Food Microbiology 57 (2016) 159–171

Contents lists available at ScienceDirect

Food Microbiology

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

Evaluation of growth, metabolism and production of potentially bioactive components during fermentation of barley with *Lactobacillus reuteri*

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ARTICLE INFO

Article history:
Received 4 July 2015
Received in revised form
19 February 2016
Accepted 27 February 2016
Available online 28 February 2016

Keywords:
Lactobacillus reuteri
Barley
Fermented cereals
1H-NMR
Bioactive compounds

ABSTRACT

Eighteen bacterial isolates from millet, buckwheat and rye flour were identified as *Lactobacillus reuteri*. Genomic fingerprinting (rep-PCR) revealed that they represented five strains and phylogenetic analyses using multi locus sequence analysis (MLSA) showed that all clustered with strains of rodent origin. Two strains (SU12-3 and SU18-3) from different phylogenetic clades were used in fermentations of six varieties of barley, both untreated and heat-treated (with inactivated indigenous enzymes) flour. They were compared with two probiotic strains of human origin (DSM 17938 and ATCC PTA 6475), one previously isolated sourdough strain (LTH 5531) and one strain of *Lactobacillus plantarum* (36E). Analyses of growth (CFU) and metabolism (1H-NMR) revealed differences at species level, with L. plantarum showing a higher capacity to assimilate nutrients without help of the cereal enzymes. Similarities were observed between *L. reuteri* strains isolated from sourdough, while the greatest differences between *L. reuteri* strains were observed between strains 6475 and 17938. Multivariate analysis of the metabolic profiles revealed clear clustering according to flour treatment, species of bacteria and barley variety and to some extent also bacterial strain. Possible bioactive compounds such as γ-aminobutyric acid (GABA), 1,3-propanediol (sign of reuterin production) and histamine were identified and quantified.

1. Introduction

Species of the genus *Lactobacillus* are usually nutritionally highly demanding and require fermentable carbohydrates, amino acids, different B-vitamins, nucleic acids and minerals to grow (Gomes and Malcata, 1999). A number of these species have been shown to grow well in different kinds of cereals, showing that cereals are a good source of the nutrients they require (Charalampopoulos et al., 2002; Müller et al., 2001). Studies have also shown that the fermentation of cereals with lactic acid bacteria, including lactobacilli, can result in many types of metabolites with putative bioactive potency. Among the metabolites found to have a bioactive effect are *e.g.* cobalamin, reuterin, riboflavin and γ-aminobutyric acid (Russo et al., 2014; Capozzi et al., 2012; Stromeck et al., 2011; Gänzle et al., 2009). Bioactive components are naturally occurring compounds which have been shown to have an effect on human health. They can be either essential or non-essential and are part of the food chain (Biesalski et al., 2009).

Barley, the fourth most common crop in Sweden during 2010–2013 (FAO, 2014), is becoming increasingly interesting as a complement to wheat flour in food due to its possible health benefits. Most studies have focused on the blood cholesterol lowering effect, with the high content of soluble fibres and, especially, β-glucan being linked to lowering of total cholesterol and low lipid density (LDL) cholesterol (Shimizu et al., 2008; Keenan et al., 2007). Fermentation of β-glucans in the colon results in the production of short-chain fatty acids (SCFA) such as acetate, butyrate and propionate (Wong et al., 2006). These inhibit the biosynthesis of cholesterol (Hara et al., 1999), but have also been shown to have an anti-inflammatory effect in both *in vitro* and *in vivo* studies (Tedelind et al., 2007). Prebiotic potential of barley and especially the β-glucans has also been demonstrated in a few studies. β-glucans from barley and oats administered to rats have been shown to promote the growth of colonic *Lactobacillus* and *Bifidobacterium* (Shen et al., 2012). An effect of barley β-glucans on the growth and

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http://dx.doi.org/10.1016/j.fm.2016.02.011
0740-0020/

probiotic features (e.g. enhanced oro-gastrointestinal stress tolerance and adhesion to enterocytes) of different *Lactobacillus* strains has recently been shown (Arena et al., 2014). The growth of these strains was improved under unstressed conditions, and after *in vitro* simulation of the digestive tract.

Lactobacillus reuteri has well-documented effects as a probiotic, *e.g.* in lowering number of days of diarrhoea in infants and improving symptoms in infantile colic (Savino et al., 2010; Weizman et al., 2005). It has also been shown to increase the eradication rate of *Helicobacter pylori* and improve the gastrointestinal symptoms associated with this infection (Emara et al., 2014). *L. reuteri* is mainly associated with the gastrointestinal tract of warm-blooded animals, but in humans it has also been isolated from breast milk, the oral cavity and the vagina. It has been shown to inhabit the gastro-intestinal tract of mammals such as humans, pigs and rodents, but also different species of poultry including chickens and turkeys (Hammes and Hertel, 2006). Moreover, *L. reuteri* has been reported in sourdoughs made from different cereals, including rye, sorghum and wheat (De Vuyst et al., 2014). The species seems to be able to dominate sourdoughs subjected to higher temperatures and prolonged fermentation and with longer time points between back-slopping (Meroth et al., 2003). It has also been shown to persist in industrial sourdough for up to ten years, even with major shifts in the population of other microorganisms during this time (Gänzle and Vogel, 2003). Despite being present in sourdoughs and being able to grow well in different kinds of cereals, the fermentation of cereals with *L. reuteri* can possibly also have effects on nutritional value. Fermentation of *e.g.* whole-grain barley flour with different species of lactobacilli, including *L. reuteri*, has been shown to increase the content of free phenolic acids six-fold (Hole et al., 2012). Formation of γ-amino-butyric acid (GABA) has also been shown to occur in *L. reuteri* when fermenting rye malt flour with added wheat gluten, but seems to be strain-specific (Stromeck et al., 2011).

The aims of this study were to isolate and determine the origin of *L. reuteri* strains from cereals and possible adaptations to the sourdough environment. The growth and metabolism of the strains was compared with that of commercially available probiotic strains, to determine these possible adaptations. Fermentations of barley varieties with small, but significant, differences in carbohydrate composition were performed to determine changes in growth and metabolism of *L. reuteri*. To determine whether any specific activities were connected to the species *L. reuteri*, one strain of *Lactobacillus plantarum* was included as a control.

2. Materials and methods

2.1. Isolation of LAB from cereals

Fifteen flours from different cereal species (barley, buckwheat, millet, rye and wheat), brands and origins (Sweden, Germany and China) and from organic and conventional production were analysed for the presence of *L. reuteri*. For this, 8 g flour were mixed with 21.3 mL sterilised water and incubated at 37 or 40 °C in a water bath without agitation. The flour/water mixture was subjected to daily back-slopping, using 1% of the old sourdough as inoculum, for up to 13 days. Enumeration of putative *L. reuteri* was performed every third day by plating on Rogosa agar (Oxoid Ltd., Basingstoke, Hampshire, UK) with added vancomycin (50 μg/mL) and incubating the plate anaerobically at 45 °C for 24 h. Since L. *reuteri* has an intrinsic resistance to vancomycin, the antibiotic was added to the medium in order to make it more selective (Romani Vestman et al., 2013). Increasing the normal incubation temperature of 37 °C–45 °C makes the growth conditions even more selective, since most *Lactobacillus* spp. are mesophilic, with optimal growth at temperatures of 30–37 °C, while *L. reuteri* grows well at 45 °C (Hammes and Hertel, 2006). The fermented flour was also analysed by extracting DNA and running an *L. reuteri*-specific PCR. The DNA extraction was performed according to the protocol for Gram-positive bacteria in the DNeasy Blood & Tissue Handbook (QIAGEN, Hilden, Germany) with a few modifications. An extra bead-beating step was performed after the first incubation with the enzymatic lysis buffer, which was extended to 60 min. The PCR was performed using the primers L-reu 1 (5′-CAGACAATCTTTGATTGTTTAG-3′) and L-reu 4 (5′-GCTTGTTGGTTTGGGCTCTTC-3′) (Song et al., 2000) and the following PCR programme: (95 °C, 5 min; 30x(95 °C, 30s; 55 °C, 30s; 72 °C, 30s); 72 °C, 10 min; 16 °C, ∞).

2.2. Identification and characterisation of Lactobacillus reuteri strains

Colonies isolated on Rogosa/vancomycin agar were identified by amplification and sequencing of 16S rRNA genes using the primers F8M (5′-AGAGTTTGATCMTGGCTCAG-3′) (Edwards et al., 1989) and 926r (5′CCGTCAATTCCTTTRAGTTT-3′) (Muyzer et al., 1993) and the following PCR programme (95 °C, 5 min; 30x(95 °C, 30s; 55 °C, 30s; 72 °C, 60s); 72 °C, 10 min; 16 °C, ∞). The PCR products were sequenced by Macrogen Inc. In order to identify the isolates to species level, a GenBank DNA database search was performed using the BLASTN algorithm. An isolate was considered as identified when the similarity to a type strain was >98%. Identified L. *reuteri* strains were analysed by a genomic fingerprinting method (rep-PCR) in order to group them at strain level. This was performed using GTG_5 primer (5′GTGGTGGTGGTGGTG-3′) and the following PCR programme: (98 °C, 5 min; 30x(90 °C, 30s; 95 °C, 30s; 40 °C, 60s; 65 °C, 4 min); 65 °C, 16 min; 4 °C, ∞) (Versalovic et al., 1994).

A reuterin production assay was performed on each of the isolates using single colonies in a grid system on MRS agar plates (Merck, Darmstadt, Germany) incubated anaerobically at 37 °C for 48 h. The plates were then overlaid with 500 mM glycerol agar and incubated at 37 °C for 30 min. Reuterin was detected by incubation for 3 min after addition of 5 mL 2,4-dinitrophenylhydrazine (0.1% in 2 M HCl) and addition of 5 mL 5 M KOH after removal of the previous solution. A colony was considered positive for reuterin production when surrounded by a red zone (Rosander et al., 2008). Presence of genes encoding urease and one of the subunits of propanediol dehydratase (involved in reuterin production) was also analysed using primers ureCF (5′-GAAAGTCTTTTTGGTGGTGG-3′) and ureCR (5′-AACGTCGTCAGGAATCTTAG-3′) and primers pduCF (5′-CCTGAAGTAAAYCGCATCTT-3′) and pduCR (5′-GAAACYATTTCAGTTTATGG-3′), respectively, and the following PCR programme: (95 °C, 5 min; 30x(95 °C, 30s; 53 °C, 30s; 72 °C, 2 min); 72 °C, 10 min; 16 °C, ∞) (Walter et al., 2011).

More in-depth characterisation was performed by multi-locus sequence analysis (MLSA) using primers for fragments of the genes *ddl*, *kpt*, *leuS*, *gyrB*, *dltA*, *rpoA* and *recA* (Oh et al., 2009). The same PCR conditions were used for all seven genes (94 °C, 2 min; 30x(94 °C, 30s; 55 °C, 30s; 72 °C, 60s); 72 °C, 7 min; 16 °C, ∞). Sequences for these seven genes were concatenated and aligned using Geneious R6 (Kearse et al., 2012). The concatenated sequences from five *L. reuteri* strains with different rep-PCR profiles isolated in this study were compared to 84 already sequenced *L. reuteri* strains of different animal origin. These strains were chosen according to their characterisation in previous studies (Oh et al., 2009). From these concatenated sequences, a phylogenetic tree was made using raxmlGUI 1.3 (Silvestro and Michalak, 2012) and iTOL v2 (Letunic and Bork, 2007).