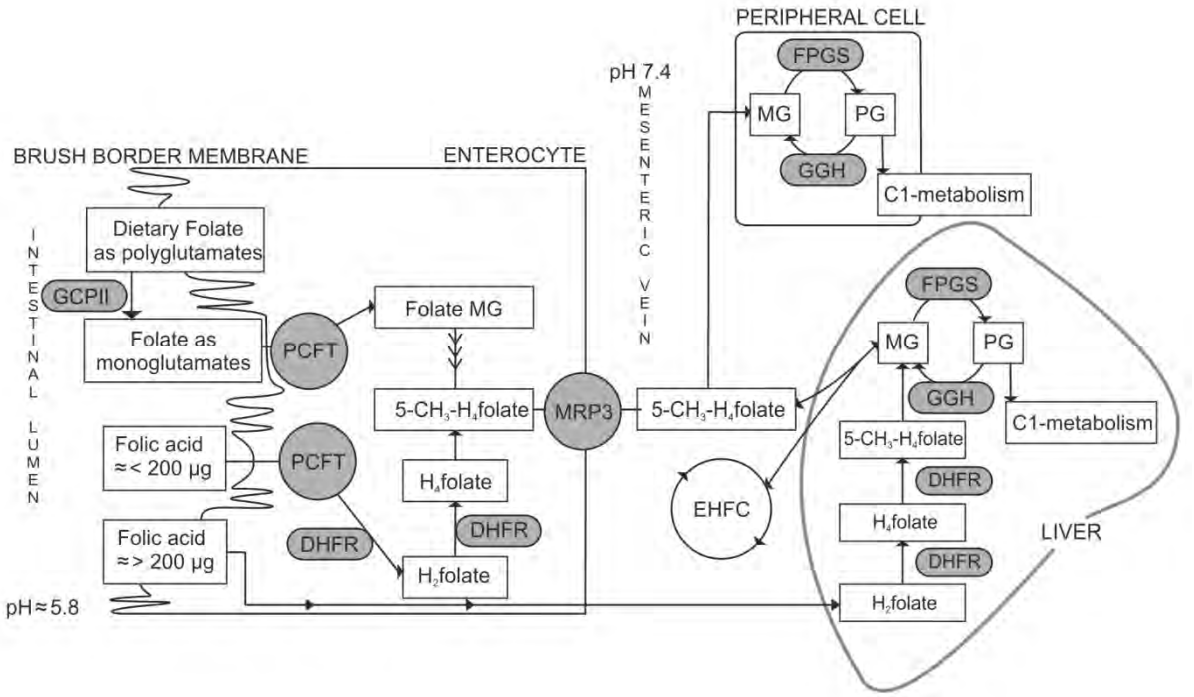


Figure 2. Folate absorption and transport. (GCPII, glutamate carboxypeptidase II; GGH, γ-glutamylhydrolase; PCFT, proton-coupled folate transporter; DHFR, dihydrofolatereductase; MG, monoglutamate; PG, polyglutamate; MRP3, multidrug resistance-associated protein; FPGS, folypolyglutamate synthase; EHFC, enterohepatic folate cycle). Combined data from Pietrzik et al. 2010; Zhao et al. 2011; Bailey and Caudill 2012; Obeid and Hermann 2012; Halsted 2013.

Transport

After absorption through the intestinal membrane, 5-CH_3-H_4folate or other folate forms as monoglutamates are released into the portal vein via protein transporters. This mechanism is not totally understood. Recently, adenosine triphosphate (ATP)-binding cassette transporter

multidrug resistance-associated protein (MRP3) has been considered to play an important role in the efflux of folate from the entrocyte through the basolateral membrane into the portal circulation (Zhao et al. 2011). The predominant form of folate in plasma is the monoglutamyl form of 5-CH_3-H_4folate. 5-CH_3-H_4folate circulates in its free form or is loosely bound to low-affinity proteins, primarily to albumin (ca. 50%) (Bailey and Caudill 2012; Ravanel and Rébéille, 2012).

Different carriers participate in the cellular uptake of folates and have different affinities for folates. Cellular uptake is mediated primarily by the reduced folate carrier (RFC), folate receptors (FRs) and also by PCFT. The RFC is the major transporter that delivers folates to tissues at its optimal, neutral pH of 7.5. RFC has a very low affinity for folic acid instead of reduced folates. High-affinity binding proteins (FRs) have high affinity for both 5-CH_3-H_4folate and folic acid and they conduct the receptor-mediated endocytosis across the cell membrane at neutral pH (Pietrzik et al. 2010; Zhao et al. 2011).

The main part of the absorbed, circulating 5-CH_3-H_4folate is transported into the liver, whose role is central to folate homeostasis. In hepatic cells, folate is converted to folylpolyglutamates and stored. Some of the folate is distributed to other tissues and some is released into the bile where it is recirculated by the enterohepatic cycle. Total folate content in the human body has been estimated to be in the range 10–70 mg (average 20–25 mg), of which approximately 50% is in the liver (Ravanel and Rébéille 2012).

After transportation into the cells, folate is converted to its polyglutamate form by folylpolyglutamyl synthase (FPGS). 5-CH_3-H_4folate is first metabolised to H_4folate via methionine synthase (MS), because 5-CH_3-H_4folate is a poor substrate for FPGS. Any unmetabolised folic acid in the circulation (i.e. not metabolised to 5-CH_3-H_4folate via H_4folate in intestinal cells) is metabolised in the liver according to present knowledge (Obeid and Hermann 2012). In the liver, folic acid is reduced to H_2folate and then to H_4folate by DHFR. Liver DHFR has a relatively low capacity for the reduction of folic acid. Therefore, after relatively high doses (> 1 mg), folic acid may still remain partly unmetabolised, which limits its utilisation (Obeid and Hermann 2012).

Biochemical functions

Folate, with its different oxidation states, plays an essential role in biosynthetic pathways as a one-carbon donor or acceptor. One-carbon groups originate from the catabolism of serine, glysine, histidine or purines. Only 5,10-CH_2-H_4folate, 10-HCO-H_4folate and 5-CH_3-H_4folate act as direct C1 donors, whereas H_4folate, 5,10-CH^+-H_4folate and 5-HCO-H_4folate play important roles as acceptors and transferors of C1 groups (Jägerstadt and Jastrebova 2013).

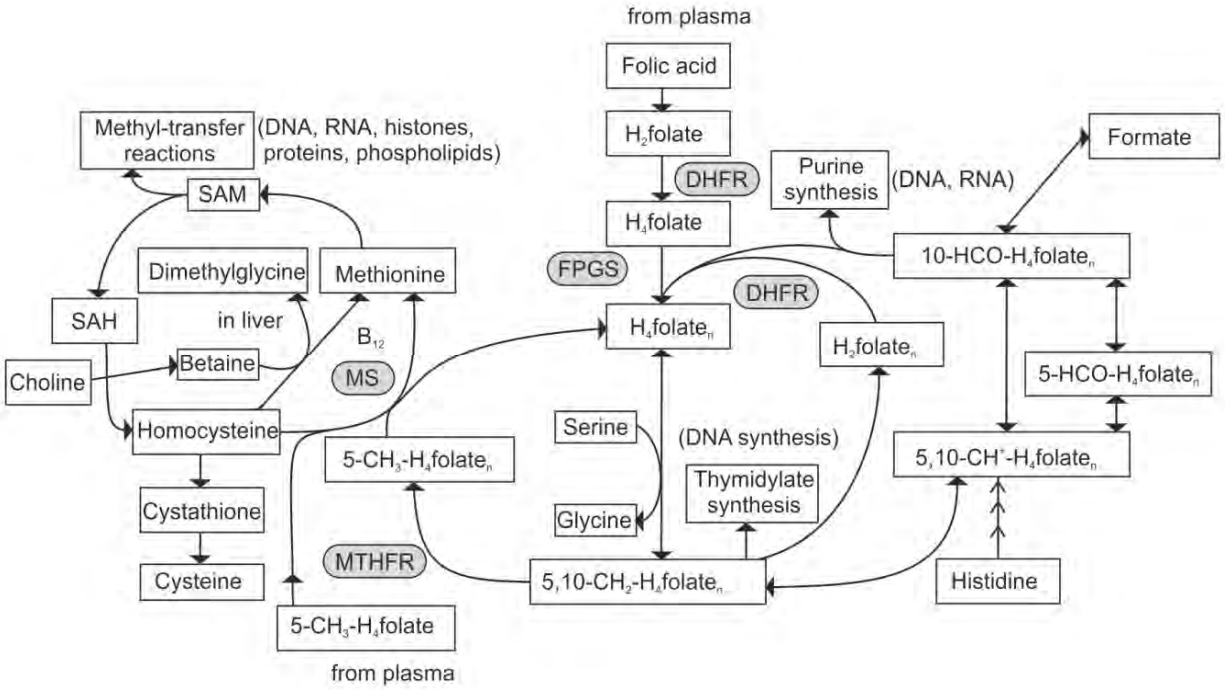


Figure 3. Intracellular folate pathway. (B_{12}, vitamin B_{12}; DHFR, dihydrofolatereductase; FPGS, folypolyglutamate synthase; MS, methionine synthase; MTHFR, methylenetetrahydrofolate reductase; SAM, S-adenosylmethionine; SAH, S-adenosylhomocysteine). Combined data from Shane 2008; Blancquaert et al. 2010; Bailey and Caudill 2012; Ramaekers et al. 2013.

The reactions of C1 metabolism can be viewed as three interdependent pathways (Figure 3). One produces deoxythymidine monophosphate, one purine precursors for DNA biosynthesis and the third produces methyl groups for S-adenosylmethionine (SAM). 5,10-CH_2-H_4folate is a critical junction between these cycles. Thymidylate synthase uses 5,10-CH_2-H_4folate as the C1 donor to convert a uracil-type base to a thymine base, which is required for DNA synthesis. This reaction results in the formation of H_2folate, which can be reduced back to H_4folate by DHFR (Blancquaert et al. 2010; Bailey and Caudill 2012). 5,10-CH_2-H_4folate converts via 5,10-CH^+-H_4folate to 10-HCO-H_4folate. Purines receive their two carbons from 10-HCO-H_4folate. 10-HCO-H_4folate is generated by the coupling of formate to H_4folate. 5,10-CH_2-H_4folate can be generated via serine, which donates its β-carbon to H_4folate and is converted to glycine (Shane 2008).

5,10-CH_2-H_4folate can be reduced to form 5-CH_3-H_4folate, a methyl donor, by methylenetetrahydrofolate reductase (MTHFR). The reaction is almost irreversible and is nicotinamide adenine dinucleotide phosphate (NADPH)-dependent. The generated 5-CH_3-H_4folate enters the methylation cycle and donates a methyl group to homocysteine to produce methionine. This reaction is catalysed by the vitamin B12-dependent enzyme, MS. Methionine is converted to SAM, which is an active methyl group donor. When there is a deficiency of vitamin B12, the action of MS is blocked. The homocysteine level in the plasma may increase and folate accumulates as 5-CH_3-H_4folate. This results in a functional folate

deficiency despite elevated 5-CH_3-H_4folate levels (Shane 2008). In the liver and in the kidneys, remethylation of homocysteine to methionine can also occur by betaine-homocysteine methyltransferase (BHMT). Betaine arises from choline oxidation in liver mitochondria (Shane 2008). In addition, the catabolism of histidine is a process of four reactions wherein the last step requires folates (Blancquaert et al. 2010).

Excretion

Whole-body folate turns over quite slowly with a half-life of 100 days under normal dietary intake and status. It is estimated that only 0.3–0.8% of the folate pool is excreted daily (Öhrvik and Witthöft 2011). Thus, folate from the diet is used effectively or stored in tissues. Much of the stored folate can be hydrolysed to monoglutamates and released into the circulation followed by reuptake again by tissues. Therefore, plasma clearance is rapid due to uptake and reuptake into tissues rather than elimination from the body (Shane 2010).

Folates are effectively reabsorbed in the kidneys. The renal excretion of folates is as low as ca. 5% after normal doses, but it increases at high folate intake (Öhrvik and Witthöft 2011). Urinary excretion represents a small percentage of normal dietary intake and it contains mainly folate catabolites. It has been suggested that folate catabolite products originate from the oxidative cleavage of the C9–N10 bond. However, recent studies have proposed that several enzyme-mediated systems may also be involved in the catabolic pathway and that formyl forms of folate may be the immediate substrate for the cleavage reaction (Anguera et al. 2006). In addition, bile secretes some folate, but it is reabsorbed in the intestine. Faecal excretion is difficult to measure due to bacterial folate production (Pietrzik et al. 2010).

Bioavailability

Bioavailable folate is a fraction of the ingested folate that is available for utilisation in normal physiologic functions and for storage (Öhrvik and Witthöft 2011; Gregory 2012). Thus, bioavailability of folate is influenced by physiological and biochemical processes during intestinal absorption, transport, metabolism and excretion (Caudill 2010). However, bioavailability is a complex and variable concept. Many factors are suggested to affect the bioavailability of dietary folate and many of them influence the efficiency of intestinal absorption. Incomplete release of folate from plant cellular structures and the instability of labile folate vitamers may decrease bioavailability during passage through the stomach and digestion. Furthermore, the extent of polyglutamation in food folate has been proposed to limit bioavailability. However, most of the bioassays on the bioavailability of food folate polyglutamates have been carried out with rodents, although their intestinal deglutamylation mechanism and enzyme system may be different from humans (Bailey and Caudill 2012; Gregory 2012; Ravanell and Rébéille 2012).

Many bioavailability studies have focused on differences between naturally occurring food folate and added synthetic folic acid. Several methodological differences in the folate analysis of clinical samples and food samples may have contributed to the variation in results. The common presumption is that the bioavailability and stability of folic acid is higher than that of

natural folate (Gregory 2012). However, there are discrepancies among the reported studies, with the bioavailability of food folate varying between 10% and 98% (Öhrvik and Witthöft 2011; Ravanel and Rébéille 2012). One recent study in which isotope labelled folates were used highlighted differences in the metabolism of folic acid and reduced folates in humans (Öhrvik et al. 2010). This finding may effect on estimation of relative bioavailability of food folate, when folic acid is used as a reference (Öhrvik and Witthöft 2011).

For dietary recommendations, the bioavailability of food folate is commonly estimated at 50% of folic acid bioavailability. In the United States, dietary recommendations are expressed in terms of dietary folate equivalents (DFEs). The DFE is defined as the quantity of natural food folate plus 1.7 times the quantity of folic acid in the diet (based on the assumption that added folic acid is 1.7 times more bioavailable than food folate). However, only controlled bioavailability studies can provide meaningful estimates of the relative bioavailability of food folate and thus confirm the DFE value (Öhrvik and Witthöft 2011).

2.2 Determination of folate in cereal samples

The determination of folate in cereal samples is challenging. Many folate vitamers exist at relatively low concentrations and with varying states of polyglutamation. Folates may be physically entrapped in the matrix and bound to carbohydrates and proteins, which limits their extractability. In addition, most of the folate forms may interconvert following changes in pH or by some reagents. Finally, folates are very sensitive to heat, light and oxygen. These factors should be considered when choosing the method of analysis and evaluating the final results.

2.2.1 Stability and interconversions of folate

The reduced forms of folate are unstable and readily undergo oxidative degradation. During sample preparation, folates are exposed to elevated temperature and pH changes. In most common sample preparation methods, several heating steps are used for the extraction and inactivation of enzymes. In addition, mobile phases with low pH (2–3) are usually used in liquid chromatographic (LC) methods. Interconversions of some folates are thus possible, as shown in the few available studies. These studies have been performed using LC methods with isotopically labelled standards, without evaluating the influence of the sample matrix (Quinlivan et al. 2006; Smith et al. 2006; De Brouwer et al. 2007; Kirch et al. 2010). The observations, however, offer valuable information on reactions of folate forms under different conditions. Based on the published data, interconversions of individual vitamers are summarised in Figure 4.

Effect of pH and heating

The presence of H_2folate, 10-HCO-H_4folate and 5,10-CH_2-H_4folate is hardly ever reported in food samples, because of their fast degradation under typical experimental conditions. H_2folate is completely lost by heating at pH values below 8 (Wilson and Horne, 1983, 1984; Quinlivan et al. 2006; De Brouwer et al. 2007) because it converts to PGA (Smith et al. 2006;

De Brouwer et al. 2007) (Figure 4A). Correspondingly, 10-HCO-H_4folate converts to 5,10-CH^+-H_4folate at low pH or, if heat treatment is included, to 10-HCO-PGA by oxidation (Figure 4B). Detection of 5,10-CH_2-H_4folate is also impossible after heating at low pH because it dissociates to H_4folates and formaldehyde (Horne 2001; De Brouwer et al. 2007) (Figure 4A). In summary, H_2folate, 10-HCO-H_4folate and 5,10-CH_2-H_4folate are more stable at high pH. In practise, this would suggest that working at a pH near to 10 without heating preserves those forms stable.

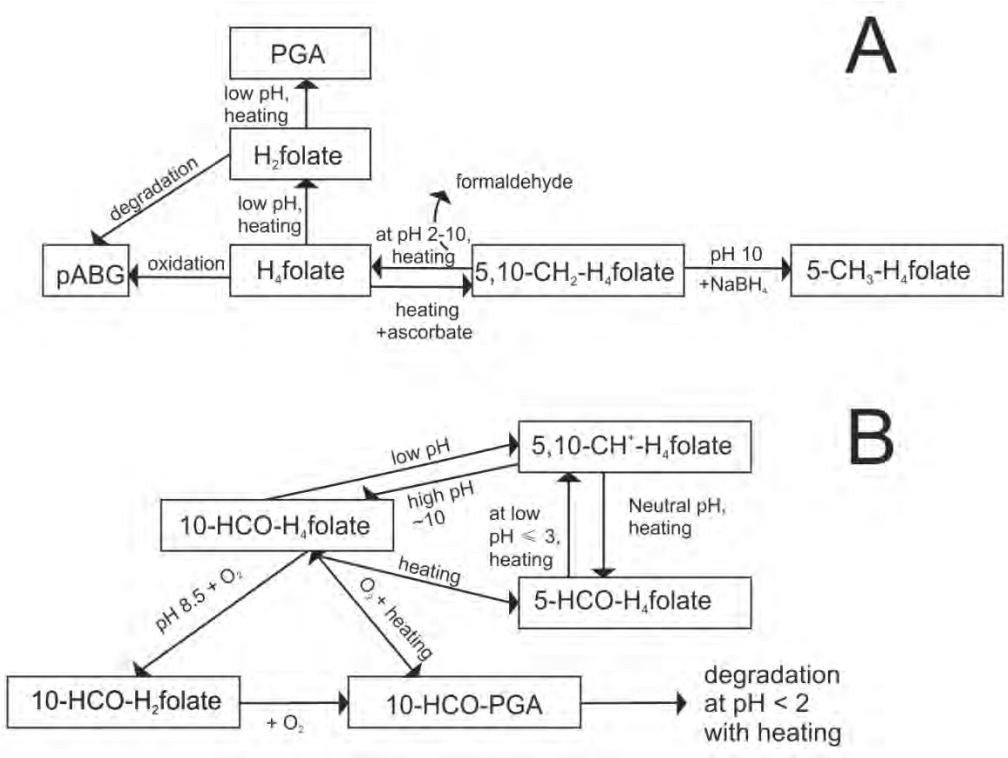


Figure 4. Interconversions of folates under different conditions: (A) interconversions between 5,10-CH_2-H_4folate and H_4folate (B) interconversions between 5-HCO-H_4folate, 10-HCO-H_4folate and 5,10-CH^+H_4folate. The scheme is of combined data based on Wilson and Horne 1983; 1984; Pfeiffer et al. 1997; Smith et al. 2006; De Brouwer et al. 2007; Kirsch et al. 2010; Ringling and Rychlik 2013.

Furthermore, it has been shown that 5-HCO-H_4folate, 10-HCO-H_4folate and 5,10-CH^+H_4folate undergo interconversion reactions under acidic conditions (Quinlivan et al. 2006; De Brouwer et al. 2007; Kirsch et al. 2010) (Figure 4B). De Brouwer et al. (2007) observed that 5,10-CH^+-H_4folate was sensitive to heat treatment (100 °C) at pH 3–9, when its conversion to 5-HCO-H_4folate was the highest. On the other hand, 5-HCO-H_4folate converted to 5,10-CH^+-H_4folate at low pH (< 3) by heating. At various pH values without heat treatment they were relatively stable (Quinlivan et al. 2006). At high pH (> 8.5), 5,10-CH^+-H_4folate dissociated to 10-HCO-H_4folate (De Brouwer et al. 2007).

In some studies of blood folate, the results have been reported as methyl versus formyl folates or methyl versus nonmethyl folate because of the potential interconversions between 5-HCO-H_4folate, 10-HCO-H_4folate and 5,10-CH^+-H_4folate (Smith et al. 2006; Smulders et al. 2007). Jägerstadt and Jastrebova (2013) suggested that formyl folates in foods should be expressed as the sum of 5-HCO-H_4folate, 10-HCO-PGA and 5,10-CH^+-H_4folate.

Effect of oxygen

Oxidation of H_4folate has been proposed to occur by at least two mechanisms, which are irreversible. The pteridine ring can sequentially oxidise to H_2folate and then to PGA through a quininoid dihydrofolate intermediate (Figure 4A). Alternatively, reduced folates undergo oxidative scission at the C9–N10 bond, producing a pterin and *para*-aminobenzoylglutamate (*p*ABG) (Reed and Archer 1980).

5-CH_3-H_4folate and PGA seemed to be relatively stable at pH 2–10 both with and without heat treatment (De Brouwer et al. 2007; Kirsch et al. 2010). In addition, Kirsch et al. (2010) showed that 5-CH_3-H_4folate and PGA were stable over 24 h at 4 °C with no evidence for interconversion to other forms.

Ascorbate is added to buffers used in extraction to avoid oxidation of the folates under analysis. However, during the heating steps or long-term incubation at 4 °C, formaldehyde is formed from ascorbate anions, causing interconversions of some folates (Kirsch et al. 2010). Wilson and Horne (1984) proposed using both mercaptoethanol (MCE) and ascorbate to avoid interconversions. This simultaneous use also seemed to improve the storage stability of folates in the freezer (Patring et al. 2005). De Brouwer et al. (2007) showed that if the buffer (pH 4–8) contained ascorbic acid and MCE, folates were fairly stable during incubation at 37 °C for 2 h. Only losses of H_2folate and 5,10-CH_2-H_4folate were marked.

2.2.2 Sample preparation for folate measurement

Analytical methods for cereal folate are currently based on microbiological assay (MA), LC and, more recently, LC-mass spectrometry (LC-MS) methods. In practice, the sample preparation is similar in all of these analytical methods, including the extraction and enzyme treatment, which are the crucial steps in folate analysis. By extraction physically entrapped folates are liberated from matrix, which makes them more susceptile for enzyme treatments. Amylase and protease are used to improve extraction from carbohydrate- or protein-rich matrices and conjugases are needed to cleave the polyglutamate forms of folate. The determination of total folate content with MA presumes deconjugation of folates at least to their triglutamates. With LC methods folate vitamers are generally analysed as their monoglutamates. Moreover, the purification of the extract is recommended in LC assays.

Extraction

Within plant cells, polyglutamyl folates are bound with high affinity to specific proteins, which protects them from oxidation (Hanson and Gregory 2011). Folates are released from binding proteins typically by heat treatment in boiling water baths. Heat treatment also

denatures enzymes that may catalyse folate degradation or interconversions. The pH of the buffer depends on the optima of the enzymes used during the deconjugation step being mostly neutral or, alternatively, mildly acidic or alkaline. The most commonly used buffers are acetate, phosphate and HEPES/CHES with ascorbic acid as a reducing agent. Wilson and Horne (1984) were the first who recommended MCE together with ascorbate to block formaldehyde formation. Thus, most of the interconversions, originating from heat treatment, are avoided.

Patring et al. (2005) tested the effectiveness of MCE, di-thiothreitol (DTT), 2,3-dimercapto-1-propanol (DMP) and 2-thiobarbituric acid as stabilizing agents in yeast samples. They showed that DMP was a better choice than the often-used MCE to protect labile H_4folate under heat treatments, storage and freezing/thawing. In addition, it is preferable owing to its lower toxicity. Whereas De Brouwer et al. (2008) did not notice any differences between DMP and DTT, they preferred DTT instead of MCE for its user-friendliness.

Enzyme treatments

Polyglutamyl forms of folate require deconjugation to their mono- or diglutamate forms prior to measurement by the MA or LC method. In addition, protease and amylase treatment are often used to increase the yield of measurable folate.

Conjugases (γ-glutamylhydrolase; EC 3.4.22.12) from different origins and with different pH optima are used for deconjugation. Chicken pancreas conjugase is used only in MAs because it is an endopeptidase producing diglutamates. Rat plasma and hog kidney (HK) conjugases are exopeptidases producing monoglutamates. Therefore, they are used in LC methods. Chicken pancreas and rat plasma conjugase have an optimum at neutral pH, whereas HK conjugase operates best at pH 4.9 (Gregory et al. 1984). Chicken pancreas and rat plasma conjugases are commercially available, whereas HK conjugase has to be isolated from fresh kidneys, cleaned up and tested for its activity. Rat plasma conjugase seems to be the most used conjugase in studies of cereal folate, perhaps due to its easy use (Pfeiffer et al. 1997; Konings et al. 2001; Yon and Hyun 2003; Gujska and Kuncewicz 2005; De Brouwer et al. 2008; Yazynina et al. 2008; Gujska et al. 2009; Patring et al. 2009; De Brouwer et al. 2010; Hefni et al. 2010; Hefni and Witthöft 2012). However, HK conjugase is also used (Gregory et al. 1984; Müller 1993; Kariluoto et al. 2001, 2008, 2010; Piironen et al. 2008; Shewry et al. 2010). Activity of the conjugase may vary between batches. Therefore, it is highly recommended to test its activity using pteroyltriglutamate ($PteGlu_3$) as a substrate (Pfeiffer et al. 1997; Patring et al. 2005) and to use significantly more conjugase than is theoretically required (Pedersen 1988).

Starch and protein content in cereal grains and products is high. Therefore, samples are generally treated with protease and α-amylase as well as conjugase. Extraction of folates trapped in complex protein or carbohydrate structures is better and thus recovery of measurable folate increases by using this so-called tri-enzyme treatment (Pfeiffer et al. 1997; Yon and Hyun 2003; Hyun and Tamura 2005). The official method 992.05 of Association of Official Analytical Chemists (AOAC) (2006) also recommends the use of three enzymes.

When Schoenlechner et al. (2010) used only pancreatin containing mainly amylase and protease without conjugase, they obtained considerably lower folate values for cereal grain samples as compared to previous reports. In contrast, lower folate contents have been obtained for cereal samples with tri-enzyme treatment than with merely conjugase treatment only in a few studies (Shrestha et al. 2000; Yazynina et al. 2008; Hefni et al. 2010). Yazynina et al. (2008) incubated gluten-free products only with rat plasma conjugase at 75 °C for 1 h instead of boiling. This treatment was effective in the prevention of gel formation and sufficient enough to release folates from the cereal matrix. They also suggested that a longer heating time and higher temperatures in tri-enzyme treatment might increase folate losses.

When the tri-enzyme treatment is used, incubation times, temperatures and the order of enzyme addition may vary. AOAC (2006) recommends incubation times of 3, 2 and 16 h for protease, amylase and conjugase treatments, respectively. However, in most studies on cereal folate, shorter hydrolysis times have been used than in the AOAC method. It has been suggested that overnight incubation may destroy vitamers (Pfeiffer et al. 1997). Cho et al. (2010) showed that optimal incubation times for protease, amylase and conjugase were 1, 2.5 and 6 h, respectively. De Brouwer et al. (2008) observed that the activity of amylase could be stopped by adding protease instead of boiling. Thus, they avoided one heating step and perhaps extra folate loss.

There are also differences in the order of enzyme addition. Hyun and Tamura (2005) recommended conjugase incubation after the treatments with amylase and protease. They also emphasised that simultaneous use of amylase and protease is not desirable because protease may destroy amylase. In the AOAC method (2006), the order of the enzymes is protease, amylase and conjugase. Martin et al. (1990) and Pfeiffer et al. (1997) first incubated food extracts simultaneously with conjugase and amylase and finally with protease. Hyun and Tamura (2005) and De Brouwer et al. (2008) recommended that the order and length of enzyme treatments should be verified for each sample material.

Cleaning step

Cleaning of the cereal extract is recommendable prior to chromatographic measurement to avoid disturbing peaks in the chromatogram. Unwelcome peaks may mask the individual vitamers and complicate the interpretation of chromatograms. However, for total folate determination with an MA, no cleaning step is required.

Most methods use either solid phase extraction with strong anion exchange (SPE-SAX) or solid phase affinity extraction (SPAE). SAX purification has been the most used method for food samples, except for cereal samples. However, in a few studies, SAX has also been used for cereal extracts (Patring et al. 2009; Hefni et al. 2010; Hefni and Witthöft 2012). Yazynina et al. (2008) purified food samples using phenyl-endcapped bonded silica cartridges and noticed that the most complex matrices, such as rice flour and crisp bread, needed additional cleaning with SAX cartridges. Chandra-Hioe et al. (2013) showed that SPE using phenyl cartridges was selective enough for cleaning extracts of folic acid fortified breads. Hence, they avoided high sodium concentrations that may disturb folic acid detection with LC-MS.

However, concentrating the extract by SPE is not possible due to the limited capacity of the cartridges and the necessity of using high elution volumes (Nilsson et al. 2004).

Affinity chromatography is a more specific cleaning system for folates. In that system, folate-binding protein (FBP) is covalently immobilized to a solid support, generally to agarose. Affinity chromatography columns are not commercially available. FBP has a high affinity for folate at pHs from 7 to 9 and has practically no binding below pH 3.5. Therefore, the folate extract is loaded under neutral conditions and eluted under acidic conditions. In addition, this concentrates the extract (10-fold or more). Due to the high specificity of FBP to most of the folate vitamers, extracts are cleaner and the background of the chromatogram is lower than after SPE purification. This is why affinity chromatography has been used for the purification of challenging cereal samples before HPLC (Pfeiffer et al. 1997; Konings et al. 1999; 2001; Kariluoto et al. 2001, 2004; Piironen et al. 2008; Kariluoto et al. 2010). Despite the special binding capacity of FBP to folate, 5-HCO-H_4folate does not attach with the same intensity as other forms (Gregory 1989). The total load should retain fewer than 25% of the column capacity to confirm an acceptable recovery of 5-HCO-H_4folate (Kariluoto 2008). Furthermore, the binding capacity significantly decreases with increased usage. Thus, the capacity of the columns should be checked regularly (Pfeiffer et al. 2010).

2.2.3 Quantification

Microbiological assay

The most commonly used method to measure total folate in food samples is MA. Several important improvements have been introduced since its initial introduction. The use of 96-well microtiter plates instead of test tubes improved the efficiency and lowered the detection limit. Cryoprotection of test organisms in glycerol has enabled the maintenance of the test organisms and increased the reproducibility. MA is still an inexpensive and sensitive method employing simple instrumentation to analyse total folate in food samples.

MA is based on the growth of the microorganism, which needs folate as a nutrient. The extent of growth can be measured turbidimetrically. The majority of the determinations are performed by *Lactobacillus rhamnosus* (ATCC7469), formerly known as *L. casei*. *L. rhamnosus* can fully utilise mono-, di- and triglutamates, but after triglutamate, its response decreases significantly with the increasing length of the glutamyl tail (Pfeiffer et al. 2010).

Although MA has maintained its status as the most popular method for total folate analysis, it has also been criticised during its history. It still takes at least two days to complete the analysis. The presence of antibiotics or antifolates can interfere with the measurement. This problem, however, has mainly concerned blood samples. MA may be exposed to microbial contamination unless sterile working conditions are adhered to. Although *L. rhamnosus* has generally been reported to exhibit a similar growth response to various folate monoglutamates, a few studies have shown some differences between them. Weber et al. (2011) showed that the response of *L. rhamnosus* to 5-HCO-H_4folate was the highest,

followed by those for 10-HCO-H_4folate, PGA, 5-CH_3-H_4folate and H_4folate. 5,10-CH^+-H_4folate had the lowest response of all studied vitamers. A difference of 15% in the calculated results was observed when using 5-CH_3-H_4folate or folic acid as a calibrator for the chloramphenicol-resistant strain of *L. rhamnosus* (Pfeiffer et al. 2010).

HPLC

LC techniques enable the separation of different folate vitamers and added folic acid. Most HPLC methods separate folate vitamers as their monoglutamates based on reverse-phase chromatography. Generally, octadecyl (C18) bonded silica has been used as the stationary phase material (Pfeiffer et al. 1997; Kariluoto et al. 2001, 2004; Freisleben et al. 2003; Gujska and Kuncewicz 2005; Gujska and Majewska 2005). In addition, octyl (C8) (Johansson et al. 2002; Jastrebova et al. 2003) or phenylbonded silica phases (Gregory et al. 1984; Lucock et al. 1995; Bagley and Selhub 2000) have been used. Retention of monoglutamates on these columns decreases rapidly above pH 4 to 5 (Lucock et al. 1995). In most methods, the mobile phase therefore consists of a phosphate buffer at a pH of around 2–3 and acetonitrile. The best separation is achieved by using a gradient elution (Pfeiffer et al. 2010).

Folate vitamers in cereal samples have generally been detected by ultraviolet (UV)/diode array detectors (DADs) and/or by fluorescence (FLR) detectors. At pH 3–5, the maximum absorption of common monoglutamates varies between 267 and 300 nm, but 5,10-CH^+-H_4folate has the maximum absorption at 355 nm. Reduced folate vitamers, such as H_4folate, 5-CH_3-H_4folate and 5-HCO-H_4folate, can be detected fluorometrically using an excitation wavelength of 295 nm and measuring emission at 365 nm. Native fluorescence of 5-CH_3-H_4folate is about ten times stronger than that of 5-HCO-H_4folate and twice as strong as that of H_4folate (Gounelle et al. 1989). The corresponding wavelengths for 10-HCO-PGA are 360 and 460 nm. Even though, with the exception of 5-CH_3-H_4folate, native fluorescence detection is less sensitive than UV detection (Lucock et al. 1996), detection by FLR is generally also used for the quantification of H_4folate, 5-HCO-H_4folate and 10-HCO-PGA due to fewer interfering peaks.

The masking of peaks for 5-HCO-H_4folate and H_4folate has been a problem in a few studies on cereal folate, even though the purification step was included in the analyses (Kariluoto et al. 2008; Hefni et al. 2010; Jastrebova et al. 2011; Hefni and Witthöft 2012). Therefore, simultaneous use of both the detection systems and verifying the peaks with a photodiode array spectra is recommended.

UHPLC utilises columns packed with small-diameter particles (1.8 µm) allowing for work under high pressures. UHPLC provides a significant improvement in the resolution per time unit and faster analysis compared to HPLC methods. In a few studies, this technique has been applied in food samples. Jastrebova et al. (2011) showed that the run time was 4-fold shorter and limit of detection (LOD) values were lower with UHPLC than with HPLC. Further, they preferred high-strength silica C18 (HSS) columns with trimethylsilane activation (T3) more than bridged ethyl hybrid (BEH) C18 columns, as they separated the late-eluting vitamers more successfully. However, 5-HCO-H_4folate could not be detected in food samples. More

recently, UHPLC has been used combined with MS. For analysing folates in rice (De Brouwer et al. 2010) and folate-fortified breads (Chandra-Hioe et al. 2011), UHPLC-MS/MS methods have been developed.

LC-MS

LC-MS methods are not limited by the low fluorescence activity of PGA and 5-HCO-H_4folate. Recently, LC combined with tandem mass spectrometry (LC-MS/MS) applications have also gained increasing attention for the analysis of cereal samples. These applications have used positive-ion electrospray for ionization (ECI) and acidic mobile phases have been composed of either formic or acetic acid with methanol and/or acetonitrile as an organic modifier (Stokes and Webb 1999; Freisleben et al. 2003; Rychlik et al. 2004; Patring et al. 2009; De Brouwer et al. 2010; Vishnumohan et al. 2011).

The limitation of LC methods has been the lack of a suitable internal standard that could compensate for losses in sample preparation and for the matrix effect. The use of isotopically labelled folate monoglutamates of folates as internal standards has opened a new window to more specific folate analysis with LC-MS. Pawlowsky et al. (2001) published the first stable isotope dilution (SIDA) method for folic acid in fortified foods by using commercially available [$^{13}C_5$] folic acid and [$^{13}C_5$] PteGlu as the internal standards. SIDA with LC-MS detection enables compensating for the loss of folate vitamers during sample preparation and, on the other hand, for matrix effects (Vishnumohan et al. 2011; Ringling and Rychlik 2013). As ^{13}C-labelled H_4folate, 5-CH_3-H_4folate, 5-HCO-H_4folate and 5,10-CH^+-H_4folate are also currently commercially available, LC-MS/MS methods using SIDA offer more accurate quantification of folate vitamers in cereal samples (Chandra-Hioe et al. 2011; Vishnumohan et al. 2011; Ringling and Rychlik 2013).

However, LC-MS/MS methods based on using isotopically labelled folates as internal standards are still expensive for routine work and are not available in every food laboratory. Therefore, the next step could be the development of methods that would utilise the less expensive but faster UHPLC system.

2.3 Folate in cereal grains and fractions

Cereal grains and cereal products are an important source of natural folate. In Finland, bread and other cereal products accounted for as much as 29 and 33% of the total dietary intake for women and men, respectively (Helldán et al. 2013). Folate levels in cereal grains vary over a wide range among cereal species and cultivars. Folate content in cereal products depends on both initial grain content and on the milling level of the grain. Based on the information mainly on wheat, bioactive compounds, such as folate, are unevenly distributed in cereal grain.

Folates in wheat and rye have been studied the most. Their total folate content ranges from approximately 300 to 800 ng/g dm. The comparison between different folate contents is

difficult because of differences in the samples (e.g., one single cultivar, a mixed cultivar sample, cultivar not specified), sampling procedures (e.g., sampling from one or several grocery stores), storage and preparation of the samples and analytical methods (MA, HPLC or LC-MS).

There is considerable interest in increasing the consumption of oat and barley food. Many positive effects of oats and barley are associated with beta-glucan, but furthermore, they contain many other beneficial compounds such as phenolic acids, tocopherols, alkylresorcinols (barley) (Andersson et al. 2008) and avenanthramides (oats) (Welch 2011). However, data on folate in oat and barley grains and their milling fractions are still limited.

2.3.1 Total folate in cereal grains

Folate in wheat

Wheat is one of the major cereal grains consumed throughout the world and has an important role in the intake of folate, especially in the Western diet. Among cereal grains, wheat is the most extensively studied as a folate source. However, folate levels in the published studies vary over a wide range.

Davis et al. (1984) reviewed wheat folate up to that time and showed a range from 160 to 810 ng/g dm in American and Canadian bread wheats. More recent studies have reported total folate content varying from 340–1140 ng/g dm (Håkansson et al. 1987; Arcot et al. 2002; Gujska and Kuncewicz 2005) and 910 ng/g fw (Müller 1993). The studies by Arcot et al. (2002) and Gujska and Kuncewicz (2005) also showed variation between wheat cultivars. The folate content of 12 Australian wheats, each from three to five locations, varied from 799 to 1143 (Arcot et al. 2002) and those of four Polish wheats harvested from the same location (Gujska and Kuncewicz 2005) from 336 to 403 ng/g dm.

In the most comprehensive study on cereal grains within Europe, the EU FP6 project Healthgrain, the average folate content of 130 winter wheat cultivars harvested from the same location was 561±102 ng/g dm and the range was from 364 to 774 ng/g dm (Piironen et al. 2008). The average folate content and range for 20 spring wheats were similar (551±108 and 323–741 ng/g dm, respectively). Furthermore, the folate content of 26 selected wheat cultivars varied within growing locations and harvesting years (Kariluoto et al. 2010).

Folate in oats and barley

Information on folate content in barley grains is limited, apart from the results in the Healthgrain (Andersson et al. 2008). In that study, the mean folate content of 10 barley cultivars, grown in the same location, was 657 ng/g dm and the content ranged from 518–789 ng/g dm. There is a large variation between other reported values, ranging from 360 and 370 ng/g by Cerna and Kas (1983) and Han et al. (2005) to 730 ng/g by Hegedüs et al. (1985).

Literature on the folate content in wholegrain oats is as scarce as that for barley. The Healthgrain project with its five oat cultivars is so far the most extensive study on folate in

oats. The folate content ranged from 495 to 604 ng/g dm and the mean content was 566 ng/g dm (Shewry et al. 2008). Previously, Cerna and Kas (1983) reported that oat grains contained 670 ng/g of folate. Only 136 ng/g dm was determined in oat grains omitting conjugase treatment in the sample preparation (Schonlechner et al. 2010).

Folate in other cereal and pseudocereal grains

Folate in rye has not been investigated as intensively as in wheat, but somewhat more than in oats and barley. According to the previous reports, folate content in wholegrain rye seems to be slightly higher than in wheat grains. In the Healthgrain study, the average folate content was 693 ng/g dm in 10 rye cultivars (Nyström et al. 2008), which is in line with the findings of Kariluoto et al. (2001). They reported a mean value of 700 ng/g fw in 10 Finnish rye cultivars. In addition, those studies showed moderate variation in folate content, being 1.2–1.3-fold among cultivars. Apart from these results, there are only sporadic reported values on folate levels in wholegrain rye. Müller (1993) and Gujska and Kuncewics (2005) found high values of 1430 ng/g fw and 1288 ng/g dm, respectively, whereas Hefni and Witthöft (2012) reported low folate content in rye cultivars (310–390 ng/g). Both the previous values of 920 (fw) and 650 (dm) ng/g reported by Cerna and Kas (1983) and Hegedüs (et al. 1985), respectively, and the value 798 ng/g dm by Gujska et al. (2009) are within the range of the results of Kariluoto et al. (2001) and Nyström et al. (2008).

Reported values of folate content in rice vary from 80 to 400 ng/g dm due to different pearling levels (Pfeiffer et al. 1997; Yon and Hyun 2003; Rychlik 2004; De Brouwer et al. 2008; De Brouwer 2010). Folate content in maize is also quite low, whereas sorghum and teff had high folate content of around 800 ng/g according to Hager et al. (2012). However, folate levels in pseudocereals have scarcely been reported. According to the limited published data, quinoa and amaranth seemed to have promising folate content, at over 500 ng/g dm (Schoenlechner et al. 2010; Hager et al. 2012), which was similar to or even higher than the level in wheat, rye, oats and barley.

2.3.2 Total folate in grain fractions

Cereal grain is a complex structure composed of different tissues: the germ, which contains the plant embryo; the endosperm, which is packed with starch granules to provide energy for germination; the thick cell-walled aleurone layer, covering the endosperm; and the pericarp and hulls (in oats and barley) (Figure 5). The purpose of the conventional grain-milling process is to dissociate and separate the starchy endosperm from the fibrous outer layers and germ. The aleurone cells, along with the other bran layers and the germ, are removed to form the bran fraction. The aim is to achieve endosperm flour with a high yield and purity. However, tissues, which are eliminated and apparently used as feed, are especially rich in micronutrients, fibre and phytochemicals (Hemery et al. 2007).

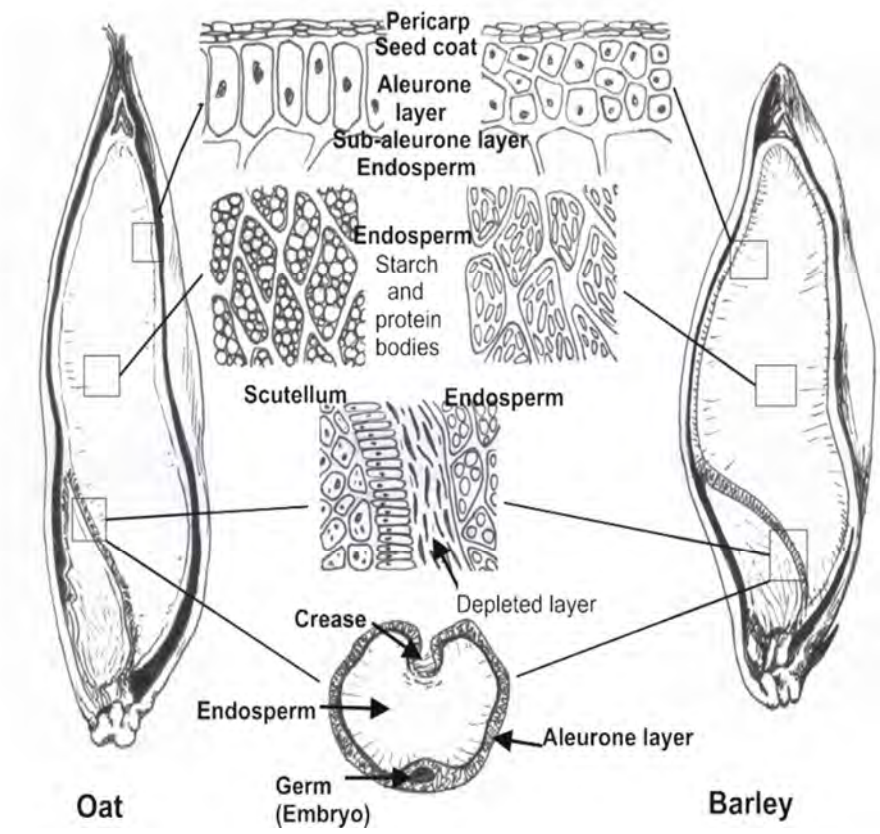


Figure 5. Oat and barley grains with enlarged cross sections. On the left is an oat grain and on the right is a barley grain with surrounding hull. Grains have been split longitudinally, showing the major tissues. At the lowest is a cross section of the lowest box. The figure has been modified from Newman and Newman 2008; Miller and Wulcher 2012.

Grain processing

Unlike wheat grain, barley and oat grains are enveloped by hulls. The tightly adhering hull consists of the lemma and palea. The hull typically accounts for about 25% and 13% of the total dry weight of the oat and barley grain, respectively. In oats, the hull is relatively loose, while the hull of barley is cemented to the pericarp and difficult to separate. Wheat and oats contain aleurone layers of single-cell thickness; barley is notable with its two- to three-cell thick layer. Further, oat kernel is soft in relation to other grains resulting in poor separation of the adhering tissues. Hence, the aleurone layer does not separate as cleanly from the endosperm as it does in wheat (Delcour and Hoseney 2010). The above-mentioned features explain some of the difficulties in oat and barley milling and fractionations.

Barley milling

As the barley hull is strongly attached to the pericarp, grains are generally pearled and further pearled, polished, flaked or ground to grits or flour before being used as a food or an ingredient. Pearling is a process of abrasive scouring that gradually removes the hull, pericarp, seed coat, aleurone, subaleurone layers and the germ. The totally pearled barley

kernel usually represents around 40–50% of the wholegrain, but the pearling level may be adjusted depending on the use of the final product (Sullivan et al. 2010).

Other abrasive methods, such as scarification, have been applied to produce phytosterol- or tocol-rich fractions from barley (Moreau et al. 2007a; Liu and Moreau 2008). Pearling and scarification are similar abrasive processes, but in most pearlers, the abrasive surface rotates, whereas in most scarifiers the abrasive surface is stationary and kernels are rotated with the stator (Moreau et al. 2007b).

Barley flour is commonly prepared by hammer milling pearled grain. It is difficult to produce white flour free of bran particles, because barley bran shatters easily during the roller-milling process. Therefore, barley flour usually contains visible specks of bran particles. Furthermore, it appears darker and it has higher ash content than wheat flour (Baik and Ullrich 2008). In Finland, commercial wholegrain barley flour is produced from 10 to 15% pearled barley grain. Barley bran is not commercially available.

Oat milling

Typical end products of oat processing are oat flakes and oat flour, but recently, oat bran has increased in favour as a fibre-rich product. Modern oat milling consists of the following steps: cleaning, grading, dehulling, hull removal, groat separation, kilning, cutting and/or flaking, flour production and oat bran production. The cleaned and graded oat grains are dehulled by a combination of impact and abrasion forces in a dehuller. The goal is to maximize hulling efficiency while minimizing groat breakage. In the next step, in kilning, dehulled oat grains are stabilized by inactivating enzymes. Otherwise, oat products rapidly develop a soapy and bitter flavour because of the action of lipase, lipoxygenase and peroxidase. Proper control of the time–temperature–moisture profile is important to the consistency of the flavour and quality of the final product. Unfortunately, kilning reduces the content of some vitamins (Girardet and Webster 2012).

Oat groats are steel-cut into two to four pieces for the production of oat flakes. The cut groat mixture contains various-sized pieces, a few uncut groats and fines (middlings and flour). Fines are removed by passing the steel-cut oat stream through a sifter. Before flaking, groats are steamed, which increases the toughness of the groats and thus reduces production of fines during flaking. Oat flour can be milled directly from stabilized groats (resulting in coarse flour) or from flakes (resulting in fine flour). Typically, oat bran is produced from the groats that have been dehulled, kilned and steel-cut. Whole groats and oat flakes can be also used for bran production. Groats are ground in an impact mill or passed through a roller mill. The resulting flour is sieved to produce a coarse bran fraction and a fine flour fraction (Girardet and Webster 2012).

By-products of milling

Hulls – with a proportion of 25% in good-milling-quality oats – represent the largest volume of by-product. They are used for animal feed and, in northern Europe, some mills utilise hulls to produce energy. Oat middlings are fine pieces of flakes, chips and flour obtained during the

steel-cutting and flaking of oat groats. The aim of oat milling is not to produce middlings of more than 3–5% of the starting material. Oat middlings can be utilised in the oat flour process or they can be added to animal feed (Girardet and Webster 2012).

Total folate in cereal grain-milling fractions

Previous studies on wheat fractions have indicated that folate accumulates in the wheat bran and germ. Wheat bran contained ca. 1000 ng/g of folate (Fenech et al. 1999; Arcot et al. 2002; Kamal-Eldin et al. 2009; Patring et al. 2009; Hemery et al. 2011). This is ca. 2-fold more than on average in native grain. However, a higher content of 2240–3370 ng/g has also been reported (Mullin and Jui 1986). A study on folate content in commercial bran fractions (Kamal-Eldin et al. 2009) confirmed that folate is concentrated in the bran. Wheat aleurone and germ fractions are especially rich in folate: the folate content is over 1000 ng/g for aleurone (Fenech et al. 1999; Buri et al. 2004; Hemery et al. 2011) and 2000 ng/g for germ (Piironen 2011; Ringling and Rychlik 2013). Respectively, a lower folate content of 160–290 ng/g has been found in commercial white wheat flours (Yon and Hyun 2003; Gujska and Kuncewicz 2005; Patring et al. 2009) and in rye flours at extraction rate of 72–75% (Hegedüs et al. 1985; Gujska and Kuncewicz 2005) than that in outer parts of the wheat grain.

Data on folate distribution in oat and barley grains is limited. Folate content data has only been published for oat flour and oat flakes. Commercial oat flakes contained 240–290 ng/g (Patring et al. 2009) and oat flour 140–480 ng/g of folate (Gujska and Kuncewicz 2005; Cho et al. 2010). In addition, one study reported that folate content in commercial oat bran products varied from 300–500 ng/g (Patring et al. 2009). Unfortunately, there are no reports of folate levels in barley fractions.

2.3.3 Folate vitamers in cereal grains and fractions

Only a few studies have been published on folate vitamer distribution in cereal grains and their milling fractions, and most of them are for wheats. Information on folate vitamers in oats and barley and their fractions is almost absent. The few published studies show that cereal grains contain a wider variety of vitamers than vegetables, in which 5-CH_3-H_4folate is the predominant folate (Vahteristo et al. 1997; Konings et al. 2001). A comparison between the occurrence and proportions of folate vitamers is difficult among cereal grain samples because of differences in the analytical methods and number of examined vitamers. Moreover, the harvesting time, growing conditions and storage time may have an effect on the vitamer pattern.

However, 5-HCO-H_4folate seems to be the main vitamer in wheat and 5-CH_3-H_4folate and 10-HCO-PGA are the next most abundant vitamers (Gujska and Kuncewicz 2005; Piironen et al. 2008; Patring et al. 2009; Kariluoto et al. 2010). These same vitamers have also been found in wholegrain rye flour (Kariluoto et al. 2001; Gujska and Kuncewicz 2005; Gujska et al. 2009; Patring et al. 2009; Shewry et al. 2010), oat flakes and bran (Patring et al. 2009), rice (Pfeiffer et al. 1997; De Brouwer et al. 2008, 2010) and wheat germ (Ringling and Rychlik

2013). Recently, 5,10-CH^+-H_4folate has been reported in wheat, rye, oats, rice and wheat germ (De Brouwer et al. 2010; Kariluoto et al. 2010; Shewry et al. 2010; Ringling and Rychlik 2013).

Previous publications have reported conflicting values for 10-HCO-H_2folate in cereal products. It has been found in relatively high amounts in wholegrain wheat (Piironen et al. 2008; Kariluoto et al. 2010), wholegrain rye (Kariluoto et al. 2001; 2004; Shewry et al. 2010), rye bread (Kariluoto et al. 2004; Gujska et al. 2009), white bread (Konings et al. 2001; Kariluoto et al. 2004) and rice (Pfeiffer et al.1997). Recently, Ringling and Rychlik (2013) detected it only in minor amounts in wheat germ and rye bread using an LC-MS/MS method. In studies where the labile H_4folate form has been detected, it has accounted for around 5–10% of total vitamers.

2.4 Folate production by microbes

2.4.1 Folate biosynthesis

The *de novo* biosynthesis of folates is restricted to plants and microorganisms (Blancquaert et al. 2010). As the folate molecule contains one pterin moiety bound to *para*-amino benzoic acid (*p*ABA), the *de novo* biosynthesis of folate necessitates the availability of *p*ABA and 6-hydroxymethyl-7,8-dihydropterin pyrophosphate (DHPPP), the precursor of pteridine. The folate-synthesis pathway in plants and microbes is basically similar, except that in plants the process is split among three subcellular compartments (chloroplast, mitochondrion, cytosol). One precursor, the pteridine moiety, is synthesised in the plant cytosol and the other, *p*ABA, is synthesised in the chloroplast. Folates are synthesised from these two precursors in the mitochondrion. In addition to biosynthetic enzymes, membrane-bound transporters for folates and various pathway intermediates must be present (DellaPenna 2007).

Figure 6 illustrates the folate biosynthesis pathway. Pteridine synthesis begins with the conversion of guanosine triphosphate (GTP) to dihydroneoptrin triphosphate (DHN-PPP). This reaction is mediated by GTP cyclohydrolase I (GCHI). DHN-P is dephosphorylated to dihydroneoptrin (DHN) and finally, the lateral side chain of DHN is released to 6-hydoxymethyldihydropterin (HMDHP) and glycoaldehyde. *p*ABA is synthesised from chorismate in two steps in plastids. Chorismate is the product of the shikimate pathway.

Synthesised *p*ABA can be esterified to glucose. The ester is often more abundant than free *p*ABA and can be reconverted to *p*ABA. H_4folate is synthesised from the precursors HMDHP and *p*ABA in mitochondria. First, HMDHP is coupled to *p*ABA to yield dihydropteroate (DHP). Next DHF synthase couples DHP to glutamate to yield DHF. Finally, H_2folate is reduced to H_4folate by DHF reductase, which in plants is fused to thymidylate synthase. The H_4folate is polyglutamated via folypolyglutamate synthase (FPGS) action (DellaPenna 2007; Hanson and Gregory 2011; Rossi et al. 2011). Genes for all of the enzymes of folate synthesis

have been identified in model organisms such as *Escherichia coli*, *Saccharomyces cerevisiae* and *Arabidopsis thaliana* (Hanson and Gregory 2011).

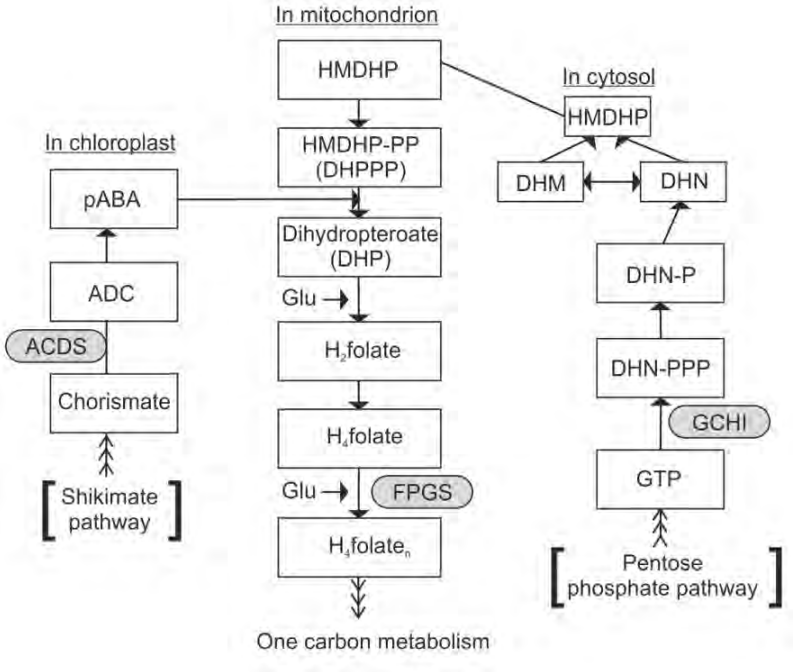


Figure 6. The folate biosynthesis pathway in plants (modified from DellaPenna 2007; Blancquaert et al. 2010). (ACDS, aminodeoxychorismate synthase; ADC, aminodeoxychorismate; DHM, dihydromonapterin; DHN, dihydroneopterin; DHN-P, dihydroneopterin monophosphate DHN-PPP, dihydroneopterin triphosphate; FPGS, folypolyglutamate synthase; GCHI, GTP cyclohydrolase; Glu, glutamate; GTP, guanosine triphosphate; HMDHP, 6-hydroxymethyldihydropterin; HMDHP-PP, 6-hydroxymethyldihydropterin pyrophosphate; *p*ABA, *para*-aminobenzoic acid).

2.4.2 Folate production by bacteria and yeasts

Fermentation with microbes has been used for many years in foods to increase the shelf life, to modify the texture, to contribute to flavour or to achieve alcoholic end products. In addition, microbes produce many nutritional components, such as vitamins. The ability of microbes to synthesise folate could be utilised for natural enrichment. Therefore, the interest in increasing the levels of natural folates by selecting high folate-producing food-grade microorganisms and to optimise processes in folate production is high. Folate-production ability has been studied mainly in milk-based media using LAB, propionic acid bacteria (PAB) and bifidobacteria. In addition, there are a few reports on folate production by yeasts. However, almost nothing is known about folate production by bacteria found in cereals endogenously and natural folate production by microbes using aqueous cereal matrices as the cultivation medium.

Theoretically, it is possible to predict the ability for folate synthesis if the genome of the microbe is available. Folate *de novo* biosynthesis necessitates both the precursors DHPPP and *p*ABA. Therefore, only those microbes that do have genes encoding the enzymes for the biosynthesis of DHPPP, chorismate and *p*ABA are capable of folate production (Rossi et al. 2011). It is known that among LAB, the genera *Lactococcus* and *Streptococcus* and, among bifidobacteria, the species *B. adolescentis* and *B. dentium* possess all the genes for both the shikimate pathway and chorismate conversion into *p*ABA based on their sequenced genomes (Rossi et al. 2011). Thus, all the sequenced lactococci and streptococci, with rare exceptions, and the mentioned species of *Bifidobacterium* are able to produce folate, to accumulate the vitamin within the cells and excrete it into the medium, even when there is no *p*ABA available in the cultivation medium.

Secondly, there are sequenced species, with a complete shikimate pathway for chorismate production, which lack the enzymes necessary for chorismate conversion into *p*ABA. These species are able to synthesise folate only if *p*ABA is available in the medium. For example, *L. plantarum* and *B. longum* produce folate when *p*ABA is available in the cultivation medium (Rossi et al. 2011). Third, there are species that lack both the genes of DHPPP synthesis and the gene encoding DHPPP transformation into DHP. These strains are therefore auxotrophic for folates, and remain incapable of folate production even in the presence of *p*ABA supplementation (Rossi et al. 2011). This inability has been confirmed in many studies on *L. rhamnosus* and *L. casei,* which mainly consume folate for growth (Sybesma et al. 2003; Crittenden et al. 2003; Hugenschmidt et al. 2010; Herranen et al. 2010).

However, even if it is proved by genome sequencing analysis that a microbe species harbours the all genes for folate biosynthesis, there may be differences among strains in terms of folate production. The extent of vitamin production, the partitioning between accumulation and excretion and the vitamer composition depend on the individual strain. In addition, culture conditions, such as pH and temperature, the growth rate and the presence of *p*ABA finally influence folate production (Lin and Young 2000; Crittenden et al. 2003; Sybesma et al. 2003; Rossi et al. 2011).

Folate production by bacteria

Data on folate production by bacteria is summarised in Table 1, Table 2 and Table 3. An exact comparison is difficult because of differences in expressing the results (per dm or fw; intracellular, extracellular, or total folate). However, the tables give an overview of different species as potential folate producers.

Production by lactic acid bacteria

LAB are a heterogeneous group of microaerophilic, gram-positive organisms that ferment hexose sugars to produce primarily lactic acid. LAB can be classified as obligate homo-fermentative (synthesising mainly lactic acid) or obligate hetero-fermentative (mainly lactic acid, acetic acid or ethanol, and CO_2) (Florou-Paneri et al. 2013). Besides having a key role in food fermentations, LAB are increasingly being paid attention to for the production of

functional foods. Thus, their potential to produce folate has also been observed. Common industrial LAB have been reported to produce folate in many studies. However, the ability of LAB to synthesise folate varies considerably among species. The variation may reflect the lack of the genes involved in folate biosynthesis, the strain differences and, finally, the effects of the different culture conditions (Capozzi et al. 2012).

According to numerous reported studies, there is strong evidence for folate production in the majority of the strains of *S. thermophilus* (Lin and Young 2000; Smid et al. 2001; Sybesma et al. 2003; Crittenden et al. 2003; Sanna et al. 2005; Kariluoto et al. 2006a; Herranen et al. 2010) and *L. lactis* (Sybesma et al. 2003; Ayad 2007; Gangadharan et al. 2010; Nor et al. 2010; Herranen et al. 2010). Their ability to produce folate may explain the folate content in fermented milk products such as yogurt. However, big differences have been noticed in the produced folate levels among individual strains of these bacteria (Smid et al. 2001; Ayad 2007; Hugenschmidt et al. 2010; Tomar et al. 2009; Laiño et al. 2012).

Folate production has also been observed in *L. plantarum* (Sybesma et al. 2003; Kariluoto et al. 2006a; Hugenschmidt et al. 2010; Nor et al. 2010), *L. delbrueckii* (Lin and Young 2000; Crittenden et al. 2003; Sybesma et al. 2003; Sanna et al. 2005; Kariluoto et al. 2006a; Padalino et al. 2012*), L. reuteri* (Santos et al. 2008; Hugenschmidt et al. 2010), *L. helveticus* (Sybesma et al. 2003; Sanna et al. 2005), *L. acidophilus* (Lin and Young 2000; Kariluoto et al. 2006a), *L. curvatus* (Hugenschmidt et al. 2010), *L. brevis* (Kariluoto et al. 2006a), *S. franciscensis* (Kariluoto et al. 2006a) and *L. crustorum* (Dana et al. 2010). In addition, a few *Leuconostoc* strains, *L. lactis* and *L. paramesentroids*, have been shown to produce folate (Sybesma et al. 2003).

Table 1. Folate production by lactic acid bacteria (continues).

Growth conditions	Net total folate production Intracellular	Extracellular	Method	Reference
***Lactobacillus* strains**				
L. plantarum				
MRS, 37 °C	18 µg/L	27 µg/L	MA	Sybesma et al. 2003
MRS, 30 °C, pH 7, 18 h	36 µg/L		MA	Nor et al. 2010
SWP, 37 °C, pH 6.6, 24 h; 28 strains		0–27 µg/L, one strain 400 µg/L	MA	Hugenschmidt et al. 2010
YPD, 28 °C, 19 h	0.66 µg/g cell mass	16 µg/L	MA	Kariluoto et al. 2006a
Sterile rye flour/ water, 30 °C , 19 h	No production		MA	Kariluoto et al. 2006a
MRS, 37 °C, 10 h	~40 µg/L		HPLC	Padalino et al. 2012
L. helveticus				
MRS, 2 strains, 37 °C	90 µg/L; −1 µg/L	−1 µg/L; 3 µg/L	MA	Sybesma et al. 2003
RSM, 37 °C	No production, some consuming		MA	Crittenden et al. 2003
Goat milk, 42 °C, 8 h	52 µg/L		HPLC	Sanna et al. 2005
SWP, 37 °C , pH 6.6, 24 h; 3 strains		±0 µg/L	MA	Hugenschmidt et al. 2010
L. acidophilus				
RSM, 37 °C; many strains	No production, consuming		MA	Crittenden et al. 2003
RSM, 37 °C, 6 h	32 µg/L		HPLC	Lin and Young 2000
SWP, 37 °C , pH 6.6, 24 h; 5 strains		±0 µg/L	MA	Hugenschmidt et al. 2010
YPD, 28 °C, 19 h,	0.13 µg/ g cell mass	50 µg/L	MA	Kariluoto et al. 2006a
Sterile rye flour/ water, 30 °C, 19 h	No production, consuming		MA	Kariluoto et al. 2006a
L. casei				
MRS, 37 °C	32 µg/L	−45 µg/L	MA	Sybesma et al. 2003
RSM, 37 °C	No production, consuming		MA	Crittenden et al. 2003
SWP, 37 °C, pH 6.6, 24 h; 19 strains		~0–25 µg/L	MA	Hugenschmidt et al. 2010

HPLC = high-performance liquid chromatography; MA = microbiological assay; MRS = Man-Rogosa-Sharpe agar; RSM= reconstituted skim milk, SWP = supplemented whey permeate; YPD = yeast extract, peptone, dextrose.

Table 1. (continued) Folate production by lactic acid bacteria (continues).

Growth conditions	Net total folate production Intracellular	Extracellular	Method	Reference
Lactobacillus rhamnosus				
RSM, 37 °C	No production, consuming		MA	Crittenden et al. 2003
MRS, 37 °C	34 µg/L	−98 µg/L	MA	Sybesma et al. 2003
SWP, 37 °C , pH 6.6, 24 h		Consuming	MA	Hugenschmidt et al. 2010
L. delbrueckii* ssp. *bulgaricus				
MRS, 37 °C	41 µg/L	12 µg/L	MA	Sybesma et al. 2003
RSM, 37 °C; 3 strains	No production, consuming		MA	Crittenden et al. 2003
RSM, 37 °C, 6 h; 2 strains	40–46 µg/L		HPLC	Lin and Young 2000
Goat milk, 42 °C, 8 h; 3 strains	5–33 µg/L		HPLC	Sanna et al. 2005
MRS, 37 °C, 10 h	~100 µg/L		HPLC	Padalino et al. 2012
FACM, 37 °C, 18 h; 41 strains	only 4 strains produced: extra 4–86 and intra 9–16 µg/L		MA	Laiño et al. 2012
Sterile rye flour /water, 30 °C, 19 h	30 µg/L		MA	Kariluoto et al. 2006a
L. delbrueckii* ssp. *lactis				
Goat milk, 42 °C, 8 h	63 µg/L		MA	Sanna et al. 2005
L. reuteri				
RSM, 37 °C	No production, consuming		MA	Crittenden et al. 2003
CDM with *p*ABA but no folic acid 37 °C, at the stationary phase	21 µg/L		MA	Santos et al. 2008
Melon juice with *p*ABA, 37 °C, at the stationary phase	123 µg/L		MA	Santos et al. 2008
SWP, 37 °C, pH 6.6, 24 h; 3 strains		0–125 µg/L	MA	Hugenschmidt et al. 2010
L. brevis				
YPD, 28 °C, 19 h	0.55 µg/g cell mass	170 µg/L	MA	Kariluoto et al. 2006a
Sterile rye flour /water, 30 °C, 19 h	No production, consuming		MA	Kariluoto et al. 2006a
SWP, 37 °C, pH 6.6, 24 h; 9 strains		0–130 µg/L	MA	Hugenschmidt et al. 2010

CDM = chemical defined medium; FACM= folic acid casein medium; HPLC = high-performance liquid chromatography; MA = microbiological assay; MRS = Man-Rogosa-Sharpe agar; *p*ABA = *para*-aminobenzoic acid; RSM= reconstituted skim milk, SWP = supplemented whey permeate; YPD = yeast extract, peptone, dextrose.

Table 1. (continued) Folate production by lactic acid bacteria (continues).

Growth conditions	Net total folate production Intracellular	Extracellular	Method	Reference
Lactobacillus johnsonii				
RSM, 37 °C	No production, consuming		MA	Crittenden et al. 2003
MRS, 30 °C, pH 7, 24 h, 0.5% glucose	28 µg/L		MA	Nor et al. 2010
L. paracasei , L. buchneri, L. fructivorans				
SWP, 37 °C , pH 6.6, 24 h	No production		MA	Hugenschmidt et al. 2010
L. fermentum				
RSM, 37 °C	No production, consuming		MA	Crittenden et al. 2003
L. curvatus				
SWP, 37 °C , pH 6.6, 24 h; 6 strains		1–11 µg/L	MA	Hugenschmidt et al. 2010
L. sanfranciscensis				
YPD, 28 °C, 19 h	1.05 µg/g cell mass	160 µg/L	MA	Kariluoto et al. 2006a
Sterile rye flour /water, 30 °C, 19 h	No production, consuming		MA	Kariluoto et al. 2006a
L. crustorum				
Skim milk, 43 °C, 3.5–7 h	56–67 µg/L		MA	Dana et al. 2010
***Lactococcus* strains**				
L. lactis* ssp. *cremoris				
M17, 30 °C	92–95 µg/L	9–46 µg/L	MA	Sybesma et al. 2003
M17, 40 °C; 5 strains	69–99 µg/L	10–46 µg/L	MA	Ayad 2007
Skim milk media, 37 °C, 7 h	13 µg/L		MA	Gangadharan et al. 2010
***L. lactis* ssp. lactis**				
M17, 30 °C	90–256 µg/L	6–11 µg/L	MA	Sybesma et al. 2003
M17, 30 °C, pH 7, 24 h	31 µg/L		MA	Nor et al. 2010
Skim milk media , 37 °C, 7 h	14 µg/L		MA	Gangadharan et al. 2010
M17, 40 °C; 9 strains	4–63 µg/L	5–26 µg/L	MA	Ayad 2007
L. lactis* ssp. *lactis biovar ***diacetylactis***				
M17, 40 °C	65–84 µg/L	14–16 µg/L	MA	Ayad 2007

CDM = chemical defined medium; FACM= folic acid casein medium ; HPLC = high-performance liquid chromatography; M17= M17 medium for *Lactococcus* strains; MA = microbiological assay; MRS = Man-Rogosa-Sharpe agar; RSM= reconstituted skim milk, SWP = supplemented whey permeate; YPD = yeast extract, peptone, dextrose

Table 1. (continued) Folate production by lactic acid bacteria.

Growth conditions	Net total folate production Intracellular	Extracellular	Method	Reference
Streptococcus thermophilus				
RSM, 37 °C, 6 h	24–36 µg/L		HPLC	Lin and Young 2000
-	12–142 µg/L		MA	Smid et al. 2001
M17, 37 °C	4–179 µg/L	23–45 µg/L	MA	Sybesma et al. 2003
RSM, 37 °C	40–50 µg/L		MA	Crittenden et al. 2003
UHT milk, 37 °C, 12 h	15–37 µg/g cell mass		HPLC	Holasová et al. 2004
Goat milk, 42 °C, 8 h	17–59 µg/L		HPLC	Sanna et al. 2005
Elliker, 37 ºC, 19 h	543 µg/ g cell mass	20 µg/L	MA	Kariluoto et al. 2006a
Sterile rye flour/water 30 °C, 19 h	50 µg/L		MA	Kariluoto et al. 2006a
RSM, 37 °C, 24 h	4–34 µg/L		MA	Tomar et al. 2009
RSM, 42 °C, 48 h (the optimised conditions)	56 µg/L		MA	Tomar et al. 2009
18 strains	up to 48 µg/L		MA	Iyer et al. 2010
SWP, 37 °C, pH 6.6, 24h		−2–27 µg/L	MA	Hugenschmidt et al. 2010
MRS, 37 °C, 10 h	~200 µg/L		HPLC	Padalino et al. 2012
FACM, 37 °C, 18 h	8–55 µg/L	4–77 µg/L	MA	Laiño et al. 2012
Leuconostoc lactis				
MRS, 30 °C	7 µg/L	37 µg/L	MA	Sybesma et al. 2003
Leuconostoc paramesenteroids				
MRS, 30 °C	10 µg/L	33 µg/L	MA	Sybesma et al. 2003

CDM = chemical defined medium; FACM = folic acid casein medium; HPLC = high-performance liquid chromatography; MA = microbiological assay; MRS = Man-Rogosa-Sharpe agar; RSM= reconstituted skim milk, SWP = supplemented whey permeate; YPD = yeast extract, peptone, dextrose.

In conclusion, the studied LAB species have not always produced folate. It seems that growth conditions and strain characteristics affect folate production. In addition, there are also LAB species, such as *L. rhamnosus* and *L. casei*, which did not show folate production under any conditions but instead, consumed folate (Crittenden et al. 2003; Sybesma et al. 2003; Herranen et al. 2010; Hugenschmidt et al. 2010).

Production by bifidobacteria

The members of the genus *Bifidobacterium* represent one major group of the human gut microbiota. Some bifidobacterial species are claimed to produce health-promoting bioactive molecules, such as conjugated linoleic acid and certain vitamins such as folate (Le Blanc et al. 2013). Several different species of bifidobacteria have been screened for their ability to produce folate in low-folate or folate-free media (Table 2). *B. adolescentis*, *B. pseudocatenulatum* and *B. catenulatum* are good folate producers (Pompei et al. 2007a; D'Aimmo et al. 2012; Padalino et al. 2012). According to these studies, folate production by bifidobacteria species was equal to the levels found in different strains of *S. cerevisiae* and other yeast species (Hjortmo et al. 2005; Patring et al. 2005; Kariluoto et al. 2006a; Hjortmo et al. 2008a). Thus, produced folate varied from 65 to 90 µg/g dm cell mass (D'Aimmo et al. 2012). In addition, some strains of *B. longum*, *B. infantis*, *B. breve* and *B. bifidum* were able to produce folate to some level (Lin and Young 2000; Crittenden et al. 2003; Pompei et al. 2007a; D'Aimmo et al. 2012). On the other hand, strains of *B. animalis* ssp. *lactis* and ssp. *animalis* hardly produced any folate (Pompei et al. 2007a; D'Aimmo et al. 2012), except in the study by Crittenden et al. (2003) where folate concentration doubled in milk-based medium.

Interestingly, the bifidobacteria strains that produced folate also released it into the medium (Deguchi et al. 1985; Crittenden et al. 2007). Rats fed with folate-producing bifidobacteria with oligofructose as a prebiotic exhibited increased plasma folate levels. This confirmed that folate was produced *in vivo* and absorbed (Pompei et al. 2007b). Further, folate concentration in the faeces increased when folate-producing strains *B. adolescentis* and *B. pseudocatenulatum* were consumed (Strozzi and Mogna 2008).

Table 2. Folate production by bifidobacteria (continues).

Growth conditions	Net total folate production Intracellular	Extracellular	Method	Reference
B. adolescentis				
FFM, 37 °C, 48 h; 10 strains	0–40 µg/L	0–65 µg/L	MA	Pompei et al. 2007a
TPY, 37 °C	~65 µg/g dm		HPLC	D'Aimmo et al. 2012
FFM, 37 °C	~89 µg/g dm		HPLC	D'Aimmo et al. 2012
MRS + cysteine, 37 °C, 10 h	~100 µg/L		HPLC	Padalino et al. 2012
B. pseudocatenulatum				
FFM, 37 °C, 48 h	8–35 µg/L	12–82 µg/L	MA	Pompei et al. 2007a
TPY, 37 °C	42 µg/g dm		HPLC	D'Aimmo et al. 2012
FFM, 37 °C	~70 µg/g dm		HPLC	D'Aimmo et al. 2012
B. catenulatum				
MRS, 37 °C, 10 h	~300 µg/L		HPLC	Padalino et al. 2012
FFM, 37 °C	~93µg/g dm		HPLC	D'Aimmo et al. 2012
B. animalis* ssp. *lactis				
TPY, 37 °C	~3 µg/g dm		HPLC	D'Aimmo et al. 2012
FFM, 37 °C	no growth		HPLC	D'Aimmo et al. 2012
B. lactis				
RSM, 37 °C	~23 µg/L		MA	Crittenden et al. 2003
B. animalis* ssp. *animalis				
TPY, 37 °C	~2 µg/g dm		HPLC	D'Aimmo et al. 2012
FFM, 37 °C	no growth		HPLC	D'Aimmo et al. 2012
B. animalis				
RSM, 37 °C	~20 µg/L		MA	Crittenden et al. 2003
FFM, 37 °C, 48 h; 7 strains	Only one strain produced 26 µg/L		MA	Pompei et al. 2007a
B. longum ssp. infantis				
TPY, 37 °C	15–25 µg/ g dm		HPLC	D'Aimmo et al. 2012
FFM, 37 °C	5 µg/ g dm		HPLC	D'Aimmo et al. 2012

FFM = folate free medium; HPLC = high-performance liquid chromatography; MA = microbiological assay; MRS = Man-Rogosa-Sharpe agar; RSM = reconstituted milk medium; TPY = containing i.a. trypticase peptone, phytone peptone, yeast extract

Table 2. (continued) Folate production by bifidobacteria.

Growth conditions	Net total folate production Intracellular	Extracellular	Method	Reference
B. longum				
RSM, 37 °C, 6 h; 2 strains	53–77 µg/L		HPLC	Lin and Young 2000
UHT milk, 37 °C, 12 h; 3 strains	8–12 µg/L		HPLC	Holasová et al. 2004
B. infantis				
RSM, 37 °C	~20 µg/L		MA	Crittenden et al. 2003
B. breve				
RSM, 37 °C	~44 µg/L		MA	Crittenden et al. 2003
FFM, 37 °C, 48 h; 15 strains		0–2.5 µg/L	MA	Pompei et al. 2007a
TPY, 37 °C	~10µg/ g dm		HPLC	D'Aimmo et al. 2012
FFM, 37 °C	~17 µg/g dm		HPLC	D'Aimmo et al. 2012
B. bifidum				
UHT milk, 37 °C, 12 h; 2 strains	6 µg/L		HPLC	Holasová et al. 2004
FFM, 37 °C, 48 h; 6 strains		0–0.6 µg/L	MA	Pompei et al. 2007a
FFM, 37 °C	35–75 µg/g dm		HPLC	D'Aimmo et al. 2012
TPY, 37 °C	28–45 µg/g dm		HPLC	D'Aimmo et al. 2012

FFM = folate free medium; HPLC = high-performance liquid chromatography; MA = microbiological assay; MRS = Man-Rogosa-Sharpe agar; RSM = reconstituted milk medium ; TPY = containing i.a. trypticase peptone, phytone peptone, yeast extract

Production by propionic acid bacteria

PAB have, besides aerobic a unique anaerobic metabolism involving several carbon rearrangement reactions. To catalyse these reactions, the PAB contain a wide variety of enzymes with specific coenzymes, such as vitamin B12, folates and biotin. These microorganisms have long been known as efficient vitamin B12 producers and a few strains have also been reported to produce folate (Hugenholtz and Smid 2002; Holasova et al. 2004; Hugenschmidt et al. 2010; Hugenschmidt et al. 2011).

Folate production by propionibacteria has been tested mainly under limiting oxygen conditions in milk-based media, which have contained lactose and lactate. The reported levels varied from 0 to 93 µg/L, which was equal to production by LAB (Table 3). However, growth conditions in those studies were optimised only for high vitamin B12 production and not for folate production.

Table 3. Folate production by propionic acid bacteria.

Growth conditions	Net total folate production Intracellular	Extracellular	Method	Reference
P. freudenreichii ssp. shermanii				
−; 9 strains	0–93 µg/L	−20–41 µg/L	MA	Hugenholtz et al. 2002
UHT milk, 1.5% fat, butter as a starter, 30 °C, 12 h ; 3 strains	Mean production 1.5 µg/L		HPLC	Holasova et al. 2004
LM , 32 °C, 72 h, pH 7.0 ; 7 strains	7.7–32.3 µg/L		HPLC	Van Wyk et al. 2011
vit. B12 medium, 88 h, in aerobic	14.2 µg/L		HPLC	Van Wyk and Britz 2012
vit. B12 medium, 94 h, in anaerobic	26.6 µg/L		HPLC	Van Wyk and Britz 2012
P. freudenreichii ssp. freudenreichii				
LM, 32 °C, 72 h, pH 7.0	17.6 µg/L		HPLC	Van Wyk et al. 2011
P. thoenii				
−	28 µg/L	8 µg/L	MA	Hugenholtz et al. 2002
P. acidipropionici				
−	58 µg/L	−22 µg/L	MA	Hugenholtz et al. 2002
P. jensenii				
−	51 µg/L	−11 µg/L	MA	Hugenholtz et al. 2002
37 strains of ***P. freudenreichii***, 14 of ***P. jensenii,*** 4 of ***P. thoenii***, 3 of ***P. acidipropionici*** and 42 of ***Propionibacterium*** sp. SWP, 30 °C, 72 h, pH 5.0		0–14 µg/L	MA	Hugenschmidt et al. 2010
P. freudenreichii				
SWP + pABA, 33 °C, 168 h		25 µg/L	MA	Hugenschmidt et al. 2011

HPLC = high-performance liquid chromatography; LM = lactate-rich medium; MA = microbiological assay; pABA = *para*-aminobenzoic acid; SWP= supplemented whey permeate

Folate production by endogenous bacteria

After harvesting, cereal grains contain bacteria mainly from the families *Pseudomonadaceae, Enterobacteriaceae, Microbacteriaceae, Micrococcaceae, Lactobacillaceae* and *Bacillaceae* (Yoshida et al. 2006). Storage contamination and processing further affect the microflora. Thus, the outer layers of cereal grain may contain various microbes. Only in a few studies has folate production of endogenous cereal bacteria been reported. Kariluoto et al. (2006a) isolated *Enterobacter cowanii* and *Pantoea agglomerans* from non-sterilised rye flour and observed that those strains were remarkable folate producers in a sterile rye flour–water mixture. Herranen et al. (2010) isolated endogenous bacteria from oat bran and rye flake products and screened their folate production capability in a medium containing 1% yeast

extract, 2% peptone, and 2% glucose (YPD medium). Bacteria with the highest cell mass folate content (> 12 µg/g fw) were *Pseudomonas* sp., *Bacillus subtilis, Janthinobacterium* sp., *E. persicina, Chryseobacterium* sp. and *P. flavus*. Some of the best folate producers such as *E. persicina, Janthinobacterium* sp. and *Pseudomonas* also excreted folate into the medium. Although the ability of endogenous cereal bacteria to produce folate has rarely been studied, the results of those few studies were encouraging. Endogenous microbiota may contribute to folate enhancement in aqueous cereal processing, even if not deliberately added.

Folate production by yeasts

Yeasts play a significant role in many indigenous fermented foods where they are deliberately added or mainly introduced by spontaneous fermentation. Yeasts are a very heterogeneous group of unicellular fungi that differ largely in morphology as well as in physiology. Commercial baker's yeast *S. cerevisiae* and many other species have, for example, fermentative and respiratory metabolism, whereas most of the other species ferment only at a negligible rate or not at all (Kurzmann et al. 2011). *S. cerevisiae* itself is a rich source of folate, containing 24–35 µg/g dm of folate (Witthöft et al. 1999; Patring et al. 2005; Patring and Jastrebova 2007).

In the previous reports, all of the studied yeast species produced folate (Table 4). Hjortmo et al. (2005) showed big differences among yeast species in terms of folate production: the total folate content ranged from 40 to 145 µg/g dm cell mass. The best producers were strains of *S. cerevisiae* and closely related species. Several species showed a 2-fold or higher folate content as compared to the commercial strain of baker's yeast. In another study, Hjortmo et al. (2008c) also found a big difference between two species of *Saccharomyces*. Kariluoto et al. (2006a) showed that the studied yeasts excreted folate in negligible amounts into the medium.

Table 4. Folate production by yeasts.

Growth conditions	Net total folate production Intracellular	Extracellular	Method	Reference
S. cerevisae strains and related species: ***S. bayanus, S. paradoxus, S. pastorianus, S. boulardii*** Synthetic medium, 30 °C, pH 5.5, 12–24 h	40–145 µg/g dm		HPLC	Hjortmo et al. 2005
Strains of heterogenous mix of yeasts such as ***Debaryomyces, Candida*** Synthetic medium, 30 °C, pH 5.5, 12–24 h	40–90 µg/g dm		HPLC	Hjortmo et al. 2005
S*accharomyces* and ***Candida*** species isolated from kefir grains Synthetic medium with 2% glucose, 30 °C, 12 h	100–117 µg/g dm		HPLC	Patring et al. 2005
S. cerevisae YPD, 28 °C, 19 h	3.4–6.5 µg/g fw	70–150µg/L	MA	Kariluoto et al. 2006a
Synthetic medium , 30 °C, pH 5.5, 12 h	~90 µg/g dm		HPLC	Hjortmo et al. 2008a
YPD, 30 °C, pH 5.5, 12 h	~30µg/g dm		HPLC	Hjortmo et al. 2008a
Molasses, 30 °C, pH 5.5, 12 h	~20 µg/g dm		HPLC	Hjortmo et al. 2008a
Malt wort, 18–30 °C	16–22 µg/g dm		MA	Pietercelie et al. 2011
Candida milleri YPD, 28 °C, 19 h	6.1–8.5 µg/g fw		MA	Kariluoto et al. 2006a
Torulaspora delbrueckii YPD, 28 °C, 19 h	3.1–4.0 µg/g fw		MA	Kariluoto et al. 2006a
S. pastorianus Malt wort, 18–30 °C	15–21 µg/g dm		MA	Pietercelie et al. 2011
S. cerevisae, Candida milleri, Torulaspora delbrueckii Sterile rye flour /water, 30 °C, 19 h	0.1–0.19 µg/g fw		MA	Kariluoto et al.2006a
S. cerevisae, Candida glabrata Kluyveromyces marxianus, Issatchenkia orientalis, Pichia anomala maize-based porridge, 30 °C, 46 h	0.2–0.7 µg/g dm		HPLC	Hjortmo et al. 2008b

HPLC = high-performance liquid chromatography; MA = microbiological assay; YPD =containing yeast extract, peptone, dextrose

2.4.3 Folate vitamer production

The vitamer distribution in folate produced by microbes has been studied mainly in LAB, bifidobacteria and yeasts. Most studies have focused on detecting 5-CH_3-H_4folate, H_4folate and 5-HCO-H_4folate after ca. 6–12 h fermentation at 37 °C in milk-based medium. A comparison of the results is difficult due to differences in analytical procedures and in the number of investigated vitamers. However, according to the available literature, 5-CH_3-H_4folate is the main vitamer produced by microbes.

S. thermophilus has mainly been observed to produce more 5-CH_3-H_4folate than H_4folate (Lin and Young 2000; Padalino et al. 2012). Gangadharan et al. (2010) reported that 5-CH_3-H_4folate was the major form synthesised by *L. lactis* during a 7-h fermentation in folic acid medium, while *L. cremoris* produced more H_4folate than 5-CH_3-H_4folate.

5-HCO-H_4folate has been determined in a few studies. Sanna et al. (2005) detected 5-HCO-H_4folate in goat milk inoculated with *S. thermopilus*, *L. delbrueckii* ssp. *bulgaricus*, *L. delbrueckii* ssp. *lactis* and *L. helveticus* after an 8-h incubation. Lin and Young (2000) also observed that a few LAB strains (*S. thermophilus*, *L. acidophilus* and *L. bulgaricus*) accumulated 5-HCO-H_4folate as well as the predominant forms 5-CH_3-H_4folate and H_4folate during a 6-h fermentation in skim milk. When *L. plantarum* and *P. freudenreichii* were co-cultured in whey-based broth, most of the produced 5-HCO-H_4folate was excreted into the medium. 5-HCO-H_4folate probably originated totally from *L. plantarum* because *P. freudenreichii* was shown to produce minimal amounts of extracellular folate as a single culture (Hugenschmidt et al. 2011).

Bifidobacterium strains have been observed to produce mainly 5-CH_3-H_4folate. Strains of *B. longum* (Lin and Young 2000) and *B.catenulatum* and *B. adolescentis* (D'Aimmo et al. 2012; Padalino et al. 2012) accumulated more 5-CH_3-H_4folate than H_4folate in milk-based medium. Lin and Young also found some 5-HCO-H_4folate, whereas Padalino and co-workers did not find any 5-HCO-H_4folate. Holasova et al. (2004) showed that *B. longum* strains were moderate 5-CH_3-H_4folate producers in UHT milk during a 12-h fermentation. However, 5-CH_3-H_4folate was the only investigated vitamer in their study. On the other hand, more H_4folate than 5-CH_3-H_4folate was found in some *B. longum* and *B. breve* strains cultivated in TPY medium (trypticase peptone, phytone peptone, yeast extract) for 6–8 h (D'Aimmo et al. 2012). Relatively low folate production was common for those strains and it was associated with high H_4folate production.

There are only a few studies on vitamers produced by propionibacteria. Van Wyk et al. (2011) reported that folate in *P. freudenreichii* strains fermented for 72 h in lactate-rich medium was composed of 5-CH_3-H_4folate, 5-HCO-H_4folate and H_4folate, but the proportion of vitamers was not given. In another study, *P. freudenreichii* accumulated mainly 5-CH_3-H_4folate followed by H_4folate and 5-HCO-H_4folate (Van Wyk and Britz 2012). Holasova et al. (2004) reported on folate production by *P. fereudenreichii* strains only as 5-CH_3-H_4folate.

As with bacteria, the predominant folate vitamer in yeast fermentations seems to be 5-CH_3-H_4folate followed by H_4folate. In a few studies, 5-HCO-H_4folate and 10-HCO-PGA have also been found. Hjortmo et al. (2005) determined vitamer distribution in almost 50 yeast strains cultivated in a synthetic medium for the late respiratory phase (12–24 h). Yeast strains were classified into two groups. One group consisting of *S. cerevisiae* and related species had a fairly constant H_4folate content. By contrast, the proportion of 5-CH_3-H_4folate varied. The other group included a heterogeneous mix of yeasts from different origins. In that group, no folate form dominated; some strains had a high H_4folate content and others were rich in 5-CH_3-H_4folate. Small amounts of 5-HCO-H_4folate were also detected in some yeast strains. Other folate forms such as 10-HCO-PGA and PGA were not observed.

Valuable data on the distribution of folate forms during a controlled batch fermentation of *S. cerevisiae* in synthetic medium was reported by Hjortmo et al. (2008c). In the respiro-fermentative phase of growth, 5-CH_3-H_4folate dominated and started to increase immediately after inoculation. When glucose was depleted, the content of 5-CH_3-H_4folate dramatically decreased. They also observed that 5-CH_3-H_4folate and total folate content increased linearly with increasing growth rate, whereas the content of H_4folate was moderate and independent of growth rate and the state of the cells.

H_4folate, 5-CH_3-H_4folate, and 5-HCO-H_4folate were found in yeast strains isolated from kefir granules and cultured in synthetic medium (Patring et al. 2005). One group of the yeast strains produced high amounts of 5-CH_3-H_4folate (52–59% of total folate content), but lower amounts of 5-HCO-H_4folate and H_4folate. The other group accumulated similarly high amounts of both 5-CH_3-H_4folate and 5-HCO-H_4folate, but significantly lower amounts of H_4folate.

2.4.4 Enhancement of folate levels by microbes

Effect of growing conditions

Different conditions in culturing may affect folate production, even though the genome of the microbe predicts the ability for folate biosynthesis. Numerous physical and nutritional factors such as the composition of the medium, temperature, pH, oxygen tension and incubation time have been reported to affect folate production.

Composition of the medium

*p*ABA is one of the folate precursors, and its addition has been shown to increase the folate-synthesis activity in both *S. thermophilus* and *L. lactis* (Sybesma et al. 2003; Wegkamp et al. 2008; Iyer et al. 2010), *L. lactis* ssp. *cremoris* (Gangadharan et al. 2011), *L. reuteri* (Santos et al. 2008) and probionibacteria (Hugenschmidt et al. 2011). Folate production was also related to *p*ABA concentration (Sybesma et al. 2003; Wegkamp et al. 2008; Tomar et al. 2009; Iyer et al. 2010; Gangadharan et al. 2011). However, higher levels of *p*ABA did not further increase the folate yield. On the contrary, Gangadharan et al. (2011) observed that higher additions even lowered folate production in *L. lactis* ssp. *cremoris*. One recent study observed that milk

and milk-based media contained enough natural *p*ABA for folate production of the tested Lactobacillus strains, *L. delbrueckii* and *L. plantarum* (Padalino et al. 2012).

Lactose affects the growth of LAB, as it is a carbon source. Based on previous studies, it also influences folate production. Lactose addition at a concentration of 1% led to a 17% increase in folate when *S. thermophilus* was incubated at 42 °C, but higher levels decreased folate production (Tomar et al. 2009). Lin and Young (2000) showed that strains of selected LAB and Bifidobacteria (*L. acidophilus*, *L. bulgaricus*, *S. thermophilus* and *B. longum*) had 2–2.5-fold folate levels in reconstituted non-fat dry milk than in a lactose-free, complex medium, whereas in the study by Hugenschmidt et al. (2011), the addition of extra lactose into the lactose-containing whey medium did not affect folate production in co-cultured *L. plantarum* and *P. freudenreichii*.

Prebiotics such as fructo-oligosaccharides (FOS), galacto-oligosaccharides (GOS) and sugar alcohols are also known to stimulate the growth of LAB and bifidobacteria. Gangadharan et al. (2011) noticed that the separate addition of sorbitol and mannitol into the skim-milk medium increased folate production slightly by *L. lactis* ssp. *cremoris*. However, Padalino et al. (2012) observed that the addition of FOS and GOS did not stimulate the synthesis in *B. catenulatum, B. adolescentis, L. delbrueckii, L. plantarum* and *S. thermophilus.* Instead, their addition only increased the rate of bacterial growth. Pompei et al. (2007a) did not notice any differences in folate production by *B. adolescentis* between glucose, fructose, raffinose, lactose and FOS as a carbon source.

Folate production may be stimulated by growing the cells under growth-limiting conditions. Consequently, the addition of NaCl decreased the growth rate and increased folate production in both *L. lactis* and *S. thermophilus* (Sybesma et al. 2003). The reason for increased folate production when growth was inhibited is not yet known. Probably, guanosine triphosphate (GTP), one of the folate precursors, accumulates because of decreased DNA and RNA synthesis (Sybesma et al. 2003).

Both methionine and glysine (Gangadharan et al. 2011) and glutamate (Hugenschmidt et al. 2011) addition elevated folate levels in *L. lactis* ssp. *cremoris* in skim milk. By contrast, glutamate addition had no effect on the folate level in co-cultured *L. plantarum* and *P. freudenreichii* (Hugenschmidt et al. 2011). Sodium thioglycholate and sodium ascorbate also increased folate production by *L. lactis* ssp. *cremoris* under agitated conditions (Gangadharan et al. 2011).

In yeasts, the effect of the growth medium on folate production is less studied. However, the addition of *p*ABA into a synthetic medium that already contained some *p*ABA did not affect intracellular folate content in *S. cerevisiae* (Hjortmo et al. 2008a). Further, when Hjortmo et al. (2008a) supplemented a synthetic medium with peptides and amino acids, folate levels decreased extensively in *S. cerevisiae*. They suggested that yeast preferred to utilise folate, nucleotides and amino acids from its surroundings instead of through its own biosynthesis. Pietercelie et al. (2011) showed that the addition of oleic acid and yeast-peptide complex

shortened fermentation times and increased total folate production by yeasts in a malt medium. They suggested that oleic acid probably influenced the fluidity of the cell membrane. This had an effect on the folate transport system or on cell multiplication. Further, yeast gained energy for its membrane synthesis from the exogenous oleic acid.

Effect of time, growth rate and temperature

Folate levels in LAB (e.g., *S. thermophilus, L. delbrueckii, L. plantarum* and *L. lactis* ssp. *cremoris*) and in bifidobacteria (e.g., *B. longum* and *B. bifidum)* were the highest after 6–12 h incubation in milk-based media and a longer fermentation time decreased folate levels (Lin and Young 2000; Holasova et al. 2004; Holasova et al. 2005; Gangadharan et al. 2011; Padalino et al. 2012). Lin and Young (2000) suggested that one reason for folate depletion might be the consumption rather than the synthesis of folate. Fermentation at 37 °C led to higher folate production than fermentation at 30 °C (Holasova et al. 2005) or at 30 °C and 42 °C (Gangadharan et al. 2011). However, Tomar et al. (2009) showed that folate production in *S. thermophilus* cultivated in skim milk was higher at 42 °C than at 30 °C or at 37 °C.

Long fermentation also decreased folate levels in yeasts. Hjortmo et al. (2008a) incubated *S. cerevisiae* in synthetic medium and showed that the total folate content in the cell mass was the highest (about 125 µg/g dm) when the yeast was fermentatively degrading glucose to ethanol and carbon dioxide; that is, during the first 12 hours. During this period, the growth rate was high. After the respiro-fermentative phase, total folate content per cell mass was continuously decreasing. When the stationary phase was reached after 20 h of fermentation, the intracellular folate content was still observed to decrease. Thus, the physiological state of the cells clearly affected the folate content. Pietercelie et al. (2011) compared the folate production of different yeast species in malt wort. For all the species, folate production correlated positively to yeast growth. Moreover, yeast cultures produced more folate at 30 °C than at 18 or 12 °C.

Effect of pH

Sybesma et al. (2003) showed that production of folate in *S. thermophilus* and *L. lactis* increased with increasing controlled external pH in continuous cultures. They assumed that because the pH optima of all enzymes involved in folate biosynthesis is between 7.3 and 9.3, their activity increased under high external pH. Under controlled pH, acidification of the medium was delayed and intracellular pH was maintained at a more optimum level for folate biosynthesis. On the other hand, Pompei et al. (2007a) did not observe any effect of pH on folate production by *B. adolescentis* in the pH range from 5.7 to 6.9.

Metabolic engineering of microbes

Metabolic engineering allows increased folate production by altering metabolite or protein levels in the cell. This can be done by over-expression or disruption of metabolic or regulatory genes. Folate production levels in microbes can be modulated by gaining insight in the genes, pathways and metabolites that are involved (Iyer and Tomar 2009).

As an example, Sybesma et al. (2003) improved the total folate production three fold in *L. Lactis* by over-expressing the folate gene cluster, which encodes GTP cyclohydrolase I, the first step in folate synthesis. Wegkamp et al. (2007) combined over-expression of the *p*ABA gene cluster and the folate biosynthesis genes. As a result, folate production in *L. lactis* increased almost 80-fold. However, due to legislative limitations and the negative perception of genetic modification by consumers, selection of natural overproducers has gained favour.

Natural enhancement of folate in cereal matrices

Information on folate production by microbes in cereal matrices is limited. Yeasts contribute to the final folate content in yeast-fermented food products, such as bread. According to Kariluoto et al. (2004), yeast-leavened white breads contained 2.5-fold more folate than breads leavened with baking powder. Folate content may also increase during sourdough fermentations. The increase is mainly due to the growth of folate-synthesising yeasts (Osseyi et al. 2001; Kariluoto et al. 2006a). Folate production by yeasts in sourdough fermentations may compensate for folate losses during baking. Yeasts increased the folate content of sterilised rye flour–water mixtures 3-fold during 19-h fermentations, whereas studied LAB decreased it (Kariluoto et al. 2006a). Hjortmo et al. (2008c) emphasised the right choice of the yeast strains for baking because folate levels were 3–5-fold higher in bread leavened with a good producer than with low producer.

When maize flour–water mixtures were fermented with different yeast species originally isolated from fermented maize porridge, the folate content increased (Hjortmo et al. 2008b). The highest folate concentration was found after 46 h of fermentation with *Candida glabrata* and *S. cerevisiae*. Fermentation with *C. glabrata* increased the folate content 23-fold compared with the unfermented matrix. In addition, Kariluoto et al. (2006a) reported that two endogenous bacteria, *E. cowanii* and *P. agglomerans*, isolated from rye flour, showed good folate production during incubation in a rye flour–water mixture.

3 OBJECTIVES OF THE STUDY

Cereal grains and cereal products are an important source of natural folates. However, little is known about folate levels in oats and barley. The intake of natural folate could be enhanced by the selection of folate-rich cereal grain fractions or by using microbes as folate producers. The overall aim of this thesis was to examine the occurrence of folate in oat and barley grains and their milling fractions, and to investigate how and to what extent it would be possible to enhance the folate content by aqueous processing of oats and barley.

The specific objectives of the individual studies were:

- To introduce and validate a UHPLC method for determining folate vitamers in the cereal matrix **(I)**.
- To study folate levels and their variation in oat and barley cultivars from three harvesting years **(I, II)**.
- To study folate levels in oat and barley milling fractions and folate vitamer distribution within oat and barley grain **(I, II)**.
- To study folate production by selected endogenous cereal bacteria and some other microbes under different cultivation conditions **(III)**.
- To study folate production by selected microbes in the aqueous processing of oats and barley **(IV)**. The selected microbes were observed as promising folate producers in study **III.**

4 MATERIALS AND METHODS

This section summarises the materials and methods presented in more detail in the original publications (**I–IV**).

4.1 Materials

4.1.1 Oat and barley cultivar samples (I, II)

In studies **I** and **II,** the total folate content in Finnish oat and barley cultivars from three harvesting years was investigated. Five oat cultivars (Julius, Venla, Fiia, Peppi, Ivory) and five hulled barley cultivars (Jyvä, Tocada, Saana, Einar, Minttu) were grown in 2006, 2007 and 2008 in the same area in southern Finland. After harvesting they were stored in the dark at 10–15 °C. The grains were obtained from Boreal Plant Breeding Ltd. (Jokioinen, Finland) not until in autumn 2008, milled (0.5-mm particle size) and stored at −18 °C until they were analysed (within one month). Total folate content was measured by MA in autumn 2008.

The samples were characterized for typical agronomical and quality parameters (hectolitre weight, fresh grain weight, hull yield, lipid content, protein content and starch content) by Boreal Plant Breeding Ltd. (Finland).

4.1.2 Fractions of oat and barley grains (I, II)

Oat fractions

The starting materials in the oat-milling processes were composed of pooled Finnish oat cultivars grown in southern Finland in summer 2008. After harvesting, the grains were stored in the dark at 10–20 °C in summertime and at ca. 0 °C in wintertime until they were fractionated and milled (0.5-mm particle size). Fractionation was carried out twice, and the first fractionation was performed in autumn 2008 and the second in summer 2009.

Grains were fractionated and processed in a conventional, commercial oat-milling process by Myllyn Paras Ltd. (Hyvinkää, Finland), as shown in Figure 7. Hulls, bran, endosperm flour, oat flakes and also by-products such as flour from oat cutting and residual flour from oat flaking were separated. Fractions were milled and stored at −18 °C until they were analysed (within one month). Total folate content was determined with an MA in both processing sets. Folate vitamer distribution was determined only in the later set with the UHPLC method.

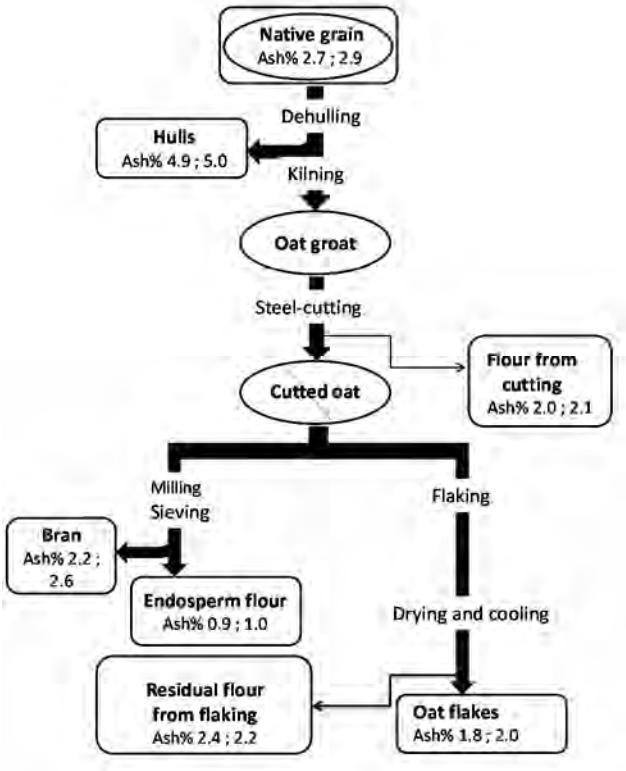


Figure 7. Schematic representation of the oat fractionation process. Fractions in the rectangular boxes were analysed for folate content. Ash contents in the fractions of the two millings are also given in the rectangular boxes.

Barley milling fractions

Scarification fractions

Two hulled Finnish barley cultivars (Minttu and breeding line Bor 03173), harvested in 2006, were subjected to abrasive milling in autumn 2007. The scarification was carried out using a laboratory seed scarifier in the USDA Regional Research Center (Wyndmoor, PA). Barley samples were scarified for a total of 100 s and fines were separated after each 20-s scarification. The scarification was repeated several times and fine fractions were pooled to obtain a representative sample. These fractions and native and scarified grains were stored at −18 °C before total folate analysis with the MA and folate vitamer-distribution analysis with the HPLC method.

Industrial-milling fractions

Wholegrain barley was fractionated in a commercial-scale roller mill process (Myllyn Paras Ltd. Hyvinkää, Finland). The starting material was a mixture of Finnish hulled barley cultivars grown in southern Finland. After harvesting, the grains were stored in the dark at 10–20 °C in summertime and at ca. 0 °C in wintertime. Grains were pearled with an industrial pearler, at 5, 10 and 20% weight intervals during the continuous grain flow (Figure 8). In addition, grains that had passed through 10% pearling were further milled to mimic

commercial barley flour products: fine barley flour (0–200 µm), coarse barley flour (200–400 µm), fine barley granule (400–700 µm) and coarse barley granule (700–1100 µm). The fractionation was performed three times.

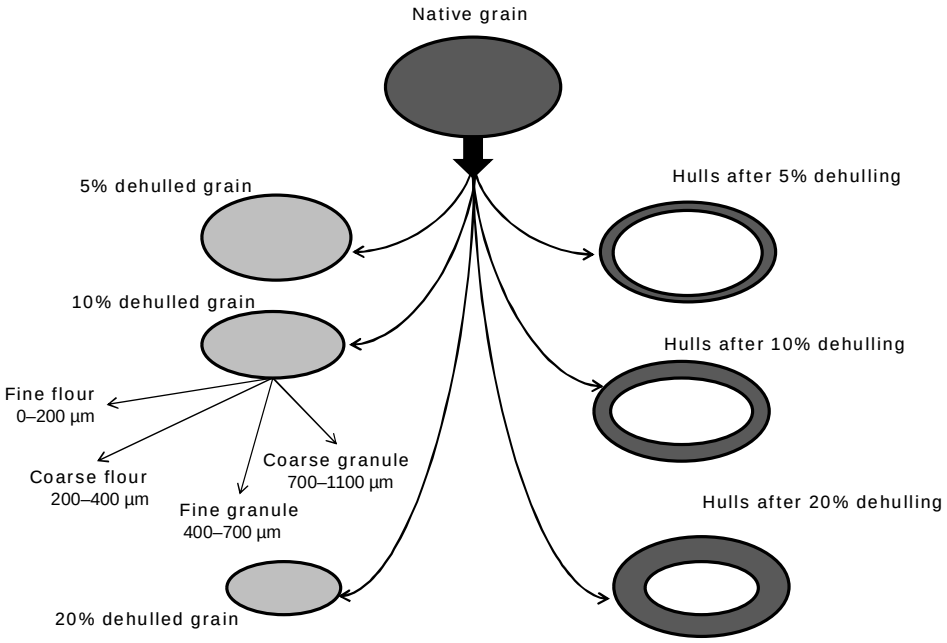


Figure 8. Fractionation scheme of the industrial-milling process of barley grain.

In the first two millings (performed in October 2008 and June 2009), barley grains harvested in August 2008 and in the third milling (performed in October 2011) grains harvested in August 2011 were used. Total folate content in all fractions was determined with the MA. In addition, folate vitamer distribution in samples of the third milling was analysed with the UHPLC method. After every milling, the 5, 10 and 20% dehulled barley kernels, together with native grains, were milled to a 0.5-mm particle size.

4.1.3 Folate production in rich medium (III, IV)

In the third study (**III**), folate production by 20 bacteria isolated mainly from oat products were investigated. First, microbes were cultured in a rich medium containing 1% yeast extract, 2% peptone and 2% glucose (YPD medium) at 28 °C and without buffering. Next, selected microbes were cultured under different controlled pH and temperature conditions. In study **IV,** folate production by selected food-grade yeasts was studied in YPD medium and the folate vitamer distribution of their cell masses during at the stationary phase was analysed.

Microbe strains

Bacteria (**III**) represented the best folate producers in an initial screening among 42 isolates (Herranen et al. 2010). The selected 14 bacteria strains had been isolated from regular oat bran (Raisio Plc, Raisio, Finland), oat fibre Natureal® OBCN15 (Finn Cereal LTD, Finland)

and oat fibre OatWell® 14% (Swedish Oatfibre, Swedish Oat fibre AB, Väröbacka, Sweden). In addition, one bacterium had been isolated from rye flakes Nalle Ruishiutale (Raisio Plc, Raisio, Finland). The isolated bacteria had been identified by sequence analysis of the 16S rRNA genes and characterized (Herranen et al. 2010). Furthermore, *S. cerevisiae* ALKO743, *Pantoea agglomerans* ABM5061, *Bacillus* sp. ABM5119, *B. cereus* ABM5122 and *B. cereus* ABM5123 were used as reference organisms in this study.

Yeasts in study **IV** represented food-origin yeasts: *S. cerevisiae* ALKO743 (baker's yeast, the control strain), *S. cerevisiae* CBS7764 (isolated from rainbow intestine), *S. cerevisiae* ABM5131 (from kefir grains), *Candida milleri* ABM4949 (from rye sourdough starter), *Kluyveromyces marxianus* ABM5130 (from kefir grains) and *Clavispora lusitaniae* ABM5147 (from fermented oat product).

Cultivation in YPD

Pre-culturing and cultivation of the microbes were presented by Herranen et al. (2010). The overnight pre-cultures were used to inoculate fresh YPD media that were incubated at 28 °C for 24 h. Aliquots (50 ml) of the growing culture were taken during the exponential ($Klett_{60}$ value 100) and the stationary phase upon 24-h incubation. Cell fractions were separated by centrifugation. Total folate was determined in the cell mass and culture media with the MA.

Growth in YPD when adjusting pH and temperature

In study **III**, the influence of pH and temperature on the folate production of seven bacterial strains was investigated. *Exiguobacterium* sp. RB3, *Janthinobacterium* sp. RB4, *P. Ananatis* ON1, *P. agglomerans* ON3, *Pseudomonas* sp. ON8, *Chryseobacterium* sp. NR7 and *Bacillus* sp. ABM5119 were grown as above in the YPD medium buffered with 0.1 M MES (2-(*N*-morpholino) ethanesulfonic acid) at pH 5.5 and 7.0 and at three different temperatures (18, 28 and 37 °C). Strains were incubated for 15 to 30 h until they were in the early stationary phase according to the turbidity (A_{525}). Total folate was determined in the cell mass and culture media with an MA. Each strain was grown in three independent experiments.

Folate vitamer distribution in the cellular biomass was determined at the exponential and stationary phase both for the strains mentioned above and for *Acinetobacter calcoaceticus* RB1, and *S. cerevisiae* ALKO743 (only in the stationary phase) with the HPLC method. In addition, folate vitamer distribution in the cellular biomass was determined during the stationary phase for food-grade yeasts with the UHPLC method.

4.1.4 Folate production in cereal matrices (IV)

Microbe strains

The promising folate producers from study **III** with a limited hydrolytic activity on beta-glucan were selected for the further studies in fibre-rich oat and barley cereal matrices. In addition, a few other bacteria and yeasts used in the food industry were included as reference strains. Strains were precultured in YPD and inoculated into the cereal matrix.

Cereal matrices

The microbe strains and the matrices where the strains were studied are presented in Table 5. Proportions of cereal materials and water was set so that the estimated content and viscosity of beta-glucan in the matrices were similar. The 3.5% (w/v in water) oat bran matrix was made from oat bran (OatWell ® 14%, Oat Fiber AB, Bua, Sweden), the 10% oat flour matrix from milled oat flakes (Raisio Plc, Finland), the 3.5% barley bran from barley bran (Bonafibre Oy, Lahti, Finland) and the 20% barley flour from milled barley groats (Myllyn Paras Ltd, Hyvinkää, Finland). Folate production in the matrices was studied without and with 1% (w/v) glucose addition. Furthermore, the 20% barley flour matrix was treated with heat-stable α-amylase Termamyl 300 L (Novozymes A/s, Bagsvaerd, Denmark) before autoclaving in order to obtain a similar consistency and viscosity as in other matrices.

Autoclaved matrices were inoculated with 1 ml of microbial culture in YPD and incubated first at 28 °C for 24 h (except for *L. reuteri* at 37 °C) and next at 4 °C for 2 weeks. An uninoculated control sample was included in each incubation. Samples for folate and glucose analysis were collected at 0 h, 8 h, 24 h and 2 weeks and stored at −20 °C. The viable counts of the microbes were determined after 0 h, 24 h and 2 weeks of fermentation. The experiment was typically repeated two or three times and samples were analysed for folate in duplicate in each incubation. In addition, the folate vitamer distribution was determined in 10% oat flour and 3.5% barley bran matrices with the UHPLC method.

Table 5. Microbes and matrices (combination is marked with **x**) where the microbe was tested in study **IV**. –glc, without glucose addition; +glc, 1% glucose addition.

Microorganism	3.5% Oat bran		10% Oat flour		3.5% Barley bran		20% Barley flour	
	− glc	+ glc	− glc	+ glc	− glc	+ glc	− glc	+ glc
Yeasts								
Saccharomyces cerevisiae ALKO743	**x**	**x**	**x**	**x**		**x**	**x**	**x**
Saccharomyces cerevisiae ABM5131			**x**	**x**				
Candida milleri ABM4949	**x**	**x**		**x**		**x**	**x**	**x**
Kluyveromyces marxianus ABM5130	**x**	**x**			**x**	**x**	**x**	**x**
Clavispora lusitaniae ABM5147	**x**	**x**				**x**	**x**	**x**
Bacteria								
Pantoea ananatis ON1	**x**	**x**				**x**	**x**	**x**
Pseudomonas sp.ON8	**x**	**x**	**x**	**x**	**x**	**x**	**x**	**x**
Janthinobacterium sp. RB4	**x**	**x**				**x**	**x**	**x**
Bacillus sp.ABM5119	**x**	**x**				**x**	**x**	**x**
Streptococcus thermophilus ABM5097			**x**	**x**		**x**		
Lactobacillus reuteri				**x**		**x**		
Lactobacillus rhamnosus LC-705				**x**		**x**		
Propionibacterium sp.				**x**		**x**		

4.2 Analytical methods

4.2.1 Standards and quantification of folate

5-HCO-H_4folate ([6S]-5-formyltetrahydrofolate, sodium salt; Merck Eprova AG, Schaffhausen, Switzerland) was used as an external standard in the MA. It was dissolved and purity-checked as other calibrants (see below). In addition, other folate vitamer standards (as their monoglutamates) were used for identification and quantification in the HPLC and UHPLC methods. 5-CH_3-H_4folate ([6S]-5-methyltetrahydrofolate, calcium salt), H_4folate ([6S]-tetrahydrofolate, sodium salt), 5,10-CH^+-H_4folate ([6R]-5,10-methenyltetrahydrofolate chlorine hydrochloride) and PGA were obtained from Merck Eprova AG (Schaffhausen, Switzerland). 10-HCO-PGA (10-formylfolic acid) was obtained from Schircks Laboratorios (Jona, Switzerland). 10-HCO-H_2folate was synthesised from [6R,S]-5,10-CH^+-H_4folate according to Pfeiffer et al. (1997) with a somewhat longer reaction time (3 h). Calibrants were dissolved as described by van den Berg et al. (1994). Their purities were calculated using molar absorptivity coefficients (Baggot et al. 1995). For the purity check of 5,10-CH^+-H_4folate, the stock solution was diluted into 0.1M K_2HPO_4, pH 1.0 and the purity was confirmed using a molar absorptivity coefficient at pH 1.0 (E_{356}= 25 x$10^3 M^{-1}$ cm^{-1} according to Rabinowitz 1963.

A combined working solution of other vitamers and a separate working solution of 5,10-CH^+-H_4folate was prepared so that the concentration of each vitamer was 200 ng/ml (except for 10-HCO-H_2folate at 400 ng/ml). A separate working solution for 5,10-CH^+-H_4folate was prepared because synthesised 10-HCO-H_2folate solution contained traces of 5,10-CH^+-H_4folate.Working solutions were prepared in 0.01M acetate buffer containing 1% (w/v) sodium ascorbate and 10mM 2-mercaptoethanol (pH 4.9) and stored at −20 °C in small aliquots for no more than three months. In addition, 5-CH_3-H_4PteGlu_2 ([6R,S]-5-methyl-5,6,7,8-tetrahydropteroyldiglutamate trihydrochloride salt; Schircks Laboratories, Jona, Switzerland) was used for identification.

4.2.2 Extraction and tri-enzyme treatment (I, II, III, IV)

The first step in the folate analysis was extraction followed by enzyme treatments (conjugase, α-amylase, protease) for analysing total folate with the MA or vitamer distribution with the HPLC or UHPLC method. Samples were weighed in duplicate as follows: cereal samples and cereal fermentation samples 0.5–1.5 g, cell fractions 0.1–0.5 g, and supernatants 2–5 g. 10–15 ml of extraction buffer (50 mM Ches, 50 mM Hepes, containing 2% sodium ascorbate and 10 mM 2-mercaptoethanol, pH 7.85) was added and samples were flushed with nitrogen and placed into a boiling water bath for 10 min. Samples were cooled and the pH was adjusted to 4.9 with HCl if hog kidney conjugase was used (all samples in **I**, **II**, **III** and **IV,** except for some of the microbe samples in **III**) or to 7 if chicken pancreas conjugase was used (some of the microbe samples in **III**). Hog kidney conjugase (HK) was prepared from fresh pork

kidneys according to Gregory et al. (1984). Its activity was tested in every batch according to Vahteristo et al. (1996).

After pH adjustment, conjugase was added: 1–1.5 ml of HK or 1 ml of chicken pancreas solution (5 mg/ml in water, Difco, Sparks, MD). In addition, α-amylase (EC 3.2.1.1, St. Louis, MO) was added (20 mg) in all samples except in the microbe samples, because treatments with amylase and protease were found unnecessary for cell and supernatant samples. Samples were incubated for 3 h at 37 °C. After that, protease (EC 3.4.24.31; Sigma, St. Louis, MO) was added (4 mg) without pH adjustment if chicken pancreas was used, and after pH adjustment to 7.0 if HK was used. Incubation was continued for 1 h at 37 °C. Enzymes were inactivated by boiling. If only conjugase treatment was used, the enzyme was inactivated after 3 h.

Samples analysed with only the MA were filled to an exact volume with 0.5% sodium ascorbate and then analysed directly with the MA without a purification step. Extracts analysed using both the microbiological and the HPLC/UHPLC assay were centrifuged twice for 10 min at 8000 rpm at room temperature, after inactivating the enzymes and cooling. The supernatants were combined and filled to an exact volume (25 ml) with 0.5% sodium ascorbate. Then the samples were filtered before total folate analysis with the MA and were purified for HPLC/UHPLC.

4.2.3 Purification of sample extract with affinity chromatography

The sample extracts were purified and concentrated before LC analysis with affinity chromatography using FBP. Affinity columns were prepared by coupling FBP from bovine milk (Scripps Laboratories, San Diego, CA) to agarose (Affi-Gel10; Bio-Rad Laboratories, Richmond, CA) as described by Konings (1999). The gel volume was approximately 1.4–1.8 ml, the average amount of FBP was 0.5 mg and the binding capacity 4–5 µg PGA/column.

Columns were equilibrated with potassium phosphate buffer (0.1 M, pH 7.0). After addition of the sample extract (10–20 ml), the columns were washed with 0.025 M potassium phosphate/1 M NaCl (pH 7.0) followed by 0.025 M potassium phosphate. Folates were eluated with 0.02 M trifluoroacetic acid/0.01 m dithiothreitol into 5-ml volumetric flasks followed by filtering.

4.2.4 Microbiological assay (I, II, III, IV)

Total folate content was determined in all samples with the MA previously described (Kariluoto et al. 2004; Piironen et al. 2008). The assay was carried out on 96-well microtiter plates using *Lactobacillus rhamnosus* ATCC 7469 as the test organism and 5-CHO-H_4folate as the calibrant. Two dilutions were made from each sample extract, and eight levels of calibrant (0–80 pg/well) were included in each plate. Plates were incubated for ca. 18 h at 35 ºC and turbidity was measured with a microplate reader at 595 nm.

4.2.5 High-performance liquid chromatography (II, III)

Before the introduction of the UHPLC method, folate vitamers were determined as monoglutamates both in scarification samples in study **II** and in cell fractions and supernatants in study **III** using an HPLC method, as described previously (Kariluoto et al. 2001, 2004). An FLR detector (Waters 470, Milford, MA) was used to detect and quantify H_4folate, 5-CH_3-H_4folate, and 5-HCO-H_4folate (excitation 290 nm and emission 356 nm) and 10-HCO-PGA (excitation 360 nm and emission 460 nm), and a dual γ absorbance detector (Waters 2487) was used to detect and quantify 10-HCO-H_2folate (290 nm), 5,10-CH^+-H_4folate (360 nm) and PGA (290nm). The vitamers were separated on a ThermoQuest (Cheshire, UK) Hypersil ODS column (30 °C) (150 mm × 4.6 mm, 3 µm) using Waters 510 and 515 HPLC pumps and a Waters 717 plus autosampler (+8 °C). Gradient elution was performed with acetonitrile and 30 mM phosphate buffer, pH 2.2, at a 0.9 mL/min flow rate. A Waters Empower 2 build 2145 was used to control the system and to collect and calculate chromatographic data.

A separate working solution of 5,10-CH^+-H_4folate and a combined working solution of other vitamers were used for the calibration curves. Peaks were identified by retention times. Identity was also confirmed by comparison of relative peak areas in both detectors. Quantification of folate vitamers was based on using a multilevel (n = 7–11) external calibration curve. Calibrants were purified with affinity columns similarly to the sample extracts.

4.2.6 Ultra-high performance liquid chromatography (I, II, IV)

In this thesis, a UHPLC method based on the previously used HPLC system (Kariluoto et al. 2001) was introduced and validated. Vitamer distribution in oat and barley cereal milling fraction samples (**I, II**), yeast cell mass and cereal matrix fermentation samples (**IV**) were analysed with the UHPLC method.

General conditions in UHPLC

In study **I**, the separation of vitamers was first tested on two Waters Acquity UHPLC columns: BEH C18 (1.7 µm, 2.1 × 100 mm) and HSS T3 (1.8 µm, 2.1 × 150 mm). A mobile phase system used in HPLC was scaled for UHPLC taking into account the parameters of both tested columns. Several different gradients were tested for both columns. Gradients were evaluated on the basis of chromatographic parameters (retention time, resolution, peak areas, selectivity, symmetry factor and theoretical plates). After optimising, the UHPLC system consisted of a Waters Acquity UHPLC system with binary solvent manager, sample manager, column manager, and PDA and FLR detectors (Waters, Milford, MA). The UHPLC separation was achieved on an HSS T3 (1.8 µm, 2.1 × 150 mm) column. The mobile phase system was comprised of 30 mM potassium phosphate buffer at pH 2.2 and acetonitrile at a constant flow rate of 0.4 ml/min. Resolution of folate vitamers was achieved with a linear gradient system of buffer and acetonitrile. The gradient started at 5% (v/v) acetonitrile, isocratically for 2.2 min, and then rose to 15.4% within 5.3 min and was maintained for 0.4

min. Thereafter, the concentration decreased to initial conditions within 0.4 min and reconditioning took 4.7 min. The Waters Empower 2 build 2145 was used to control the system and data handling.

Folate vitamers were detected at the FLR detector and at the PDA detector similarly as in the HPLC method described above, with one exception: 5-HCO-H4folate was detected and quantified with the PDA at 290 nm, as the acetonitrile gradient of the FLR detector at 290/356 nm led to a sharp slope in the baseline. Quantification of folate vitamers was based on an external standard method. As with the HPLC method, a combined working solution and separate working solution of 5,10-CH^+-H_4folate were used for the calibration curves. Peaks were identified by retention times and by diode array spectra. Spiking was used to confirm the peaks, if necessary. In some high folate-content cereal samples, an unknown peak eluated immediately after 5-CH_3-H_4folate monoglutamate. It was identified as 5-CH_3-H_4-$PteGlu_2$, quantified using the standard curve of 5-CH_3-H_4folate monoglutamate and was taken into account in the 5-CH_3-H_4folate content.

Validation of the optimised UHPLC method

The optimised UHPLC system was validated (**I**). The limit of detection (LOD) and the limit of quantification (LOQ) for vitamers were determined. Since no blank matrix was available, the working solution was serially diluted, and the LOD and LOQ were defined as the lowest analyte concentration yielding a signal-to-noise (S/N) ratio of > 3 and > 10, respectively. In addition, the linearity of the response for each folate vitamer, and the interday and intraday precision and accuracy of the retention times and detector response (peak areas) were evaluated.

The recovery test for UHPLC was performed by spiking the in-house reference (n = 6, amount 1.5 g) with one level (100 ng) of each folate vitamer before extraction. The recovery samples were handled similarly to the other samples for UHPLC determination. Furthermore, the in-house reference and Certified Reference Material BCR 121 were analysed both with the UHPLC method and MA as with normal samples.

4.2.7 Quality assurance

General procedures

Analytical procedures were carried out under yellow or subdued light. Moreover, sample extracts and standard solutions were kept under nitrogen atmosphere whenever feasible. Reference samples were stored at −70 °C and they, as well as the samples, were allowed to temper at room temperature in a desiccator for 1 h before weighing.

Microbiological assay

The total folate content of the duplicates was not allowed to differ by more than 10%. In addition, Certified Reference Material BCR 121 (wholemeal flour, Institute for Reference Materials and Measurements, Geel, Belgium) or in-house reference flour (commercial wholemeal wheat flour, Myllyn Paras, Finland) were analysed as a quality control sample in

each incubation. Action limits in the control charts were 500±70 ng/g dm (the certified value) for BCR 121 and 521±55 ng/g fw (average ±1.5 × standard deviation) for the in-house reference. The results of the set of analyses were rejected if the folate content of the references was outside the action limits. Further, criteria for the minimum and maximum turbidity of the highest and the lowest calibrant levels were also set in order to follow the repeatability of the growth of *L. rhamnosus*.

HPLC and UHPLC methods

The HPLC method had been previously validated for folate analysis (Kariluoto 2008). In addition, the capacity of the affinity chromatography columns had been tested for different vitamers in the previous study by Kariluoto (2008). Based on her results, the total folate load was kept at under 25% of the column capacity. The capacity of affinity columns was checked by the FBP-binding test with PGA after preparing them, and between the sample batches (on average monthly). The column was discarded if the capacity was lower than 90% for PGA.

The recovery test for UHPLC was performed by spiking the in-house reference (n = 6, amount 1.5 g) with one level (100 ng) of each folate vitamer before extraction. The recovery samples were handled similarly to the other samples for UHPLC determination.

In this thesis, samples that were analysed with the HPLC or UHPLC methods were also analysed with the MA. The same sample extract was always used in both methods. The total folate result obtained with MA was compared with the sum of vitamers obtained with the HPLC or UHPLC method.

4.2.8 Other analytical methods

Dry matter and ash content (I–II)

Oat and barley cultivar and fraction samples were analysed for moisture content with the AACC 44-15A method. Samples as duplicates (2–4 g) were dried overnight at 102 °C. Ash content was determined gravimetrically by incinerating oat and barley fraction samples as duplicates in the presence of nitric acid in a furnace oven at 550 °C, according to the AACC 08-01 method.

Viable cell count (IV)

The viable cell count was determined after 24 h and 2 weeks of storage in fermented cereal matrices. Viable bacteria and yeast cells were plate counted in YPD agar after incubation. The samples were serially diluted with phosphate-buffered saline, and 50 µl aliquots were spread plated in duplicate in YPD plates. After incubation for 1 to 6 days at 28 ºC/37 ºC, the colonies were counted and the viable cell number was expressed as cfu/ml.

Glucose (IV)

The glucose consumption by microbes was studied in selected cereal matrices during the fermentation. Glucose was determined from each incubation in duplicate with a glucose

oxidase kit (Megazyme K-GLUC D-glucose, Megazyme International, Wicklow, Ireland). Glucose results were not reported in paper **IV** but they have been included in this thesis.

Data analysis

In this thesis, total folate and folate vitamer concentrations are presented as averages of replicate samples on a dry matter (dm) basis in studies **I–II**, and on a fresh weight (fw) basis in studies (**III–IV**) if not otherwise stated.

In studies **I–II,** statistical analyses were performed using Statgraphics Plus 4.0 (Manugistics Inc., Rockville, MD). The correlation of total folate levels in oat and barley cultivars harvested in 2008 with typical agronomical and quality parameters (hectolitre weight, fresh grain weight, hull yield, lipid content, protein content and starch content) was tested using Pearson's correlation coefficients. In all analyses, p-values lower than 0.05 were considered as statistically significant.

5 RESULTS

5.1 Validation of the UHPLC method (I)

The UHPLC method was validated for the HSS T3 column. The chosen conditions offered satisfactory separation of the seven folate vitamers. The validation data is presented in Table 6. Good linearities (R^2 value (coefficient of determination) = 0.90 or higher) were shown for all vitamers within the routine working concentration range of 20–200 ng/ml. The detection limits were 0.2 ng/ml for H_4folate, 5-CH_3-H_4folate and 10-HCO-PGA at the FLR detector and 4–8 ng/ml for the other vitamers at the UV detector. Intraday variations in the detector response and retention times expressed as relative standard deviation (RSD) were from 1.1% to 3.8% and from 0.1% to 0.3%, respectively, whereas interday variation was slightly greater and it varied from 3.1% to 4.9% (detector response) and from 0.7% to 2.7% (retention time). Recoveries of vitamers were 85–99% in wholegrain wheat flour (n = 6).

Table 6. UHPLC method validation parameters established with the folate calibrants.

Folate vitamer (detector used for validation)	Coefficient of determination, R^2 Linear range 20–200ng/mL	LOD[a], ng/mL	LOQ[a], ng/mL	Intraday variability (n=12)		Interday variability (n=72)		Recovery[b] (%, n=6)
				Retention time (RSD[a], %)	Peak area (RSD, %)	Retention time (RSD, %)	Peak area (RSD, %)	
H_4folate (FL 290/356 nm)	0.9547	0.2	0.7	0.09	1.76	2.73	3.12	85±13
5-CH_3-H_4folate (FL 290/356 nm)	0.9954	0.2	0.7	0.09	1.34	2.34	3.53	94±5
5-HCO-H_4folate (UV 290 nm)	0.9924	4	13	0.28	1.78	0.86	4.14	98±4
10-HCO-PGA (FL 360/460 nm)	0.9953	0.2	0.7	0.13	1.14	0.72	4.59	88±6
folic acid (UV 290 nm)	0.9942	4	13	0.16	3.77	0.52	4.90	99±4
10-HCO-H_2folate (UV 290 nm)	0.9875[c]	8	27	0.11	2.84	1.38	4.14	87±10
5,10-CH^+-H_4folate (UV 360 nm)	0.9916	4	13	0.13	1.08	0.71	4.16	91±5

[a] RDS, relative standard deviation; LOD, limit of detection; LOQ, limit of quantification

[b] Recovery (%) in wholegrain wheat flour (100 ng of each calibrant/1.5 g flour) = 100 × (concentration of spiked sample−concentration of unspiked sample)/calculated concentration of analyte added to the test sample

[c] Linear range 40–400 ng/mL

The sum of vitamers (414 ng/g dm) in the reference material BCR 121 was 92% from the total folate content analysed with the MA. The certified value of BCR 121 is 500±70 ng/g dm. In addition, the total folate content in the in-house reference determined with UHPLC (408±17 ng/g dm) was 78% of the long-term average 521±55 ng/g dm of the MA.

The total folate content analysed with the UHPLC method was well in line with the results of the MA. The sum of the vitamers was, on average, 80±10% and 84±10% of the microbiologically analysed values in the oat and barley samples (**I, II**), respectively, and was as high as 98±16% for yeast cell mass samples in YPD medium (**IV**). The UHPLC results for oat flour fermentation (**IV**) were 88±8% of the total folate achieved with the MA. However, the vitamer sum was only 50–70% of the microbiologically determined total folate content when the scarification samples (**II**) were analysed with HPLC. Much better agreement, 90±8%, was achieved in microbe samples in YPD medium (**III)**.

5.2 Total folate content in oat and barley cultivars (I, II)

Five oat and five hulled barley cultivars were grown over three years (2006–2008) in the same area in southern Finland and were analysed for total folate content in autumn 2008. Figure 9 shows the total folate content of the oat and barley cultivars. In the oat cultivars harvested in 2008, the total folate content ranged from 579 to 740 ng/g dm, while the corresponding ranges were 531–615 ng/g in 2007 and 442–517 ng/g in 2006. The barley cultivars harvested in August 2008 had, on average, similar folate content to the cultivars harvested in 2007 (mean content of 773 and 712 ng/g dm, and ranges of 625–918 and 597–824 ng/g, respectively), but were approximately 30% higher than the same cultivars harvested in 2006 (563 ng/g dm, range 501–663 ng/g).

The folate-richest oat and barley cultivars in 2008 were Venla and Jyvä, containing 740 and 918 ng/g dm folate, respectively. On the other hand, the lowest total folate was found in oat cultivar Peppi (579 ng/g) and barley cultivar Tocada (625 ng/g) in 2008.

When correlations between folate content and available agronomical quality parameters were studied in the oat and barley cultivars in 2008, starch content correlated negatively with folate content in the barley cultivars ($r = -0.897$, $p < 0.05$). In the oat cultivars, no statistically significant association with any of these parameters was observed.

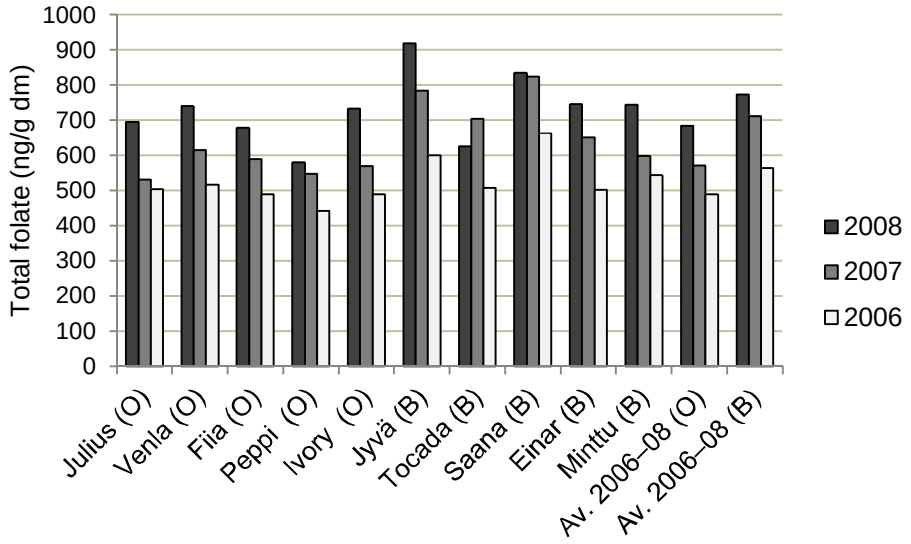


Figure 9. Total folate content of five oat and barley cultivars harvested from 2006–2008 and determined in 2008 with the MA in duplicate. O = oat cultivar; B = barley cultivar; Av. = average value of folate content.

5.3 Total folate in milling fractions

5.3.1 Oat fractions (I)

In study **I,** conventional oat processing produced fractions with total folate content ranging from 368 to 837 ng/g dm and from 240 to 989 ng/g dm in the first and the second milling, respectively (Figure 10). The highest folate level was found in residual flour from flaking. Oat bran and flour from cutting were also rich in folate. The lowest folate content was found in endosperm flours and flakes. Interestingly, hulls contained nearly as much folate as endosperm flour and flakes.

Although folate levels in the grains and milling products of the first milling were higher than in the first milling, the ash content of the fractions was rather similar (Figure 7). The lowest ash content was found in endosperm flour (mean 1.1%) and the highest in hulls (mean 5.5%). Flakes, bran, flour from cutting and residual flour from flaking had roughly equal ash content, at 2.5%, although residual flour from flaking was the folate-richest fraction. Hence, no statistically significant relationship appeared between the ash content and total folate in the oat fractions ($r = -0.099$).

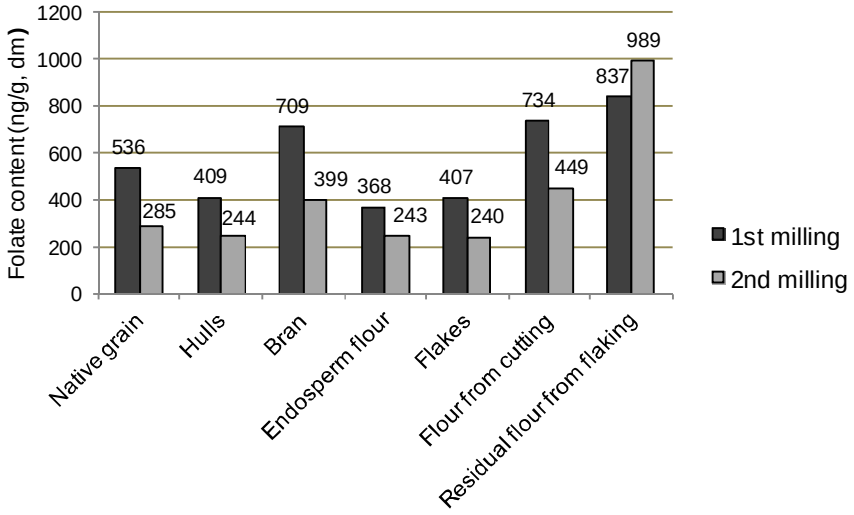


Figure 10. Average total folate content (ng/g dm) of oat-milling fractions determined with the MA in duplicate. The milling process was performed twice (in autumn 2008 and in summer 2009) for pooled oat cultivars grown in summer 2008.

5.3.2 Barley fractions (II)

In study **II,** barley fractions were produced by a typical pearling process with an industrial-scale roller mill and also by abrasive milling with a small-scale scarifier. Remarkably, folate-rich fractions were achieved by both techniques (Figure 11). The average folate content in native grains was 631±33 ng/g dm. The outer fraction, 5% dehulled hull, contained 1061±235 ng/g dm folate. The total folate concentration was the highest in the 10% dehulled (1326±77 dm) and in the 20% dehulled hull (1313±207 ng/g dm) fractions. The total folate content also varied among flours and granules. The folate content in both coarse flour and in fine and coarse granules was higher than in the 10% dehulled grains, which was the starting material for them. Fine and coarse flour had a folate content of 284±31 and 551±60 ng/g dm, respectively, and fine and coarse granule had a folate content of 427± 115 and 723± 140 ng/g, respectively.

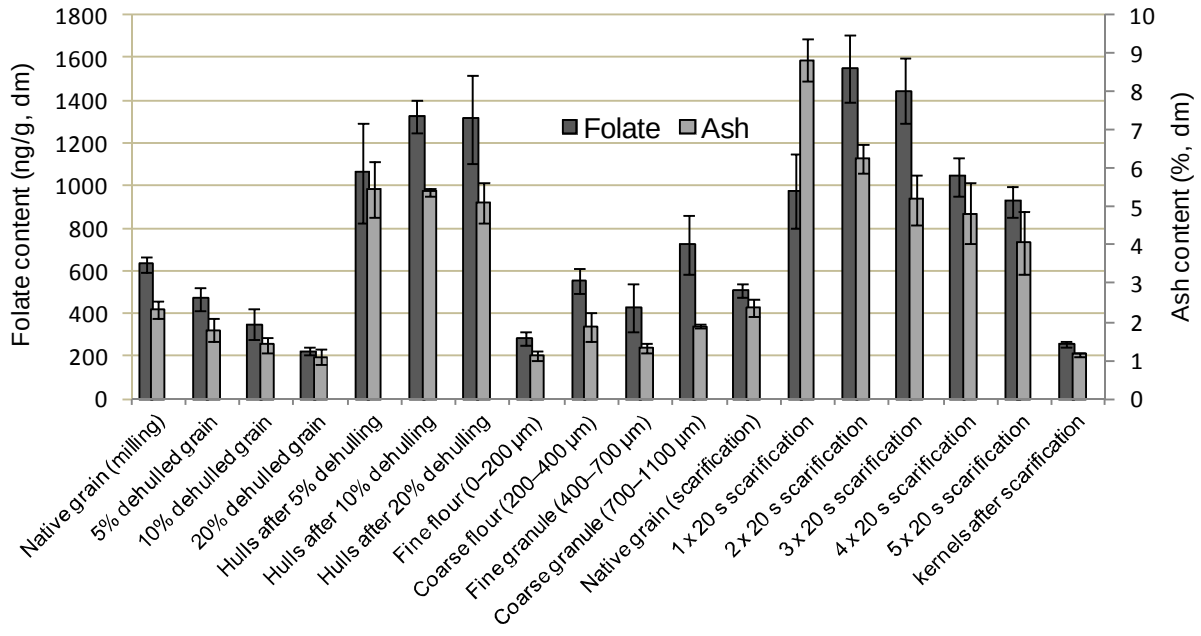


Figure 11. Average total folate content (ng/g, dm) determined with the MA and ash (%, dm) of the barley industrial-milling fractions (average ± SD) and scarification fractions (average of two cultivars ± range). The industrial milling was performed and analysed three times for pooled barley cultivars and scarification once for each of two cultivars. All samples were analysed in duplicate.

Figure 11 also shows the average folate and ash content in the scarification fractions of the two cultivars, Minttu and Bor 03173. The folate content of the first fraction was around 2-fold compared with that of native grain, and its ash content was high, at 8.8%. The next fractions (2 × 20 s and 3 × 20 s) were the folate-richest, containing 1553 ng/g to 1444 ng/g dm folate, respectively; that is 2.6–3.6-fold more folate than in native grain. The next two fractions (4 × 20 s and 5 ×20 s) still had a reasonably high amount of folate compared with native grain, at 1042 and 926 ng/g dm, respectively. After the scarification process (i.e. after 100 s), the scarification grains contained only 256 ng/g dm of folate.

The folate content in both the industrial and scarification fractions correlated with their ash content in the corresponding samples, if the first high fibrous scarification fraction (1 × 20 s) with high ash (8.8%) was omitted. Thus, the correlation coefficients (r) were 0.945 for the industrial and 0.971 for the scarification samples.

5.4 Folate vitamers in oat and barley milling fractions (I, II)

Vitamer distribution was analysed with the UHPLC method in the oat products of the second milling process (**I**) and in the barley industrial-milling fractions of the third milling batch (**II**).

In addition, the HPLC method was used to determine vitamers in barley scarification fractions. 5-CH_3-H_4folate, 5-HCO-H_4folate, 5,10-CH^+-H_4folate, 10-HCO-PGA (an oxidation product of 10-formyltetrahydrofolate), H_4folate and traces of folic acid were identified in the samples (Figure 12). 10-HCO-H_2folate was not found in any fraction.

5-CH_3-H_4folate was the main vitamer in oat samples, and its proportion was the greatest (> 50%) in the fractions with high folate content; that is, in flour from cutting and residual flour from flaking. In the endosperm flour, the proportion of 5-CH_3-H_4folate was also high (48%). The next most abundant vitamers were 5-HCO-H_4folate, 5,10-CH^+-H_4-folate and 10-HCO-PGA. The contribution of 5-HCO-H_4folate and 10-HCO-PGA to the total folate content was the highest in flakes, ca. 50% of the vitamer sum. The native oat grain contained more 5-CH_3-H_4folate than 5-HCO-H_4folate. H_4folate was found mostly in the native grain, hulls and bran. In the other oat fractions, its proportion was smaller.

On the contrary to oat samples, the main vitamer in almost all barley samples was 5-HCO-H_4folate, contributing, on average, one-third (range 27–42%) to the sum of the identified vitamers. The next abundant vitamers were 5-CH_3-H_4folate and 5,10-CH^+-H_4folate. The proportion of 10-HCO-PGA was remarkable in some industrial-milling fractions, ranging from 9–22%, whereas it was distributed more evenly over all scarification fractions, contributing, on average, 11–12% of the sum of vitamers. In addition, H_4folate and minor amounts of PGA were identified both in the scarification and in the industrial-milling fractions.

The proportions of the three most abundant vitamers (5-HCO-H_4folate, 5-CH_3-H_4folate and 5,10-CH^+-H_4folate) were quite similar across the native barley grains used in industrial milling, accounting for 31, 29 and 27%, respectively, of the sum of the vitamers. In the native grains used in scarification, the proportion of 5,10-CH^+-H_4folate was slightly lower; thus, the main vitamers contributed 36, 28 and 11% of all analysed vitamers. Furthermore, vitamer distributions of the Bor03173 and Minttu cultivars were similar.

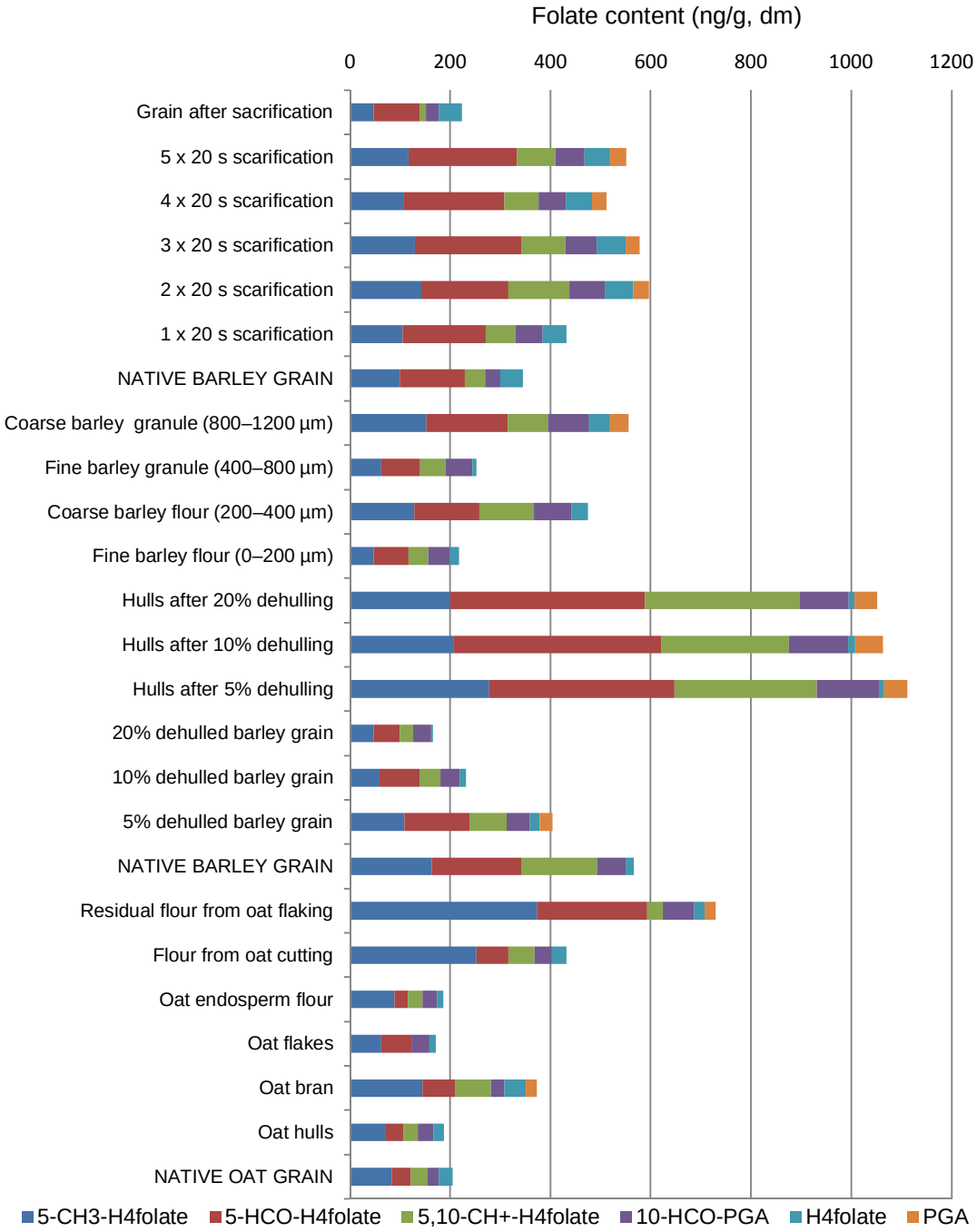


Figure 12. The folate vitamer content (ng/g, dm) in the oat and barley milling fractions performed once for pooled cultivars and analysed with the UHPLC method in duplicate.

The concentration of each vitamer increased when the total folate content increased, but their proportions varied in different fractions. This was noticed particularly in the industrial-milling fractions of barley and in certain oat products (bran, flour from cutting and residual flour from flaking). 10-HCO-PGA was more concentrated in the barley fractions derived from the inner part of the grain; that is, in the endosperm. Its proportion was the highest (22%) in the 20% dehulled grains and the lowest (9%) in the 20% dehulled hull fraction. 5,10-CH^{+}-H_4folate was

associated with hull fractions: in 20% dehulled barley hulls, its proportion was almost two-fold compared to its proportion in the dehulled grain. In oat bran, its proportion was also higher than in the other fractions. Further, the folate-rich 2 × 20 s and 3 × 20 s scarification fractions were rich in 5,10-CH^+-H_4folate (on average, 20% of the sum of the vitamers).

The proportion of especially labile vitamer H_4folate ranged from 1 to 23% in the samples, and it was found to be slightly higher in the endosperm fractions than in the outermost layers in barley and oats. On the other hand, PGA was found mainly in the hull fractions of barley, oat bran and oat flour from flaking. The coarse granule fraction of barley had slightly more PGA (7%) than the other flour and granule fractions.

5.5 Enhancement of folate by microbes in aqueous processing

5.5.1 Folate production by microbes in rich medium (III, IV)

Total folate production in YPD

Selected bacteria isolated mainly from oats, selected food-grade yeasts and a few bacteria as well as reference organisms were cultivated in YPD to their exponential growth phase (klett60 =100) (only bacteria strains) and to their stationary phase (24 h). Figure 13 shows the folate levels in the cell biomasses and in the supernatants of bacterial and yeast strains after a 24-h incubation; that is, during the stationary phase. Substantial strain-to-strain differences in intracellular concentration, ranging between 2.4 and 20.7 µg/g fw, were observed. The group of poor folate producers (< 5.0 µg/g fw) included *Enterococcus durans* ON9, *B. licheniformis* ON6, *Paenibacillus* sp. ON10, *Curtobacterium* sp. ON7, *C. citreum* RB2, *Acinetobacter calcoaceticus* RB1 and most of the *Staphylococcus* species. *Micrococcus* sp. RB6, *Staphylococcus kloosii* RB7, *P. agglomerans* (ON2, ON3), *Dietzia papillomatosis* RB5, and *Exiguobacterium* sp. RB3 were moderate producers (range from 6 to 12 µg fw). *Janthinobacterium* sp., *Chryseobacterium* sp. NR7, *Bacillus subtilis* ON4 and *Pseudomonas* sp. had high folate concentrations in their cells (> 12 µg/g fw). All studied yeast strains produced quite high folate concentrations (Figure 13). *S. cerevisiae* ALKO743 and *S. cerevisiae* CBS7764 had the highest total folate levels, at 16.5 and 16.7 µg/g fw, respectively. The other four yeasts produced less than half of that, ranging from 7.0−8.1 µg/g fw.

Most of the bacteria strains were observed to increase their intracellular folate content after the exponential phase, and higher folate levels were found after a 24-h fermentation. The increase in folate concentrations from the exponential to the stationary phase varied from 1.3- to 3.7-fold. The highest increases were found for *P. agglomerans* ON3 (3.7-fold), *S. pasteuri* OW1 (3.5-fold), *P. agglomerans* ON2 (2.4-fold), *Pseudomonas* sp. ON8 (1.9-fold), and *Janthinobacterium* sp. RB4 (1.7-fold). As is noticed in Figure 14, there were strains with higher folate levels when in the exponential phase than when in the stationary phase. The folate concentration found in the cells of *P. ananatis* ON1, *Exiquobacterium* sp. RB3,

Acinetobacter calcoaceticus RB1, *Chryseobacterium* sp. NR7, and *Propionibacterium* sp. RB9 depleted after the exponential phase (i.e. after ca. 8–10 h).

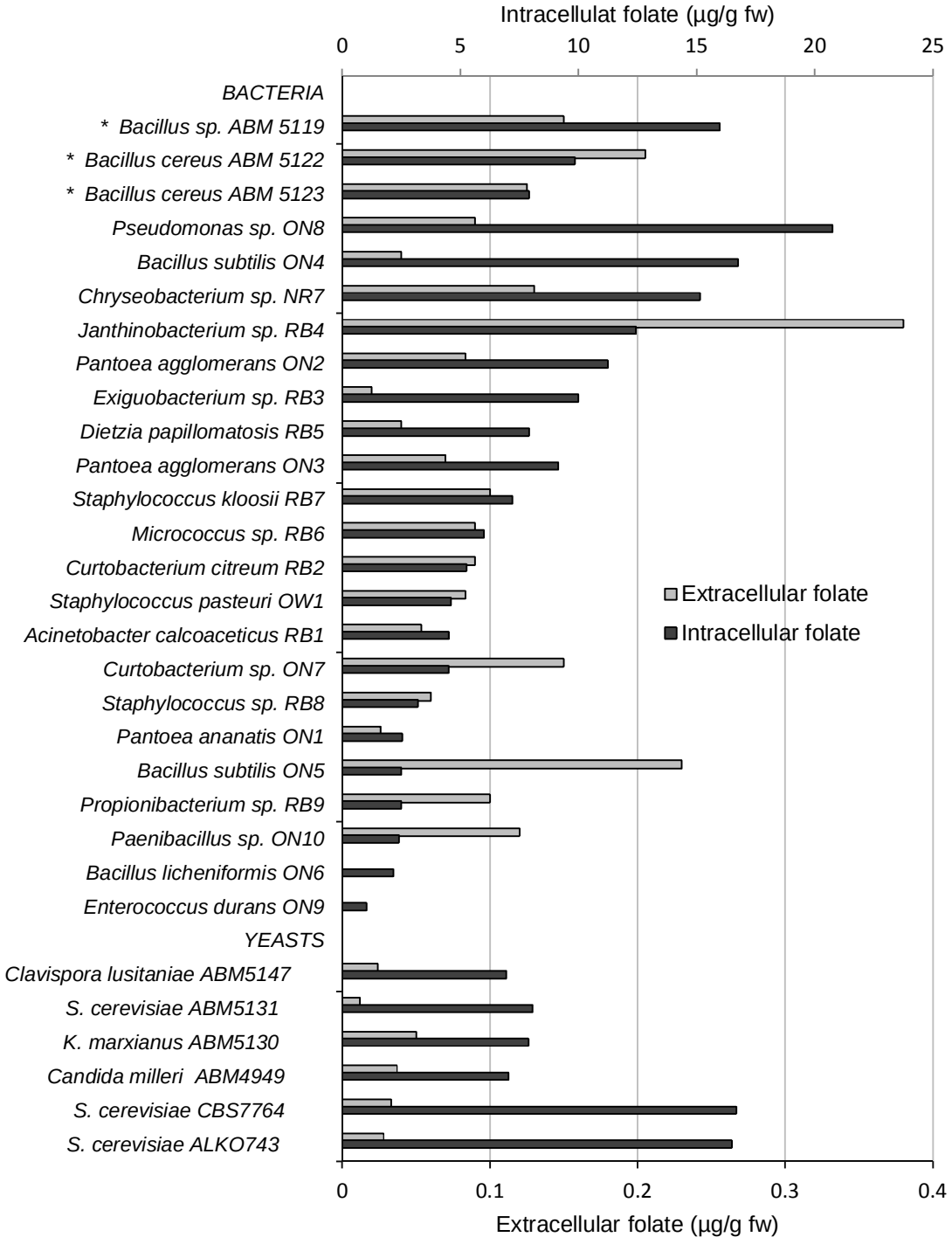


Figure 13. Average total intracellular (cell biomass) and extracellular folate concentration (µg/g fw) of bacteria, reference microbes (marked with the asterisk,*), and food-grade yeasts. Microbes were grown in YPD at 28 °C for 24 h. Total folate was determined with MA from 2 or 3 separate incubations (intracellular of bacteria), or from one separate incubation (intracellular of yeasts; extracellular of bacteria and yeasts) in duplicate.

Yeasts retained folate mainly in the cells, but some bacteria released it (Figure 13). However, folate levels in the cell biomass and in the spent medium were generally not associated. Although *B. subtilis* ON4 and *Pseudomonas* sp. ON8 had high cellular folate, their folate release into the medium was low. On the other hand, certain strains, such as *B. subtilis* ON5, *Curtobacterium* sp. ON7 and *Paenibacillus* sp. ON10, with low cellular folate, released folate into the medium (0.10–0.20 µg/g). There were, however, some exceptions. Moderate folate producers *Janthinobacterium* sp. RB4 and *S. kloosii* RB7 excreted folate, reaching concentrations of 0.1 and 0.38 µg/g in the medium.

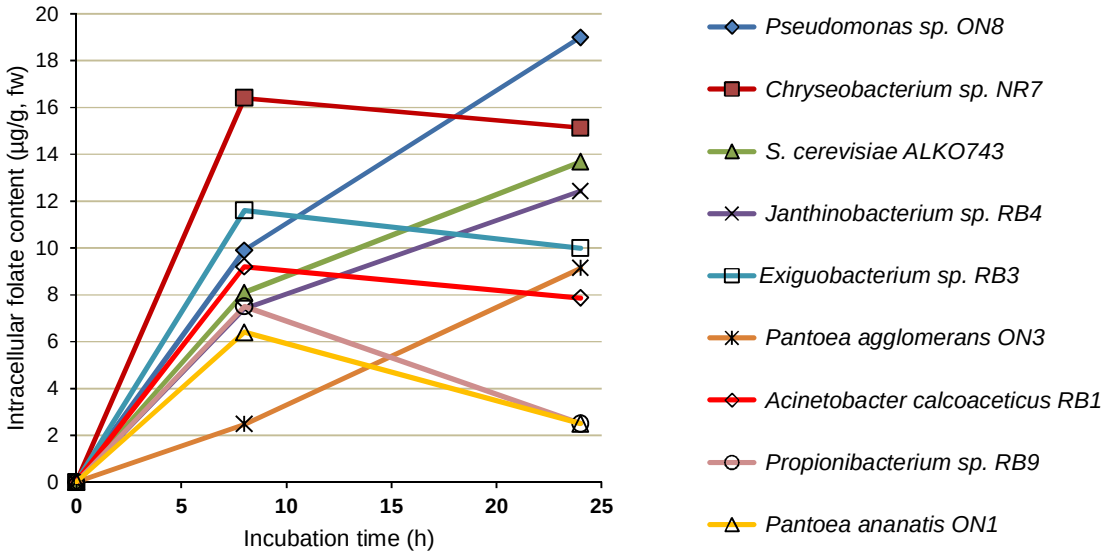


Figure 14. Intracellular folate content (µg/g fw) in selected strains at times ca. 8 h (exponential phase) and 24 h (stationary phase) in YPD incubation (Assumption: folate content at time 0 h = 0 µg/g). Total folate was determined with MA from 2 or 3 separate incubations in duplicate.

Vitamer production in YPD (III, IV)

Folate vitamers were determined in the cell masses and culture media of eight selected bacterial strains, which were proved to be good folate producers during YPD fermentation. In addition, vitamers were determined in the cell masses and culture media of six food-grade yeasts. Vitamer distribution was remarkably different between bacteria and yeast strains, and furthermore, the proportions of vitamers changed upon the transition from the exponential to the stationary growth phase in bacteria (Figure 15). The main identified vitamers in the cellular biomass were H_4folate, 5,10-CH^+-H_4folate, 5-CH_3-H_4folate and 5-HCO-H_4folate. In addition, *Exiguobacterium* sp. RB3 had a notably high level of 10-HCO-PGA.

Elevation of 5-CH_3-H_4folate and a decrease in 5-HCO-H_4folate were associated with the overall increase of the total folate content from the exponential phase to the stationary phase

in the strains *Janthinobacterium* sp. RB4, *Pseudomonas* sp. ON8 and *Bacillus* sp. ABM5119 (Figure 15). On the other hand, 5-CH_3-H_4folate was found at notably low levels in both growth phases of the strains *Exiguobacterium* sp. RB3, *P. ananatis* ON1 and *P. agglomerans* ON3 and *Chryseobacterium* sp. NR7.

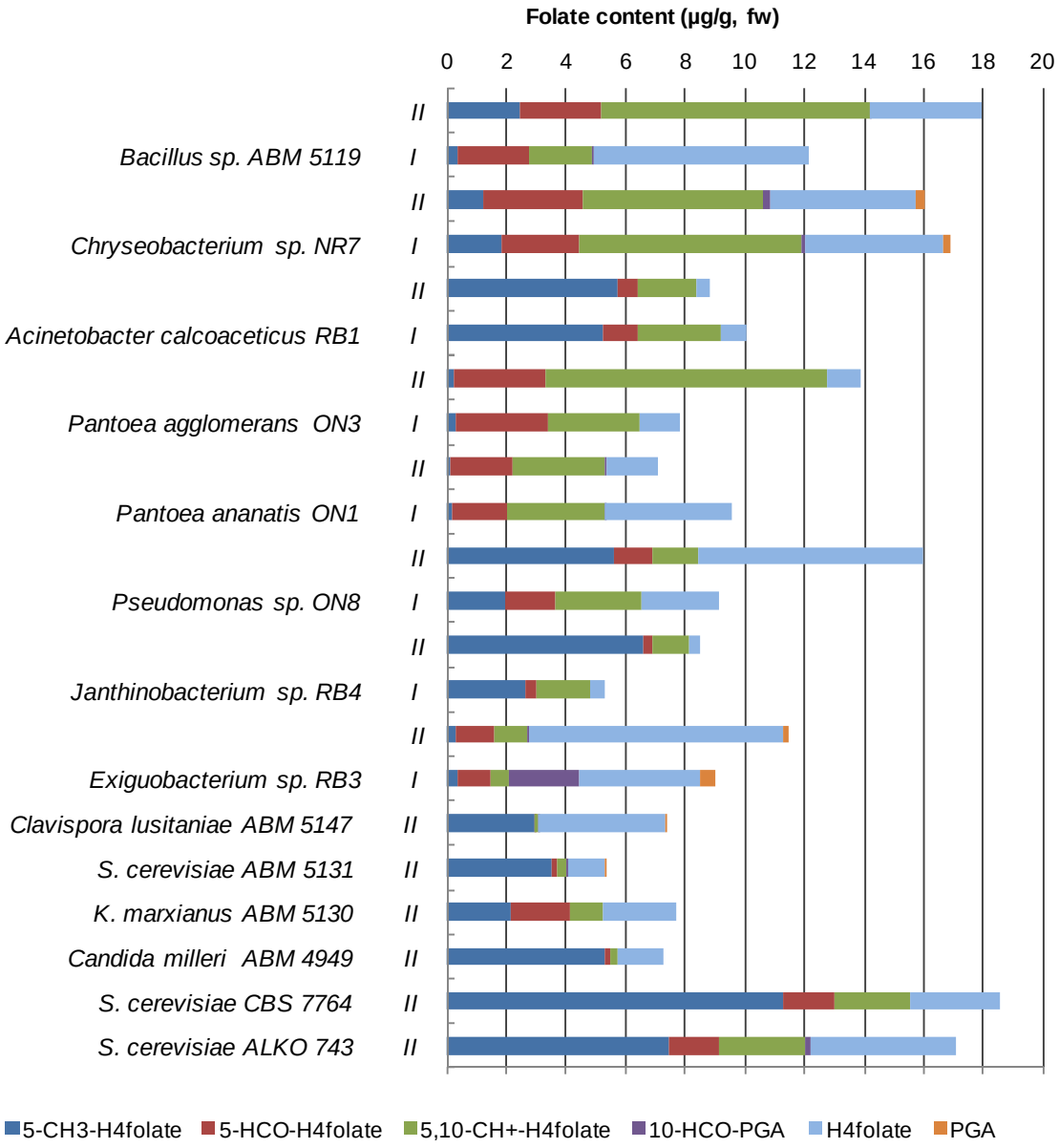


Figure 15. Folate vitamer content (µg/g fw) in the cellular biomass of the bacteria and yeast strains in the exponential growth phase (I) and in the stationary phase (II) after the 24-h incubation at 28 °C in YPD. Vitamer concentrations are from one experiment analysed in duplicate with HPLC (bacteria strains) or with UHPLC (yeast strains).

While the distribution of vitamers among bacterial strains was variable, in yeasts, 5-CH_3-H_4folate and H_4folate were the two main vitamers after a 24-h incubation. Together, they accounted for 60 to 97% of the vitamer sum. Proportions of 5-HCO-H_4folate and

5,10-CH^+-H_4folate were lower than in bacteria, accounting for ca. 10% and 17% of the vitamer sum in *S. cerevisiae* ALKO743 and *S. cerevisiae* CBS7764, respectively. In other yeasts with a lower folate content, their proportions were smaller.

Effect of pH and temperature on folate production in YPD (III)

Folate production and growth of seven bacteria strains were examined at two pH values and three temperatures in YPD medium after a 24-h incubation. In general, the studied bacteria produced the highest folate levels at temperatures of 18 °C and 28 °C (Figure 16). Intracellular folate levels ranged from 3.3 to 15.7 µg/g fw. The highest folate levels (11.1–15.7 µg/g fw) were found in *Pseudomonas* sp. ON8 in those conditions. For some strains, 37 °C and pH 5.5 was an unfavourable combination, leading to notably low intracellular folate content (< 3.0 µg/g fw) in *Exiguobacterium* sp. RB3, *Janthiobacterium* sp. RB4, *Pantoea agglomerans* ON3, *Chryseobacterium* sp. NR7 and *Bacillus* sp. ABM 5119. Cultivation at pH 7 instead of pH 5.5 improved the folate synthesis of those strains. For instance, *P. agglomerans* ON3 did not produce folate at 37 °C and pH 5.5 (0.1 µg/g fw), while at pH 7.0, the folate content in its cell biomass was 14.0 µg/g fw.

Overall, cultivation at pH 7.0 produced on average slightly higher intracellular folate content (mean 9.2 µg/g fw) compared to pH 5.5 (mean 7.5 µg/g). The growth of *Pseudomonas* sp. ON8 and *Janthinobacterium* sp. RB4 correlated with folate production with all combinations, r = 0.9546 and r = 0.9052, respectively. On the other hand, the good growth of *Pantoea agglomerans* ON3, *Pantoea ananatis* ON7 and *Bacillus* sp. ABM5119 did not predict good folate production (r = 0.4963–0.5602).

The strains *Exiguobacterium* sp. RB3, *Bacillus* sp. ABM5119 and *P. agglomerans* ON3 were observed to excrete some folate into the medium at pH 5.5 at all three temperatures (**III**: Table 2). *Janthinobacterium* sp. RB4, *P. ananatis* ON1 and *Bacillus* sp. ABM 5119 excreted folate at pH 7.0, but only at 37 °C. In addition, the high intracellular folate concentration of these three strains at pH 7.0 associated well with higher folate content in the culture media.

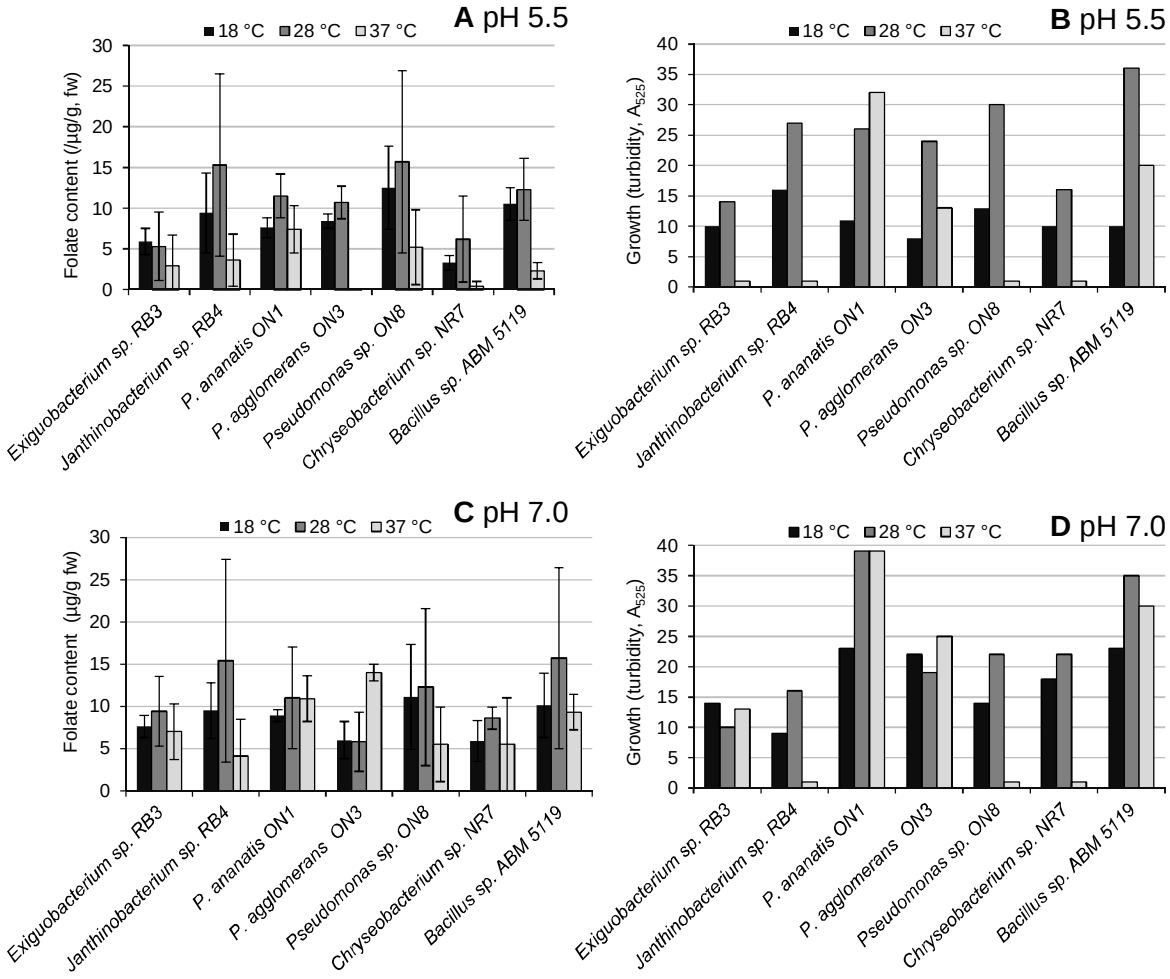


Figure 16. Total folate content in cell biomass (µg/g, fw) (A and C) and growth (OD_{595}) (B and D) in the stationary phase of bacteria strains (i.e. after a 24-h incubation) grown in MES-buffered YPD at pH 5.5 and 7.0. Folate values are averages of three independent experiments (average ± SD) analysed with MA in duplicate. OD-values are from a single experiment.

5.5.2 Folate production by microbes in oat and barley matrices (IV)

Total folate production

Yeasts and bacteria (excluding LAB) showed moderate growth in the oat and barley matrices (**IV**: Figures 2 and 3) after a 24-h fermentation. The endogenous bacterial strains from cereal grains reached maximum populations of 1.5×10^7–1.2×10^{10} CFU/ml, the yeasts, 8.6×10^6–2.9×10^8, LAB 4.6×10^4–7.0×10^8 and one *Propionibacterium* sp. at 1.1–1.9×10^9 in 24 h. Folate production, however, was not straightforwardly associated with growth.

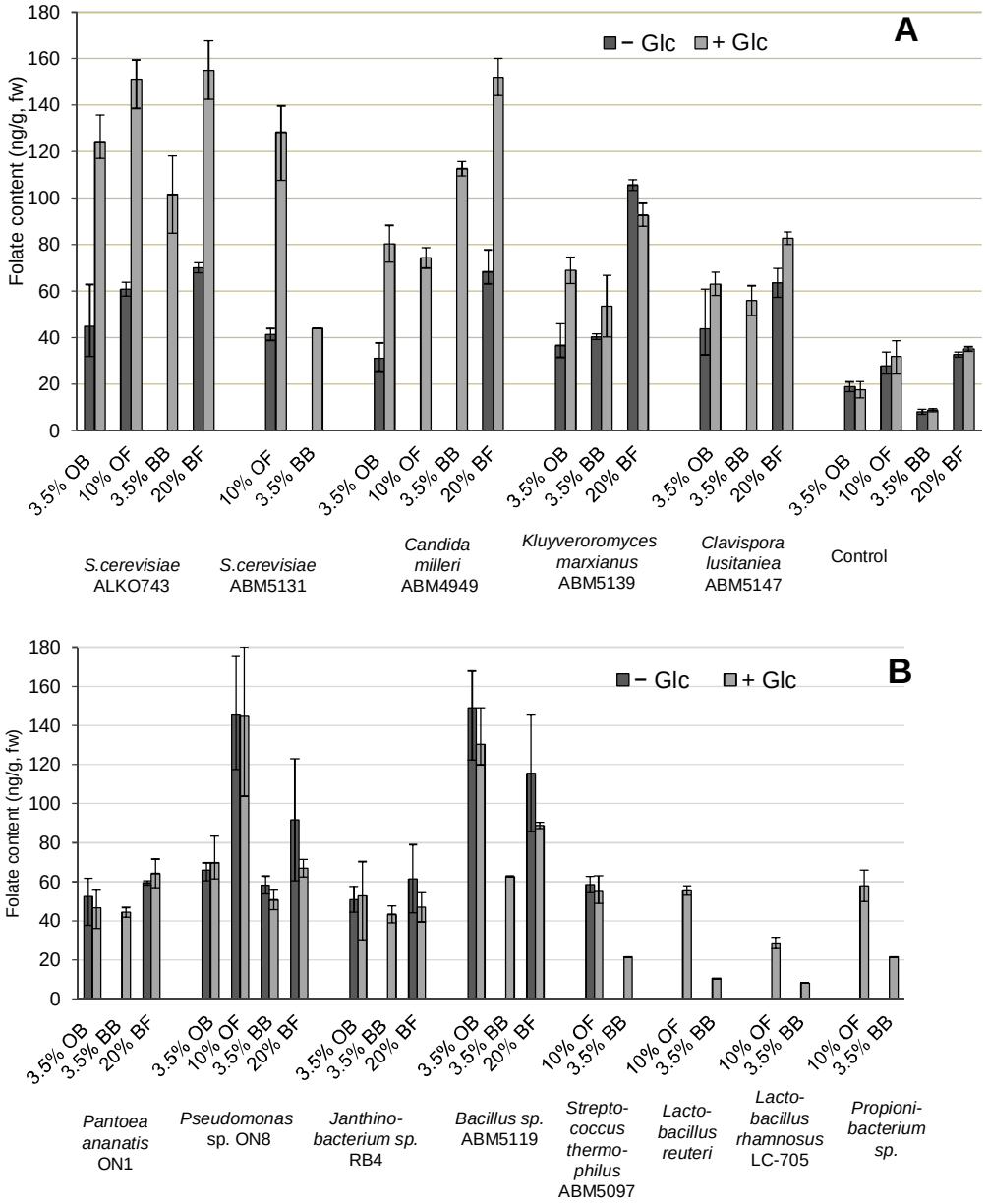


Figure 17. Average folate production by yeasts (A) and bacteria (B) in different oat and barley matrices after a 24-h fermentation at 28 °C with (+Glc) or without (–Glc) addition of 1% glucose. Controls (same for both yeasts and bacteria but shown only in fig. 17A) were uninoculated samples that were handled similarly to the inoculated samples. Error bars represent the range of 2–5 separate incubations. OB = oat bran, OF = oat flour, BB = barley bran, BF = barley flour.

Addition of glucose (1%) increased folate production by yeasts, although the effect was not that pronounced in *K. marxianus* ABM5130 and *C. lusitaniae* ABM5147 (Figure 17A). In addition, the folate production of *K. marxianus* ABM5130 and *C. lusitaniae* ABM5147 was generally weaker than for *S. cerevisiae* ALKO743 and *C. milleri* ABM4949 in all matrices with and without glucose addition. With added glucose, the folate content in the barley flour

matrix reached around 150 ng/g fw both by *S. cerevisiae* ALKO743 and *Candida milleri* ABM4949. This corresponded to a net production of approximately 120 ng/g fw.

In bacteria, glucose addition had no effect or it usually decreased the folate biosynthesis (Figure 17B). The folate production of *Pseudomonas* sp. ON8 was remarkably high, reaching a folate content of 145 ng/g fw in 10% oat flour both with and without glucose addition during the 24-h fermentation. The folate production of *P. ananatis* ON1 and *Janthinobacterium* sp. RB4 was lower, reaching approximately 50–60 ng/g fw in the barley flour matrix with and without added glucose.

The folate synthesis by bacteria and yeasts was higher in oat and barley flour matrices than in bran matrices during the 24-h fermentation (Figure 17 and Figure 18). Fermentation of the 10% oat flour matrix with 1% glucose by *Pseudomonas* sp. ON8 led to a folate content of 145 ng/g fw, while the folate content in the 3.5% oat bran matrix was only 70 ng/g fw. With glucose addition, the folate content in the 10% oat flour matrix was slightly higher (151 ng/g fw) than in the 3.5% oat bran matrix (124 ng/g fw) fermented with *S. cerevisiae* ALKO743. Respectively, *C. milleri* ABM4949 synthesised slightly more folate in the 20% barley flour matrix with 1% glucose (152 ng/g fw) than in the 3.5% barley bran matrix (113 ng/g fw). As an exception, the folate production of *Bacillus* sp. ABM5119 was high in oat bran (149 ng/g fw) with and without glucose addition.

Figure 18 shows the folate content obtained with selected microbes in barley flour and oat bran matrices during the 24-h fermentation and 2 weeks of subsequent cold storage. The highest folate content of 240 ng/g fw was observed in 20% barley flour fermented with *Pseudomonas* sp. ON8 after two weeks in cold storage. All of the studied matrices that were inoculated with *Pseudomonas* sp. ON8 contained, on average, 2-fold more folate after 2 weeks of cold storage than after a 24-h fermentation at 28 °C. *Bacillus* sp. also continued folate production in the barley flour matrix during the storage period, reaching a folate content of 127 ng/g fw. By contrast, in the 3.5% oat bran matrix its folate synthesis decreased (Figure 18). Yeast strains did not continue their folate synthesis as much as *Pseudomonas* sp. ON8 in cold storage. The folate content in yeast-inoculated matrices increased ca. 20–30% during the storage period with added glucose and by only ca. 10% without added glucose. In matrices that were fermented with *C. milleri* ABM4949, the folate concentration remained stable or slightly decreased in storage.

Folate production by LAB and *Propionibacterium* sp. was low (Figure 17B). Oat flour matrices inoculated with *S. thermophilus* ABM5097, *L. reuteri* and *Propionibacterium sp.* contained folate of around 55 ng/g fw after a 24-h fermentation. *L. rhamnosus* LC-705 did not produce any folate in the 10% oat flour and 20% barley flour matrices.

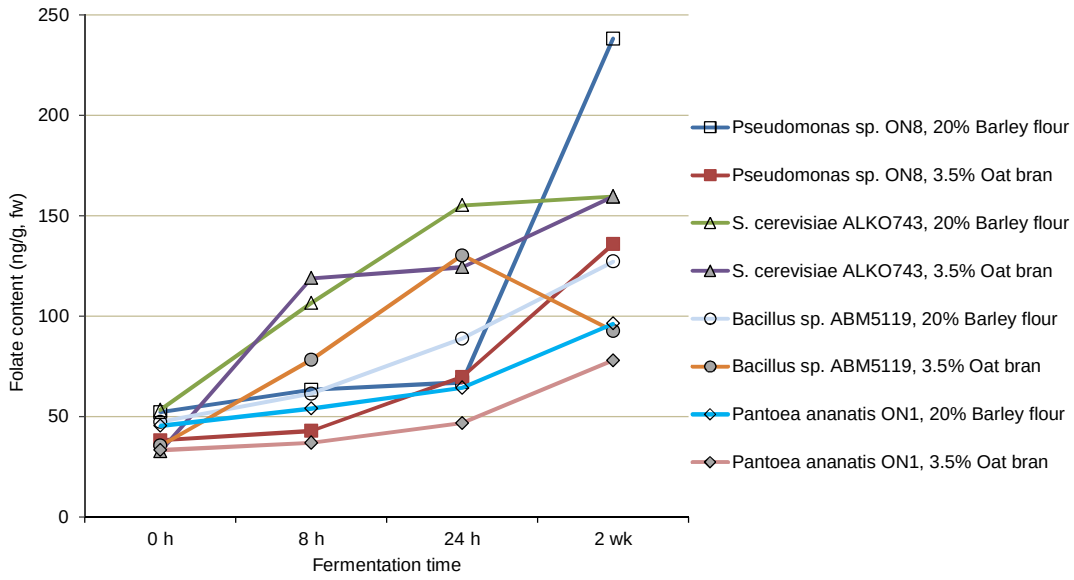


Figure 18. Total folate content during fermentation (0, 8, 24 h) and subsequent storage at 4 °C for 2 wk in 3.5% oat bran and 20% barley flour matrices inoculated with selected microbes with 1% glucose addition. The folate content is the average of 2–5 separate incubations analysed with the MA in duplicate.

Yeasts effectively consumed the added glucose, often totally during the first 24 hours (Figure 19), while bacterial strains *Pseudomonas* sp. ON8 and *P. ananatis* ON1 consumed only around 20–30% of the added glucose during cold storage. *Bacillus* sp. ABM5119 and *Janthinobacterium* sp. RB4 and the LAB (not shown in Figure 19) did not consume added glucose at all.

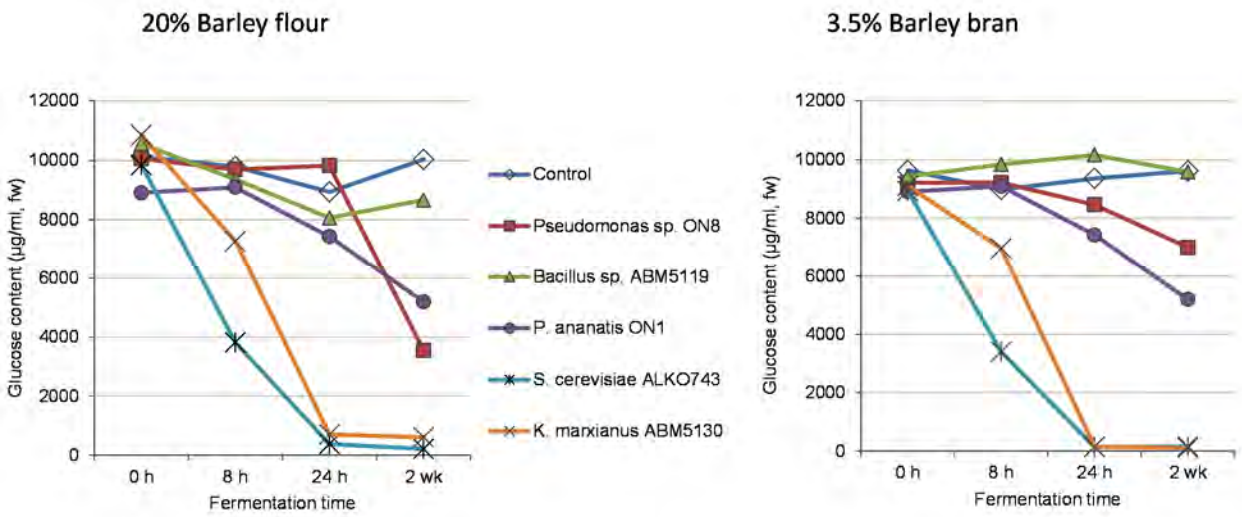


Figure 19. Glucose consumption of the selected strains during the 2-wk fermentation in 20% barley flour and 3.5% barley bran matrices with 1% glucose addition. The glucose content is the average of two separate incubations analysed in duplicate. The control is the uninoculated sample that was incubated as with the inoculated samples.

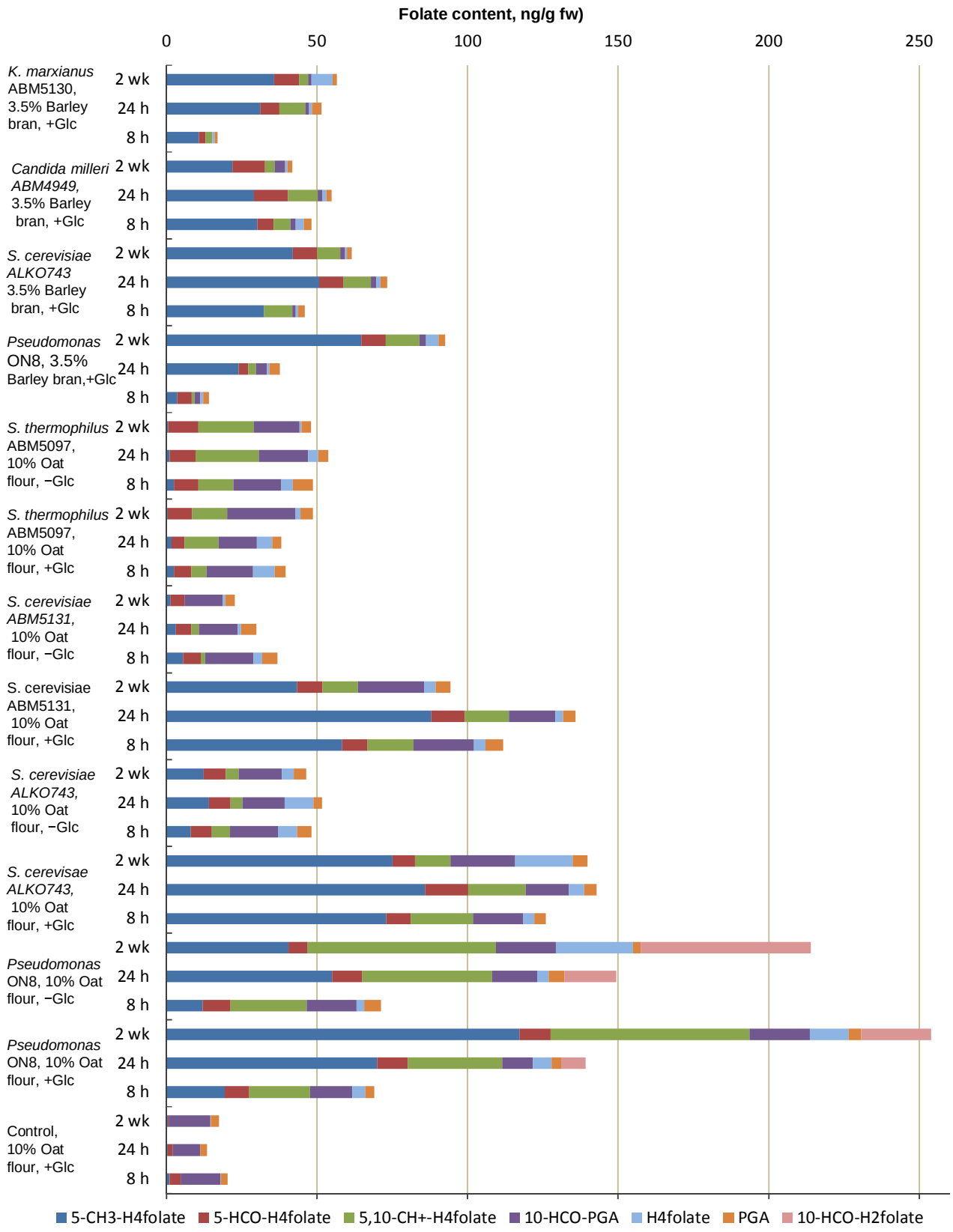


Figure 20. Vitamer distribution analysed with the UHPLC in the 10% oat flour and 3.5% barley bran matrices during fermentation at 28 °C (8, 24 h) and subsequent 2-wk storage at 4 °C. Results are from the one separate incubation analysed in duplicate. +Glc, with 1% glucose addition; −Glc, without glucose addition.

Folate vitamer production

Folate production by selected microbes at vitamer level was studied in the 3.5% barley bran and 10% oat flour matrices during the 24-h fermentation and 2 weeks of cold storage. 5-CH_3-H_4folate, 5,10-CH^+-H_4folate, H_4folate, 5-HCO-H_4folate and 10-HCO-PGA were the major vitamers in fermented matrices (Figure 20). Glucose clearly increased production of 5-CH_3-H_4folate. It accounted for over 50% of the sum of the vitamers of yeasts and *Pseudomonas sp*. ON8. In addition, synthesis of 5,10-CH^+-H_4folate by *Pseudomonas sp*. ON8 contributed significantly to the total folate content in the oat flour and barley bran matrices. H_4folate appeared at a low concentration level, on average, of < 10 ng/g fw, and its content did not increase further during the fermentation. Only in *Pseudomonas sp*. ON8 and *S. cerevisiae* ALKO743 fermented oat flour matrices (*S. cerevisiae* only with glucose) did its amount become enhanced to some extent in cold storage.

The amount of 5-HCO-H_4folate and PGA remained rather stable during the fermentation and cold storage in all samples. Interestingly, 10-HCO-H_2folate was detected in *Pseudomonas* sp. ON8 inoculated 10% oat flour samples after an 8-h fermentation and its amount increased during storage. Its proportion from the sum of the vitamers was notably higher in the oat flour matrix without glucose (26%) than with glucose (9%). Fermentation with *S. thermophilus* ABM5097 resulted in considerably low folate levels and the vitamer-distribution pattern differed from those of the other microbes. 5-CH_3-H_4folate was found only in minor amounts in oat flour matrices. In addition, the amounts of the vitamers and thus the total folate content calculated from their sum remained stable during the fermentation.

6 DISCUSSION

6.1 Evaluation of the UHPLC method for folate analysis in cereal samples

Good separation of the seven folate vitamers was achieved with the HSS T3 column at UV detector. As compared to our previous HPLC method (Kariluoto et al. 2004), the total analysis time decreased from 40 min to 12 min. This is highly beneficial when labile compounds such as folates are analysed. The LOD and the LOQ were about 100-fold lower than determined previously with our HPLC method (Kariluoto et al. 2004). The LOD values were in good agreement with those of Jastrebova et al. (2011). 10-HCO-H_2folate was easier and more confidently identified and quantified than when using the HPLC system, where it eluted very near to 5-CH_3H_4folate. The use of a PDA detector helped in the identification of the peaks via the comparison of the spectrum to that of the standards.

5-HCO-H_4folate could not be detected at the FLR detector with the validated UHPLC method, as was done in the HPLC method (Kariluoto et al. 2004). A sharp slope in the baseline at 290/356 nm excluded its accurate quantification. Thus, it had to be quantified at the PDA detector, although its LOQ was lower at the FLR detector. The slope was likely raised because of the acetonitrile gradient. Conversely, the peak of 5-HCO-H_4folate was clean and sharp enough also at UV and its identity was confirmed by the UV spectrum. The detection of 5-HCO-H_4folate has not always been self-evident in previous vitamer studies. Impurities often masked the 5-HCO-H_4folate peak, especially in cereal-based samples (Pfeiffer et al. 1997; Gujska and Kuncewicz 2005; Kariluoto et al. 2006b; Hefni et al. 2010; Jastrebova et al. 2011; Hefni and Witthöft 2012).

The recoveries of the individual vitamers were generally good, but somewhat low for H_4folate. The results were not corrected with recoveries, because the recovery tests were performed with folate monoglutamates. The majority of endogenous folates in the samples are, however, known to exist as polyglutamates. Further, endogenous folates are mostly bound to proteins, possibly giving them better stability during analysis, but restricting their extrability. Without recovery corrections the values obtained with HPLC or UHPLC, may in some cases be underestimated and should be considered rather as minimum values.

Vitamer analyses in cereal samples with the UHPLC method summarised the total folate content, which was generally well in line with the microbiological results. The folate content in cereal fractions calculated as a sum of the vitamers was closer to the MA results (on average 82%) than has typically been seen when comparing HPLC and MA. However, the HPLC method in this study gave lower content levels for the scarification samples than MA; hence, the HPLC results calculated as a sum of vitamers were 50–70% of the total folate results achieved with MA. This has been a typical difference between HPLC and MA results for cereal matrices (Konings et al. 2001; Doherty and Beecher 2003; Ginting and Arcot 2004; Kariluoto et al. 2008; Hefni et al. 2010). Generally, cereal samples are complicated matrices

and they contain several folate forms when compared, for example, to vegetable samples. There are often many interfering peaks in the chromatogram, despite the purification step. Thus, identification of vitamers is difficult and some vitamers may remain undetected, leading perhaps to underestimation.

Better uniformity of the UHPLC results with the MA results may be explained by better separation and the lower LOD of the UHPLC method than is generally achieved with HPLC. The HSS T3 column separated 5-CH_3-H_4folate and 10-HCO-H_2folate better at UV 290 nm, while they eluated near to each other in the column (Hypersil ODS column) used in our previous HPLC system. With the HPLC method, there was a risk that 10-HCO-H_2folate was masked at UV 290 nm with the diglutamate form of 5-CH_3-H_4folate, which probably originated from incomplete conjugase treatment. The diglutamate form separated better with the UHPLC, and it was summarised with the results of the monoglutamate form of 5-CH_3-H_4folate in some cereal samples. In a few previous studies, 5,10-CH^+-H_4folate has not been detected at all. Therefore, it has been potentially one reason for the lower results found by HPLC.

A small gap between the UHPLC and MA results for cereal samples indicated that unidentified folate forms may have existed in the samples. In addition, during the purification step by affinity chromatography or under the acidic conditions in UHPLC, vitamers may convert or totally decompose. In this study, for instance, we did not pay attention to *para*-aminobentzoylglutamate (*p*ABG), which is the oxidative cleavage product of folates.

On the other hand, the MA may give too high or too low results. *L. rhamnosus* is generally reported to have a similar growth response to various monoglutamates (O'Broin and Kelleher 1992). However, a difference of 15% was shown in the total folate results between 5-CH_3-H_4folate and PGA as the calibrator (Pfeiffer, 2010). Weber et al. (2011) showed that among the studied vitamers, 5,10-CH^+-H_4folate had the lowest response to *L. rhamnosus*. Further, it has been proposed that some non-folate factors in food samples could stimulate the growth of *L. rhamnosus*, leading to an overestimation of the folate content (Pfeiffer 2010).

6.2 Folate in oats and barley

6.2.1 Total folate in oat and barley cultivars

The average folate content of the barley cultivars (778, 712 and 563 ng/g) was slightly higher than those in oat cultivars (685, 570 and 488 ng/g) in each of the three harvesting years. Further, the content was lower with longer storage times. Although environmental factors cause a variation in folate levels, as has been reported for wheat (Kariluoto et al. 2010), folate loss during storage was probably the main reason for the difference among the harvesting years. Folate was determined for all samples in 2008. Hence, it is possible that folate was partly lost during storage, even though grains were stored in normal storage conditions. In

addition, the results indicate that folate loss in oat grains was faster than in barley grains. No previous reports on the stability of folate in intact grains are available. Storage of wholewheat flour and rye flour for 3 months at 20 °C and at 50% relative humidity led to total folate losses of 45% and 37%, respectively. In the flour of pseudocereals, the corresponding loss was 19–41% (Schoenlechner et al. 2010).

Optimal storage temperature and humidity are important factors for the quality of cereal grains. The metabolic activity of oat grains might be faster than in barley grains, because of their weaker water-binding capacity (Gansmann and Vorwerck 1995). In addition, grains are exposed to non-enzymatic breakdown of folate induced by heat, UV light and oxygen. Enzyme-mediated cleavage of folates could also be possible during storage. The original focus of the present study was not to investigate storage loss. However, the observations made emphasise the need to know the background of the samples for folate analysis. It is not unusual for cereal grains to be stored ca. 3 years in silos before processing and losses may take place even more rapidly in milling products than in native grains.

Similar folate losses in the cultivars of each year may be expected because of their similar storage period and conditions. A comparison of the different cultivars as folate sources is therefore possible within each harvest year. Hence, the variation in folate content was on average 1.3-fold and 1.4-fold within oat and barley cultivars, respectively, in each harvest year. The genetic variation was similar to that in 10 rye cultivars grown at the same location (Nyström et al. 2008), but smaller than that reported in 130 wheat cultivars (2-fold, grown at same location) (Piironen et al. 2008) and in 12 wheats (1.4-fold, each from three to five locations) (Arcot et al. 2002).

A comparison of the cultivars also showed that certain cultivars belonged to the folate-poorest or -richest in all years. Oat cultivar Venla and barley cultivars Jyvä and Saana were the richest in folate, while oat cultivar Peppi and barley cultivars Tocada and Minttu had the lowest content in all three harvesting years. A similar trend was observed among 10 rye varieties over two years (Kariluoto 2008) and in 130 wheats over three years (Kariluoto et al. 2010) grown in the same location.

There are only a few studies in which total folate content has been analysed without the long storage of the grains. In the diversity screen of the Healthgrain project, the folate content of oat and barley cultivars was analysed shortly after harvesting (Shewry et al. 2008; Andersson et al. 2008). In the present study, the average total folate content in the oat and barley cultivars of 2008 was 685 ng/g and 773 ng/g dm, respectively. Our values were thus slightly higher, but well in line with the reports of the Healthgrain project for five oat cultivars (mean 566 ng/g dm) (Shewry et al. 2008) and for 10 barley cultivars (mean 657 ng/g dm) (Andersson et al. 2008). Folate levels of oats and barley in this study were higher than the levels in wheats (mean 551 ng/g dm, number of cultivars = 130; Piironen et al. 2008) and close to those in rye (mean 693 ng/g dm, n = 10; Nyström et al. 2008).

Other published data on folate levels in oat and barley are limited and samples are often single and unspecified. In addition, the dissimilarity in sample preparation and methods (HPLC or MA) cause a variation in the results. The available values for oat grains are 136 ng/g dm (Schoenlechner et al. 2010) and 670 ng/g dm (Cerna and Kas 1983) and for barley 210 ng/g dm (Gujska and Kuncewics 2005), 370 ng/g dm (Han et al. 2005) and 730 ng/g fw (Hegedüs et al. 1985).

There were no correlations between folate content and available agronomical quality parameters in the oat cultivars of 2008. Shewry et al. (2008) found no association with bran yield and folate in five oat cultivars. However, a negative correlation between the average kernel size and folate was observed in wheats (Piironen et al. 2008). In the present study, a negative correlation was noticed between starch and folate in barley cultivars in 2008. Andersson et al. (2008) also showed that low folate content was associated with high starch content in ten barley cultivars. Probably, barley grains with a high starch content have low dietary fibre content (i.e. low hull content) with low levels of fibre co-passengers such as sterols and phenolics, together with folate. This was also observed in the study by Andersson and co-workers (Andersson et al. 2008).

Even though only a small number of cultivars were included in this study, the results show some variation in folate content among modern Finnish oat and barley cultivars. If the folate-richest genotypes were selected for processing, folate intake from the cereal product would probably increase at some level. Further, based on the data on folate variation among oat and barley genotypes, it might be possible to exploit plant-breeding strategies for improving folate content along with grain yield and agronomic performance. The Healthgrain project studied wheat cultivars with differences among sequences in six genes involved in the biosynthesis of folate. However, none of them showed a significant association with grain folate content, which suggests that folate enhancement by breeding may be limited (Shewry et al. 2012).

6.2.2 Total folate in oat and barley milling fractions

In the present study, oat fractions were chosen from a typical oat-milling process in which the main end products were oat bran, oat flour (endosperm flour) and flakes. Barley fractions were taken from an industrial-milling process to produce flour and barley grits. In addition, the scarification technique was chosen to give more detailed information of the folate localisation in barley grain. Each dry process yielded fractions with a high folate content. The relative folate content was up to 2–3-fold higher in comparison with native grain (Figure 21). As could be expected, and based on the earlier studies on other cereal grains, the results clearly demonstrated that folate was concentrated in the outermost layers and germ in the oat and barley grain. In addition, it was shown that some of the folate is lost during the milling processes.

Oat fractions

In oats, surprisingly, the best sources of folate were by-products such as flour from oat cutting and residual flour from flaking. In oat cutting and flaking processes, various-sized groat pieces and fines are formed. These middlings and flours were concentrated with the bran layer pieces, including the aleurone layer and germ particles. Particularly, residual flour from flaking probably contained germ particles, as indicated by its stickiness. Breakage of oat flakes is a problem in mills and it results in nonhomogenous products (Rhymer et al. 2005). The edges of the flakes and even the germ can drop off easily. This creates the residual flour from flaking, which stays in a dryer and a cooler as a by-product, the fines.

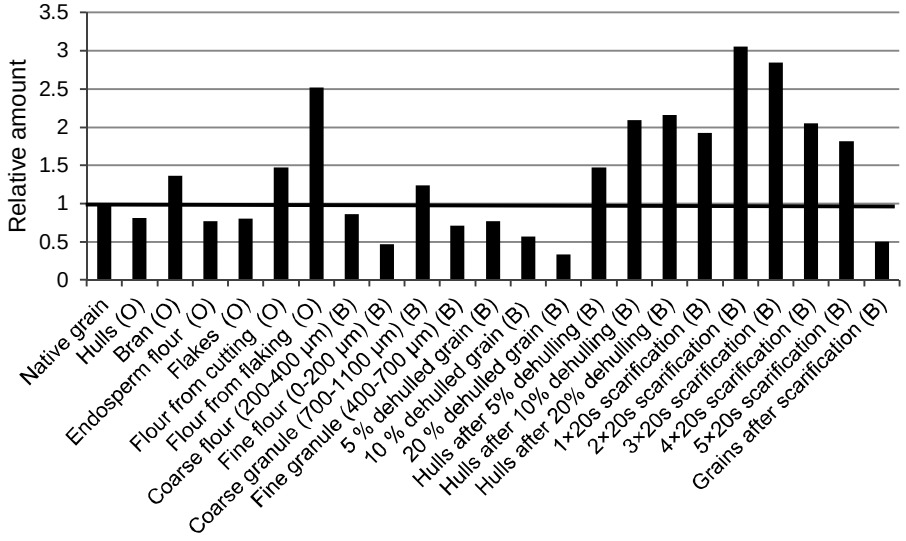


Figure 21. Comparison of total folate content (MA) of different oat (O) and barley fractions (B) to that of native grains (relative amount 1.0). Relative amounts are based on averages of two separate millings in oats, three separate industrial millings in barley, and scarifications of Minttu and Bor cultivars.

As expected, the folate level in oat bran was higher than in native grain. However, the folate content in oat bran was slightly lower than in the bran of other cereals, such as in wheat bran. In previous studies, the folate content has ranged from 704 to 1600 ng/g dm in wheat bran (Fenech et al. 1999; Arcot et al. 2002; Kamal-Eldin et al. 2009; Patring et al. 2009; Hemery et al. 2011; Piironen 2011). However, Mullin and Jui (1986) reported high amounts, of 2240–3390 ng/g (Mullin and Jui 1986), in wheat bran and, by contrast, Schoenlechner et al. (2010) reported a low amount of 186 ng/g dm in wheat bran omitting conjugase treatment during sample preparation. For oat bran, they also reported quite a low folate level of 136 ng/g dm.

Oat flakes and starchy endosperm flour (i.e. oat flour) had the lowest folate content, but still the content was around 80% of the folate in the native grain. However, the folate content in flakes could be higher if the loss into the middlings and by-products were not so high. Oat hull contained nearly as much folate as endosperm flour and flakes, but clearly less than the first milling fraction (hulls after 5% dehulling) of barley. The oat hull fraction from conventional milling always contains small amounts of the outermost layers of the grains

originating from broken groats during the dehulling, but probably not many germ pieces (Girardet and Webster 2011). This may explain the moderate folate level of the oat hull.

The oat-milling process was carried out twice. The folate content in the starting material of the first milling was nearly 2-fold compared to the second batch. In consequence, the folate content was lower in all of the fractions of the second milling. However, the relative amounts compared to native grain were quite similar in the two millings (**I**: Figure 3). As suggested earlier in the cultivar comparison, folate losses during storage may lead to a variation in folate content. Grains used in the second milling had been stored for almost one year in the grain silo following the normal storage practices of the mill. Differences in milling practice may also affect the quality of bran fractions. Oat kernel is softer than other cereal grains and has a poor separation of adhering tissues. The aleurone layer does not separate as cleanly from the endosperm in oats as it does in wheat. Thus, it is difficult to produce uniform batches of oat bran (Miller and Fulcher 2011).

Barley fractions

In this study, it was not possible to know exactly which tissues were present in the barley fractions. However, we were able to make assumptions based on previous reports on fractionation by scarification and pearling (Moreau et al. 2007a; Moreau et al. 2007b; Liu and Moreau 2008).

The folate content of the first fraction in scarification (1 × 20 s) was 2-fold and that in industrial milling (hulls after 5% dehulling) was 1.5-fold compared to that of native grain, although these fractions consisted mainly of lignocellulosic material with high mineral content (Baik et al. 2011). This composition was also confirmed by their high ash content. Folate probably originated mainly from the germ in these fractions. As the germ is located at the proximal end of the barley kernel and because fibrous hull is tightly cemented to the surface of the pericarp, tiny germ fragments were apparently removed in the first millings. This was also confirmed visually, as there were white germ particles among the fibrous hulls in both the 1 × 20 s scarification fraction and in the 5% dehulled hull fractions.

Scarification fractions 2 × 20 s and 3 × 20 s were the folate-richest, containing 2.6–3.6-fold more folate than native grain. According to Moreau et al. (2007b), the entire germ was removed following scarification for 60 s, both in hulless and hulled cultivars. Based on the study of Moreau et al. (2007b) and other previous observations on barley fractionation (Flores et al. 2007; Liu and Moreau 2008; Baik et al. 2011), the folate in these fractions probably originated mainly from the germ and partly from the aleurone.

The folate in the next fractions (4 × 20 s and 5 × 20 s) was likely to have originated in the aleurone and subaleurone layers, because most of the germ was eliminated in the previous fraction. In regard to industrial-milling fractions, it could be suggested that both the germ and most of the aleurone and subalerone layers accumulated in the hull fraction after 20% dehulling. The tissue compositions of 100-s scarificated (5 × 20 s) and 20% dehulled grains

were similar. Their almost equal folate and low ash contents also proved that the bran layer with the germ and aleurone layers was practically removed.

Flour and granule fractions imitated the production of commercially available wholegrain barley flour. These fractions were produced from the 10% dehulled grains by further milling through roller mills and sifters. In Finland, industrial mills further re-mill and re-sieve these flour and granule fractions, which were included in the present study. As a result, commercial wholegrain barley flour mainly consists of particles < 250 µm. Results obtained in this study, however, indicated that folate is lost further by resieving, because coarse flour and granules contained ca. 2-fold more folate than fine flour (0–200 µm). The coarse granule fraction might have contained more germ, pericarp and testa particles, whereas the fine flour fraction probably consisted mainly of endosperm tissue.

There is only one early study on folate content in barley milling (Hegedüs et al. 1985). In that study, pearling to an extraction rate of 81% led to folate losses of 32% compared with native grain, while in this study, folate loss after 20% dehulling was greater, at 60% (determined with MA). Hegedüs et al. (1985) also reported a folate loss of 54% in pearling to an extraction rate < 75%, which is closer to our folate loss after 20% dehulling.

Utilisation of folate-rich fractions

In the literature, the richest sources of folate are the wheat aleurone layer and wheat germ. A patented milling method separated the aleurone layer, which contained 1580 ng/g dm folate (Buri et al. 2004). Fenech (1999) reported a folate content as high as 5150 ng/g in wheat aleurone flour that also contained the germ. Hemery et al. (2011) showed that a novel electrostatic separation process for wheat produced a loss fraction that was rich in aleurone cells and contained 1190 ng/g dm folate. In addition, wheat germ contained 2400 ng/g dm folate (Piironen 2011). As the majority of folate was accumulated in the aleurone layer and germ-containing fractions in this study, it would be valuable to determine the folate content in the pure aleurone layer and germ of oats and barley. However, it would require careful and laborious hand-dissection or the development of novel dry-milling processes for oats and barley.

Due to normal milling practises in the mills, a consumer normally eats refined oats and barley products, which have lost a significant level of bioactive compounds from their outer parts (pericarp, testa, aleurone layer and germ). The present study showed that milling processes for oats and barley yielded fractions that had a higher folate content than in the available barley and oat products, such as oat flakes, oat bran and barley flour. The incorporation of folate-rich fractions or by-products into other cereal products could increase the nutritional value and benefit human health. In addition to folate, the outermost cereal layers contain numerous other bioactive components, such as phenolic acids, betaine, choline, phytosterols and minerals (Anson et al. 2012). In Western countries, consumers have favoured cereal products made of refined wheat flours over wholegrain wheat or oats, barley and rye-containing foods. The colour, flavour and taste mark more than health-promoting effects. However, the awareness of

consumers towards the benefits of high-fibre diets is increasing. Therefore, there is a need to develop palatable cereal products, which also contain high levels of bioactive compounds.

A few studies focus on barley fractions rich in beta-glucan or phenolic compounds that have been successfully incorporated into bakery products (Izydorczyk and Dexter 2008; Skrbi and Cvejanov 2011; Sullivan et al. 2011; Verardo et al. 2011). Previous studies also show that the inclusion of folate-rich fractions or middlings should be at a low or moderate enough level; otherwise, they have a detrimental effect on the quality of the product. Too high a concentration of barley flour in bread dough led to reduced baking quality, firmer dough and rapid staling. A high barley flour level decreased the volume of the bread (Sullivan et al. 2010). However, Sullivan et al. concluded, based on textural and sensory studies, that bread containing 50% barley flour or 30% of barley middlings together with wheat flour would have potential as a commercial product (Sullivan et al. 2010, 2011).

6.2.3 Distribution of vitamers in oats and barley grain

The same vitamers, 5-CH_3-H_4folate, 5-HCO-H_4folate and 5,10-CH^+-H_4folate, were detected in oat and barley fractions and native grains. However, in oat samples, 5-CH_3-H_4folate was the most abundant vitamer, whereas all barley samples contained more 5-HCO-H_4folate than 5-CH_3-H_4folate.

There are no previous reports available on the folate vitamers in oat and barley fractions from the same milling process. However, the present results were in line with previous publications, in that the vitamer profile in cereal grains varied more than in most vegetables. The predominance of 5-CH_3-H_4folate in vegetables has been shown in numerous reports and the contribution of other vitamers to total folate has been shown to be only 0–10% (Vahteristo et al. 1997; Konings et al. 2001; Wang et al. 2011). Cereal grains and other cereal samples have been shown to contain, in addition to 5-CH_3-H_4folate, remarkable amounts of formylated vitamers such as 5-HCO-H_4folate and 10-HCO-PGA (Kariluoto et al. 2004, 2006b; Gujska and Kuncewicz 2005; Piironen et al. 2008; Patring et al. 2009; Kariluoto et al. 2010; De Brouwer et al. 2010).

Equal amounts of 5-CH_3-H_4folate and 5-HCO-H_4folate have been analysed in commercial oat flakes and oat bran (Patring et al. 2009), in oat flour (Gujska and Kuncewicz 2005) and in rye flour (Kariluoto et al. 2004). 5-HCO-H_4folate was the main vitamer in winter wheat cultivars (Piironen et al. 2008; Kariluoto et al. 2010) and it was also abundant in rye cultivars (Shewry et al. 2010). Those studies observed that a higher amount of 5-CH_3-H_4folate was associated with a lower amount of 5-HCO-H_4folate. In the present study, such a relationship was not observed among the fractions. 5-HCO-H_4folate was the main vitamer in wheat grains (Gujska and Kuncewicz 2005; Patring et al. 2009) and in rye flour (Gujska et al. 2009), while more 5-CH_3-H_4folate than 5-HCO-H_4folate was found in rice (De Brouwer et al. 2010; Pfeiffer et al. 1997).

5-CH_3-H_4folate is considered to have an active role in the methylation cycle in cells. It provides the methyl group required for the transformation of homocysteine into methionine (Rébeillé et al. 2006), while the role of 5-HCO-H_4folate in cells is still somewhat unclear. 5-HCO-H_4folate is the most stable form of folate, but this molecule does not serve as a one-carbon donor. It is thought to play a role as a storage form or to have a regulatory function inhibiting some enzymes of one-carbon metabolism, such as serine hydroxymethyltransferase (SHMT). In cells, the main source of 5-HCO-H_4folate is from the hydrolysis of 5,10-CH^+-H_4folate. On the contrary, 5-HCO-H_4folate cycloligase catalyses the irreversible ATP-dependent conversion of 5-HCO-H_4folate to 5,10-CH^+-H_4folate (Roje et al. 2002; Rébeillé et al. 2006).

In some dormant organs, such as seeds and fungal spores, 5-HCO-H_4folate has been thought to act as a stable storage form of folate, which is metabolised rapidly during germination (Kruschwitz et al. 1994; Rébeillé et al. 2006). Based on the vitamer results in this study and also those from the available literature, it could be suggested that 5-HCO-H_4folate also has a storage role in cereal grains. Its proportion has been shown to be around 20–40% of the vitamer sum in cereal grains. Hence, it would be logical that during germination it is converted via 5,10-CH^+-H_4folate by 5-HCO-H_4folate cycloligase back to the C1 metabolism. Kariluoto et al. (2006a) observed that the proportion of 5-CH_3-H_4folate increased during the germination of rye grains and O'Hare et al. (2012) showed a decline in 5-HCO-H_4folate and a slight increase in 5-CH_3H_4folate during storage of *Brassica rapa* sp. (a dark green leafy vegetable).

5,10-CH^+-H_4folate was detected in almost all samples and its contribution to the sum of vitamers was moderate. It was reported also in wheat, rye, rice and wheat germ (De Brouwer et al. 2010; Kariluoto et al. 2010; Shewry et al. 2010; Ringling and Rychlik 2013). It is also possible that part of it originated from 5-HCO-H_4folate or 10-HCO-H_4folate in acidic conditions in our analysis (Figure 4) (Smith et al. 2006; De Brouwer et al. 2007; Kirch et al. 2010; Ringling and Rychlik 2013). However, its recovery from the wholegrain matrix was good and there were no extra peaks in the chromatogram at UV 360 nm. The presence of 5,10-CH^+-H_4folate is reasonable because it has an active role in the synthesis of purines. 5,10-CH_2-H_4folate converts to 5,10-CH^+-H_4folate, which further converts to 10-HCO-H_4folate. 5,10-CH^+-H_4folate is also the source for 5-HCO-H_4folate (Figure 3).

10-HCO-PGA was detected in all samples at some level and a few samples also contained PGA. These forms are not produced during biosynthesis in the plants. They are oxidation products that might arise from degradation of 10-HCO-H_4folate and H_4folate (De Brouwer et al. 2007), respectively, during sample milling and fractionation and during the analytical procedures such as heating. 10-HCO-H_4folate is necessary for the biosynthesis of purines and formyl-methionine tRNA, and hence is expected to be present in plant tissues. 10-HCO-PGA was concentrated in the oat bran fraction and endosperm-containing fine and coarse flour samples in barley. The contribution of PGA was the highest in oat bran and residual flour from oat flaking and in the hull fractions of barley.

In this study, 10-HCO-H_2folate was not detected in any of the cereal samples. It is an oxidation product of 10-HCO-H_4folate and may oxidise further to 10-HCO-PGA. Its HPLC analysis has led to confusing results. It has been determined in some rye flour and bread samples and wholegrain wheat (Pfeiffer et al. 1997; et al. 2001; Piironen et al. 2008; Gujska et al. 2009; Kariluoto et al. 2010). Patring et al. (2009) speculated that 10-HCO-H_2folate was reported in those studies that used affinity chromatography for purification before HPLC. A recent study with LC-MS/MS and without affinity chromatography purification, however, reported that its proportion in cauliflower and carrot was 2–3% and in carrot greens, it was 17% (Wang et al. 2011). In addition, a small amount of 10-HCO-H_2folate was detected in some vegetables and bread samples using isotopically labelled folate as an internal standard and an LC-MS/MS method for detection (Ringling and Rychlik 2013). Baggot and Tamura (2010) postulated that 10-HCO-H_2folate has a metabolic role as a substrate for an enzyme involved in purine biosynthesis.

All folate forms are converted mostly to 5-CH3-H_4folate and hydrolysed to monoglutamates in the intestinal mucosa prior to release into the bloodstream (Bailey and Caudill 2012). Therefore, the total folate level is more important for human nutrition than levels of single vitamers. On the other hand, labile folate forms, such as H_4folate, which degrade easily, losing their vitamin activity, are not desirable regarding folate enhancement.

6.3 Enhancement of folate by microbes in aqueous processing

6.3.1 Total folate production by microbes in rich medium

Cultivation in YPD

The studies **III** and **IV** elucidated the differences in the folate-production ability of different microbe species and strains. Substantial differences in the folate levels in the cell mass of bacteria and yeast strains were observed by culturing them in YPD at 28 °C. In addition, it was shown that some endogenous bacteria from cereal grains produce as much or even higher amounts of folate than yeasts.

The best folate producers among the studied bacterial strains achieved similar folate concentrations to yeast strains. During a 24-h incubation, *Pseudomonas* sp. ON8 had the highest cellular folate content (21 µg/g fw). Folate production of the best yeasts (*S. cerevisiae* ALKO743 and *S. cerevisiae* CBS7764) were at the same level, at around 10–16 µg/g fw, as folate production by *B. subtilis* ON4, *Chryseobacterium* sp. RB4, *Janthinobacterium* sp. RB4, *P. agglomerans* ON2 and *Exiguobacterium* sp. RB3.

Folate production has mainly been studied for LAB and bifidobacteria. Recently, differences in their folate-production ability have been possible to be explained based on their genome. The published studies showed that *S. thermophilus* (Lin and Young 2000; Smid et al. 2001;

Sybesma et al. 2003, Crittenden et al. 2003; Sanna et al. 2005; Kariluoto et al. 2006a; Herranen et al. 2010) and *L. lactis* (Sybesma et al. 2003; Ayad 2007; Nor et al. 2010; Gangadharan et al. 2010; Herranen et al. 2010) and bifidobacterium *B. adolescentis* (Pompei et al. 2007a; D'Aimmo et al. 2012; Padalino et al. 2012) are good folate producers. These species possess all the genes for *de novo* biosynthesis of the folate precursors, DHPPP and *p*ABA (Rossi et al. 2011). However, there are LAB and bifidobacterial species that harbour all the genes for biosynthesis of DHPPP but lack the gene for *p*ABA synthesis. These species are able to produce folate only in the presence of *p*ABA (Rossi et al. 2011). Many LAB and bifidobacteria lack all of the genes encoding DHPPP and *p*ABA synthesis and remain incapable of folate production even in the presence of *p*ABA supplementation (Rossi et al. 2011).

The promising folate results achieved in YPD culturing of certain endogenous bacterial strains indicate that those strains harbour all of the genes for folate biosynthesis, or at least, possess the genes and enzymes of the DHPPP *de novo* biosynthetic pathway. In the latter case, the concentration of *p*ABA in YPD was enough for folate production. On the other hand, some tested strains, such as *B. licheniformis* ON6, consumed folate from the culture media. It may lack the genes for the biosynthesis of folate precursors and is therefore auxotrophic for folate.

Folate production by yeasts has also been studied to some extent in synthetic media. Based on our results and on previous reports, yeasts are able to produce folate and there is also a variation in production between yeast species. Hjortmo et al. (2005) showed that folate content in the cell masses of yeast species from different sources varied from 40 to 145 µg/g dm (8–29 µg/g fw, if an estimated dm content of 20% was used). Kariluoto et al. (2006a) reported a folate content of 3.1–8.5 µg/g fw in cell masses in four yeasts. Our results show that the folate production of *S. cerevisiae* was the highest compared with the other yeast species. This is in accordance with the studies of Hjortmo et al. (2005) and Kariluoto et al. (2006a).

Although some microbe species have been recognised as folate producers or consumers according to the literature, the different strains may differ in terms of folate production. This was also shown in this study. For instance, the folate content in the cell biomass varied among *Bacillus* sp. and among *S. cerevisiae* strains. Variation in folate production has also been shown between the strains of *B. adolescentis* (Pompei et al. 2007a), *Propionibacterium freudenreichii* (Hugenschmidt et al. 2011; Van Wyk et al. 2011), *S. thermophilus* (Sanna et al. 2005) and *L. lactis* (Ayad 2007). The extent of folate production was also influenced by the culture conditions, such as pH, the growth rate and the presence of *p*ABA. Culture conditions may also have an influence on the number of glutamyl residues and on the vitamer composition (Rossi et al. 2011).

Effect of pH and temperature

The studied endogenous bacteria produced more folate at 28 °C than at 18 °C or 37 °C. The effect of temperature on the folate production could be largely explained by the growth of the

bacteria. Cultivation at 37 °C was especially detrimental for growth and folate biosynthesis of psychotrophic *Janthinobacterium* sp. RB4, *Pseudomonas* sp. ON8 and C*hryseobacterium* sp. NR7 (Männistö and Häggblom 2006). On the other hand, folate synthesis of LAB, bifidobacteria and PAB was higher at 37 °C, which is generally a more optimum temperature for their growth (Holasova et al. 2005; Tomar et al. 2009; Gangadharan et al. 2011; Padalino et al. 2012).

The folate content was also higher when the strains were cultivated at pH 7.0 compared to pH 5.5. This is in good agreement with previous findings that the production of folate by *L. lactis* and *S. thermophilus* increased with increasing pH (Sybesma et al. 2003). Sybesma et al. suggested that delaying the acidification of the medium by controlling the pH is beneficial for the synthesis, because the pH optima of enzymes involved in folate biosynthesis are between 7.3 and 9.3.

In this study, better growth at a certain temperature and pH was not always associated with better folate production. However, the increased growth of *S. thermophilus* (Iyer et al. 2010) and different yeast strains (Pietercelie et al. 2011) was accompanied with elevated folate content. Thus, if the basic cultivation conditions (culture medium, pH, temperature, oxygen) are optimal for the microbe and the microbe has the genes for folate biosynthesis, it would be possible to achieve high levels of folate production.

Extracellular folate levels were not clearly associated with pH and temperature in this study (**III**: Table 2). Rather low folate content in the culture media makes it difficult to draw conclusions. In some cases, autolysis of the cells may have contributed to the analysed extracellular folate. However, the strains *Exiguobacterium* sp. RB3, *Bacillus* sp. ABM5119 and *P. agglomerans* ON3 were observed to excrete a low amount of folate to the medium at pH 5.5 at all three temperatures, as the folate level in the medium was higher than that of the initial level in the YPD medium. Further, *Janthinobacterium* sp. RB4, *P. ananatis* ON1 and *Bacillus* sp. ABM5119 excreted folate at pH 7.0 but only at 37 °C. This higher folate content in their culture media was associated with their high average intracellular folate concentration at pH 7.0.

S. thermophilus has been proved to release folate into the medium more than many other LAB (Sybesma et al. 2003; Herranen et al. 2010; Laiño et al. 2012). Hugenschmidt et al. (2010) showed that LAB excreted more folate into the medium than PAB strains. In addition, some good folate producers of bifidobacterial strains released notable amounts of folate into the medium (Deguchi et al. 1985; Crittenden et al. 2007; Pompei et al. 2007a). Sybesma et al. (2003) explained the extracellular folate production by the length of the glutamyl tail. The longer glutamyl chain probably improves folate retention in the cell because it may have more negative charges in its carboxyl groups depending on the pH. In addition, Sybesma et al. observed that the excretion of folate was dependent on the pH. Cells of *S. thermophilus* grown at low pH had a higher extracellular folate fraction than cells that were cultured at a high pH. Their explanation was based on the assumption that when the extracellular pH was decreased,

the intracellular pH also decreased and, consequently, the folate was protonated and electrically neutral. Hence, transport across the membrane was enhanced.

6.3.2 Total folate production by microbes in oat and barley matrices

After studying the folate production by selected endogenous bacteria in YPD cultivation (**III**), the most promising folate producers with a limited hydrolytic activity on beta-glucan were selected for further studies in fibre-rich oat- and barley-based cereal matrices. Folate production of the bacteria was compared to that of food-origin yeasts and well-known LAB.

Folate production was similar and moderate in yeasts and cereal-associated bacteria fermentations without the addition of glucose. The addition of glucose clearly increased folate content in matrices fermented with *S. cerevisiae* ALKO743 and ABM5131, *C. milleri* ABM4949 and to some extent in *K. marxianus* ABM5130 and *C. lusitaniae* ABM5147 fermented matrices during the 24-h fermentations. Yeasts such as *S. cerevisiae* and *C. milleri* effectively consumed the added glucose during the first 24 h of metabolism and used it for their growth and ethanol fermentation. *K. marxianus* ABM5130 and *C. lusitaniae* ABM5147 are less fermentative yeasts (Kurtzmann et al. 2011). They also consumed added glucose, but consumption was not associated with folate production. Especially, *C. lusitaniae* ABM5147 is a more aerobic yeast that uses sugars only for growth. It was also observed to grow well in this study. Glucose was consumed by yeasts during the 24-h fermentation and their growth and folate production was minimal during the 2 weeks of cold storage. Oat and barley matrices also contained a small amount of initial glucose due to inoculation with YPD medium, but it was not enough for good growth of the yeasts. In the matrices that were fermented with bacteria strains, the folate content was quite similar with and without glucose addition. The initial glucose in the matrices as a carbon source was probably sufficient for their growth (**IV**: Figure 3).

More folate was synthesised by bacteria and yeasts in oat and barley flour matrices than in bran matrices with and without glucose addition. As the proportion of brans was much lower (3.5% w/v) compared to whole flour (10% or 20%) in water, the flour matrices were obviously richer in starch and amino acids than the bran matrices, even though this study used ethanol-washed oat bran, which itself was richer in proteins (21%) than endosperm flours (14%). The studied microbes did not exhibit beta-glucanase activity, hence they did not utilise the high beta-glucan content of brans as a carbon source. The outer layers of barley grain are a poor source of amino acids and starch compared to endosperm-containing wholegrain flour (Sullivan et al. 2010). Kedia et al. (2008) showed that *L. plantarum* grew better in a white oat flour–water matrix than in an oat bran–water matrix. A barley malt–water matrix was a better medium than a wholegrain barley flour–water matrix for the growth of *L. plantarum* and *L. acidophilus* (Rathore et al. 2012).

Previously, the folate-synthesis capability of yeasts has been shown in the fermentation of a maize flour–water matrix (46 h at 30 °C). The highest folate concentrations were found in

matrices fermented with *C. glabrata* TY26 (70 ng/g fw) and with *S. cerevisiae* TY08 (40 ng/g) (Hjortmo et al. 2008b). These folate levels were close to the levels found in this study without glucose addition. Further, Kariluoto et al. (2006a) found similar folate production in the fermentation of sterilised rye flour–water mixtures for 19 h at 30 °C: approximately 160–170 ng/g fw for *C. milleri* CBS8195 and *S. cerevisiae* ALKO743, and 90 ng/g fw for *S. cerevisiae* TS146 and *Torulaspora delbrueckii* TS207.

Folate production by LAB and *Propionibacterium* sp. was poor or insignificant in cereal matrices as compared to folate levels achieved using endogenous bacteria and yeasts. *L. rhamnosus* LC-705 consumed minor amounts of folate, instead of synthesising it. This finding was in agreement with the earlier studies (Sybesma et al. 2003; Crittenden et al. 2003; Hugenschmidt et al. 2010). The inability of *L. rhamnosus* LC-705 in terms of folate production can be explained by its genome. It lacks the genes and enzymes for the *de novo* biosynthesis of the folate precursors DHPPP and *p*ABA and is thus strictly auxotrophic for folate (Rossi et al. 2011).

S. thermophilus harbours the genes for the pathways of folate biosynthesis (Rossi et al. 2011). Its folate-production capacity was also shown in this study in the 3.5% barley bran and 10% oat flour matrices with a net production of 10–30 ng/g fw. This production was in accordance with previous studies, in which its net folate content varied from 17 to 60 ng/ml fw (Lin and Young, 2000; Crittenden et al. 2003; Sanna et al. 2005; Tomar et al. 2009). Iyer et al. (2010) reported optimised folate production of 60 ng/ml. Higher values, from 12 to 142 ng/ml (Smid et al. 2001; 34 strains) and from 29 to 202 ng/ml (Sybesma et al. 2003), have also been reported.

After 2 weeks in cold storage, the net production for the studied *Propionibacterium* sp. was at the same level (40 ng/g fw) as that of *S. thermophilus* ON8 and *L. reuteri* in the 10% oat matrix. This agrees with the screening results by Hugenholtz et al. (2002), where total folate production among probionibacteria varied from 9 to 78 ng/g fw in a milk medium. With *p*ABA supplementation into a whey-based medium, extracellular production of *P. freudenreichii* was 25 ng/ml (Hugenschmidt et al. 2011).

In the present study, we screened bacteria and yeasts for their folate production under the same conditions. The main focus was on studying the potential folate synthesis of endogenous, cereal-associated bacteria and food-grade yeasts. Hence, the cultivation conditions employed were not optimal for the growth of food-grade LAB and *Propionibacterium* sp. For instance, the cereal matrices did not contain any lactose and lactate, although LAB and PAB, respectively, prefer them as a carbon source (Piveteau 1999). Further, the cultivation temperature of 28 °C was not optimal for the growth of LAB. In addition, many yeasts generally grow better at higher temperatures (Holasova et al. 2005; Tomar et al. 2009; Gangadharan et al. 2012). In addition, metabolites such as lactic acid might have increased the acidity of the matrices, leading to decreased folate production.

The highest folate concentrations in the studied matrices were achieved by fermentation with *Pseudomonas* sp. ON8. It even continued its growth together with folate production during 2 weeks in cold storage. From the level reached in 24 h, the folate content still increased from 45% to 250% depending on the matrix. After 2 weeks of fermentation with the *Pseudomonas* sp. ON8, the folate level was, on average, 300 ng/g fw in the 10% oat flour matrix. This corresponds to around a 9-fold increase in folate concentration compared to the unfermented oat matrix. We can estimate that by consuming a portion of 200 g of this type of fermented product a day, the folate intake would be 60 µg, which is around 20% of the recommended daily intake (300 µg) (NNR 2012).

In conclusion, in this study, we showed that endogenous bacteria are potential folate producers in cereal matrices not affecting the beta-glucan structure. Further, the studied food-grade yeasts, *S. thermophilus* and *Propionibacterium* sp., were also noted as potential microbes for natural folate enhancement in the aqueous processing of cereals. Through the comprehensive optimisation of the production conditions in beta-glucan-rich matrix food-good folate producers, it is possible to achieve still higher folate concentrations.

6.3.3 Folate vitamer production by microbes

Production in a rich medium

The proportion of vitamers was remarkably different between bacteria and yeasts and also among bacteria strains. The proportions of vitamers varied extensively depending on the growth state of bacteria. To our knowledge, folate produced by the endogenous bacteria of cereals has not yet been studied at the vitamer level, but a few reports on vitamer production by LAB, bifidobacteria and yeasts are available.

It is difficult to find any clear uniformity when the production of folate vitamers by bacteria was compared between two growth phases. If the total folate content was at the same level during both the exponential phase and the stationary phase (*P. agglomerans* ON3), the amount of 5-CH_3-H_4folate remained low. On the other hand, the accumulation of 5-CH_3-H_4folate increased along with the rise in total folate content (*Pseudomonas* sp. ON8 and *P. ananatis* ON1). Accumulation of H_4folate (*Exiquobacterium* sp. RB3) or 5,10-CH^+-H_4folate (*Janthinobacterium* sp. RB4, *P. agglomerans* sp. ON3 and *Bacillus* sp. ON1) seemed to be associated with an increased total folate content. Thus, the production of 5-CH_3-H_4folate, H_4folate and 5,10-CH^+-H_4folate affected the total folate content the most and 5-HCO-H_4folate and 10-HCO-PGA had a smaller role among the vitamers. The production of 5-HCO-H_4folate and 10-HCO-PGA by yeasts was also low in the stationary phase. Yeast strains mainly accumulated 5-CH_3-H_4folate, and *S. cerevisiae* ALKO743 and *S. cerevisiae* CBS7764 also produced a marked amount of H_4folate.

It was likely that bacteria and yeasts were at different growth states at the moment of the expected stationary phase; that is, 24 h. Hjortmo et al. (2008a) suggested that the physiological state of the cells clearly affects the folate content and also the vitamer

distribution. She and her co-workers examined the content of 5-CH_3-H_4folate and H_4folate produced by *S. cerevisiae*. During the respiro-fermentative phase, the amount of 5-CH_3-H_4folate increased rapidly and it dominated compared to H_4folate (ca. 5:1). After around 10 h of fermentation, during the stationary phase when glucose was depleted, the content of 5-CH_3-H_4folate and H_4folate decreased. During the respiro-fermentative phase, the requirements for nucleotides, methionine and other amino acids is high for the growth in the cells. When the cell number did not increase any more, the requirements for folate decreased (Hjortmo et al. 2008a).

Production in cereal matrices

In fermented cereal matrices, the same vitamers as in YPD cultivation were observed. However, vitamer composition in the YPD medium did not predict vitamer production in the cereal matrices. Contrary to YPD cultivation, an increase or decrease in 5-CH_3-H_4folate affected the total folate content the most in bacteria- and yeast-fermented oat and barley matrices. Furthermore, this observation strengthened with glucose addition, especially in yeasts. The only exception was *S. thermophilus* ABM5097, which produced folate only in low amounts. The results indicate that if folate synthesis is low, the microbes do not accumulate 5-CH_3-H_4folate.

10-HCO-H_2folate was detected only in the 10% oat flour matrix fermented with *Pseudomonas* sp. ON8 for 24 h and its amount still increased during cold storage. The identity of 10-HCO-H_2folate was confirmed by comparing its spectrum in the sample to the spectrum of its pure calibrant. 10-HCO-H_2folate has previously not been detected in microbe fermentations. It is an oxidation form of 10-HCO-H_4folate and may have been converted from it, especially during the long cold storage. In this study, the proportion of 5,10-CH^+-H_4folate was also notable among vitamers produced by microbes. Sybesma et al. (2003) showed that 5,10-CH^+-H_4folate together with 5-HCO-H_4folate were the main vitamers produced by *S. thermophilus*. As 5,10-CH^+-H_4folate has a role in purine synthesis as a precursor for 10-HCO-H_4folate, it is obviously present in microbe cells. As suggested earlier in this study, some of the 5,10-CH^+-H_4folate, however, might originate from 10-HCO-H_4folate or 5-HCO-H_4folate, because they may convert to 5,10-CH^+-H_4folate at low pH (De Brouwer et al. 2007).

The proportion of 5-HCO-H_4folate was low and was produced by all of the studied microbes both in YPD cultivation and in fermented cereal matrices. Its proportion was much lower than in cereal grains and its level remained similar during the fermentation. In section 6.2.3, it was concluded that in cereal grains and in seeds, 5-HCO-H_4folate potentially acts as a storage form (Kruschwitz et al. 1994; Rébeillé et al. 2006). Thus, when the microbe is in an active growth state, it mainly synthesises the active folate forms for purine, nucleotide and methionine synthesis, and does not store folate.

Based on the previous literature, the most studied vitamers in microbe fermentation have been 5-CH_3-H_4folate, H_4folate and 5-HCO-H_4folate. LAB strains (*L. delbrueckii*, *L. plantarum* and *S. thermophilus*) and bifidobacteria strains (*B. adolescentis* and *B. catenulatum*) accumulated

more 5-CH_3-H_4folate than H_4folate after a 10-h incubation in milk culture medium (Padalino et al. 2012). 5-CH_3-H_4folate was accumulated the most by *S. thermophilus*, *B. longum* and *L. bulgaricus*, while *L. acidophilus* strains accumulated mainly H_4folate after a 6-h incubation at 37 °C in milk medium (Lin and Young 2000). When the determined vitamers in LAB and bifidobacteria cultivations were 5-CH_3-H_4folate, H_4folate and 5-HCO-H_4folate, the most abundant vitamer was 5-CH_3-H_4folate or H_4folate followed by 5-HCO-H_4folate (Lin and Young 2000; Sanna et al. 2005).

Based on the results in this study, microbes produced different folate vitamers. The most fundamental vitamers were 5-CH_3-H_4folate, H_4folate and 5,10-CH^+-H_4folate from the point of view of total folate production. These are the same vitamers that have an active role in folate metabolism. In cereal fermentations, especially the production of 5-CH_3-H_4folate affected the total folate concentration. In addition, glucose addition increased the production of 5-CH_3-H_4folate in yeasts. It was also noticed that the growth state affected the produced vitamer profile from bacteria. The composition of the growth medium also influenced vitamer distribution; hence, production in the YPD medium did not predict vitamer production in the cereal matrices.

7 CONCLUSIONS AND FUTURE RESEARCH PERSPECTIVES

In this thesis, the total folate content in oat and barley grains and their dry-milling fractions was studied. In addition, the occurrence of different folate forms in the fractions were investigated with the HPLC method and the UHPLC method, which was first validated in this study. Microbes have a potential for folate production. Total folate production by selected microbes and food-grade yeasts was first tested in rich medium and finally in aqueous oat and barley matrices. Further, the distribution and significance of folate vitamers was studied.

The validated UHPLC method allowed for the markedly faster separation of folate forms than the previously used HPLC methods, which is a particular advantage in analysing labile folate vitamers. The validation data demonstrated that UHPLC offered excellent repeatability and linearity for analysing endogenous folate in cereal grains and cereal products. When folate analysis in the same sample extract was carried out with the MA and with the UHPLC method, the total folate content from the sum of the vitamers matched the results achieved with the MA, with 82% in cereal matrices and 98% in microbe biomasses. The gap between the UHPLC and MA total folate results was smaller than generally has been between HPLC and MA. Furthermore, the validated UHPLC method permitted the determination of seven different folate vitamers produced by microbes in the cultivation medium as well as in a challenging cereal matrix.

New data was obtained on the folate content and its variation in oat and barley cultivars. This study proved that oats and barley are good sources of folate. The total folate content in barley was slightly higher, at 770 ng/g dm, than the folate content in oats, at 690 ng/g dm, determined shortly after harvest. There was a moderate variation in the folate content of the cultivars, which could be utilised in plant breeding for developing folate content in oat and barley cultivars. In this study, we also observed that oat and barley grains lose folate during storage. Therefore, folate losses and stability should be studied further.

Dry-fractionation of oats and barley yielded fractions with high folate content. The folate concentrations differed markedly among the conventional oat-milling samples as well as among the scarification and industrial pearling fractions of barley. Oat milling produced folate-rich by-products, which are normally discarded. The folate content in the residual flour from flaking was 2.5-fold higher than in native oat grain. Fractionation of barley indicated that almost half of the folate in native grain is normally lost in industrial dehulling and pearling processes. The folate content in 20% pearled hull fractions was 2-fold compared with the intact grain.

The folate content in the barley fractions correlated with their ash content. The total folate content of the scarification and industrial-milling fractions demonstrated that folate was localised in the outer layers and germ in barley grains. In oats, the high folate content in the bran, by-products and middlings confirmed that folate is concentrated in the bran layer and germ. More detailed knowledge regarding the localisation of folate in these fractions would require careful hand-dissection or development of novel milling techniques. Findings in this

thesis support the use of wholegrain and bran-containing oat and barley products for increasing natural folate intake. The possibility of introducing folate-rich fractions of oat and barley into baking should be studied more.

This thesis showed that a few endogenous bacteria isolated from cereal products produced folate even more than baker's yeast, *S. cerevisiae*, known as a good folate producer, and other studied food-grade yeasts such as *C. milleri*. On the other hand, the folate-production ability of food-grade LAB was not so high in cereal matrices. Folate levels achieved in cereal matrices during the 24-h fermentation did not decrease in 2 weeks of cold storage, which was encouraging. On the contrary, cereal-associated *Pseudomonas* sp. even continued folate production in cold in oat and barley flour matrices resulting in a 9-fold higher folate content than in control samples.

The results of this study thus showed that there is a potential for using cereal-associated bacteria and food-grade yeasts for folate enhancement in aqueous cereal processing. Particularly, the *Pseudomonas* sp. proved to be a promising candidate for further research. However, microbes without previous food use should be first studied for their potentially adverse effects on food quality and human health.

The predominant folate forms in oat and barley grains were 5-CH_3-H_4folate, 5-HCO-H_4folate, 10-HCO-PGA and 5,10-CH^+-H_4folate. However, there might be some unidentified folate forms in cereal samples, because there was still a small gap between the MA and UHPLC results. The vitamer pattern in folate produced by bacteria and yeast strains was quite different than that in oat and barley fractions. Bacteria and yeasts mainly accumulated 5-CH_3-H_4folate followed by H_4folate and 5,10-CH^+-H_4folate. The level of 5-HCO-H_4folate was lower than in oat and barley grains and fractions. The distribution of these vitamers was different during different growth phases in bacteria. In yeasts, the production of 5-CH_3-H_4folate affected the total folate content the most. The knowledge of vitamer distribution and their specific detailed content during the cultivation process of microbes will offer an interesting topic for further experimental research.

In summary, these findings support the increasing use of oats and barley and their folate-rich milling fractions in cereal products such as breads, biscuits, pasta and breakfast cereals as a natural source of folate. Even a small increase in the folate content in cereal products may improve the folate intake if consumed regularly. Cereal-associated endogenous bacteria together with food-origin yeasts are promising as potential folate producers. This could open the door to novel food applications that are rich in folate produced by microbes and rich in beta-glucan, with its beneficial effects on human health.

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