International Journal of Food Microbiology 155 (2012) 185–190

Contents lists available at SciVerse ScienceDirect

International Journal of Food Microbiology

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

Biodiversity of antifungal lactic acid bacteria isolated from raw milk samples from cow, ewe and goat over one-year period

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ARTICLE INFO

Article history:
Received 18 September 2011
Received in revised form 23 January 2012
Accepted 5 February 2012
Available online 12 February 2012

Keywords:
Antifungal lactic acid bacteria
Biodiversity
Milk
Cow
Ewe
Goat

ABSTRACT

Antifungal lactic acid bacteria (ALAB) biodiversity was evaluated in raw milk from ewe, cow and goat over one year period. Lactic acid bacteria were enumerated using 8 semi-selective media, and systematically screened for their antifungal activity against 4 spoilage fungi commonly encountered in dairy products. Depending on the selective medium, between 0.05% (Elliker agar) and 5.5% (LAMVAB agar) screened colonies showed an antifungal activity. The great majority of these active colonies originated from cow (49%) and goat (43%) milks, whereas only 8% were isolated from ewe milk. *Penicillium expansum* was the most frequently inhibited fungus with 48.5% of colonies active against *P. expansum* among the 1235 isolated, followed by *Mucor plumbeus* with 30.6% of active colonies, *Kluyveromyces lactis* with only 12.1% of active colonies and *Pichia anomala* with 8.7% of active colonies. In the tested conditions, 94% of the sequenced active colonies belonged to *Lactobacillus*. Among them, targeted fungal species differed according to the *Lactobacillus* group, whose presence largely depended on year period and milk origin. The *Lb. casei* and *Lb. reuteri* groups, predominantly recovered in summer/fall, were overrepresented in the population targeting *M. plumbeus*, whereas isolates from the *Lb. plantarum* group, predominantly recovered in spring, were overrepresented in the population targeting *K. lactis*, the ones belonging to the *Lb. buchneri* group, predominantly recovered in spring, were overrepresented in the population targeting *P. anomala*. Raw milk, especially cow and goat milks from the summer/fall period appeared to be a productive reservoir for antifungal lactobacilli.

1. Introduction

Milk and fermented dairy products are favorable environments for yeasts and molds growth that can be involved in fermentation or food spoilage (Rohm et al., 1992). They may ferment lactose and sucrose, utilize lactate, hydrolyze lipids and proteins and may grow at refrigeration temperatures (Huis in't Veld, 1996). Food spoilage fungi may be responsible for production of gas, alcohol and undesirable aromatic compounds leading to flavor and texture defects, and economic losses (Rohm et al., 1992; Torkar and Vengust, 2008). Certain molds may also synthesize a wide panel of mycotoxins detrimental to the consumer health (Nelson, 1993; Pitt, 2000; Tantaoui-Elaraki and Khabbazi, 1984). To avoid fungal spoilage, numerous chemical preservatives are used, including organic acids, sodium benzoate, potassium sorbate, potassium benzoate and pimaricin (Brul and Coote, 1999; Smith and Hong-Shum, 2003). However, increasing resistances of fungi toward chemical preservatives, consumers demand for healthy and natural products and legislation evolution (Brul and Coote, 1999), have lead industrials to find new means of preservation such as bioprotective cultures like MicroGARD® and HOLDBAC™ (Danisco, Niebûll GMBH, Germany).

Lactic acid bacteria (LAB) are good candidates for fermented dairy food biopreservation. They are naturally present in yogurts, creams, fresh and mature cheeses and some of them possess antimicrobial activities (Pfeiler and Klaenhammer, 2007; Schnurer and Magnusson, 2005). Moreover, most of them belong to the qualified presumption of safety (QPS) and generally recognized as safe (GRAS) lists which insure their safe use in food (Bernardeau et al., 2008; Rossetti et al., 2009). Only few lactic acid bacteria such as enterococci remain excluded from this status and require more vigilance because of their role in infection cases and spreading of antibiotic resistance genes (Mathur and Singh, 2005). Based on literature data, 0% to 75% LAB would have antifungal properties (De Muynck et al., 2004; Gerez, et al., 2009; Magnusson et al., 2003; Rouse et al., 2008; Schillinger and Villarreal, 2010; Valerio et al., 2009; Voulgari et al., 2010). This huge variation in the percentage of active LAB is explained by differences in methods for activity testing including medium utilized for LAB growth and targeted fungi. Until now, no study has systematically described, in a specific niche such as milk, the prevalence and the periodical variations of antifungal LAB, tested with the same method, and compared these data with the total number of LAB present in the same sample.

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doi:10.1016/j.ijfoodmicro.2012.02.003

LAB represent 20–30% of total bacterial counts in raw milk, but conditions of production, season, breeding and the animal origin of milk influence their abundance and diversity (Drakoularakou et al., 2003; Gaya et al., 1999; Michel et al., 2001; Verdier-Metz et al., 2009). According to Medina et al. (2001), enterococci represent the majority of LAB (48%) in ewe raw milk, followed by lactobacilli (*Lactobacillus casei* and *Lactobacillus plantarum)* (30%), lactococci (14%) and *Leuconostoc* spp. (8%). Franciosi et al. (2009) showed that 94% of isolated LAB in cow's milk included lactococci, enterococci and streptococci, the remaining 6% isolates were lactobacilli (mostly *Lb. casei*, *Lactobacillus delbrueckii*, *Lactobacillus paracasei* and *Lb. plantarum*), *Leuconostoc* and pediococci. LAB commonly encountered in goat's milk are *Lactococcus lactis, Lactococcus gravieae, Enterococcus faecalis, Leuconostoc mesenteroides* and *Lb. casei* (Callon et al., 2007). LAB from raw milk may originate from various sources, which can explain diversity found among seasons, animal species, etc. They can directly come from milk, but also from animals' environment. Indeed, *Leuconostoc* spp. come from vegetation and roots but can easily propagate and persist in various niches such as raw materials. Their presence in milk is due to contamination (Hemme and Foucaud-Scheunemann, 2004). The ubiquitous genera *Lactococcus* and *Lactobacillus* may come from plants, feces or udder skin (Kagkli et al., 2007; Mofredj et al., 2007; Tailliez, 2004).

The objective of this work was to evaluate the biodiversity of antifungal LAB in different raw milk samples, coming from ewe, cow and goat and collected at three different periods (winter, spring and summer/fall) over one year in three collect areas in France. To reach our goal, bacteria were enumerated in eight semi selective media used for LAB enumeration and overlaid with four targeted fungi commonly involved in the spoilage of dairy products.

2. Materials and methods

2.1. Raw milk samples

Raw milk was sampled during year 2009 in refrigerated bulk tanks from milk collecting trucks coming from restricted areas in three French "Départements" according to the three tested species: ewe (Aveyron), goat (Deux Sèvres) and cow (Ille-et-Vilaine). For each species, nine raw milk samples were collected during three successive weeks at three periods of the year (winter, spring and summer/fall). They corresponded to January, April and July for ewes; February, June and November for goats; and February, June and September for cows. These periods were different depending on species; they corresponded to different lactation periods (beginning, middle and end) for ewes and goats as well as different feeding conditions. In winter, ewes were kept in sheep-folds and they were fed with dried food (grains and hay), they did not receive silage. They started to graze outside in spring. Cows and goats were mainly kept in stalling during winter and fed with dried and humid food (hay, silage and grains); they started to graze outside in spring. Milk samples (500 ml) were aseptically taken in refrigeration tanks, kept at 4 °C, sent to the laboratory and analyzed within 48 h. Milk pH was recorded and 10 ml were immediately used for bacterial enumeration.

2.2. Enumeration of total and lactic acid bacteria

Raw milk samples (10 ml) were serially diluted 10-fold in peptone water (0.1%, pH 7) and plated in quadruplicate on both non selective and semi-selective media. Total bacteria were enumerated on plate count agar (PCA) supplemented with 1% milk (AES Chemunex, Bruz, France) (Michel et al., 2001) and Elliker agar (Sigma-Aldrich, Saint-Quentin Fallavier, France) (Carr et al., 2002; Randazzo et al., 2002; Teuber, 1993). Lactobacilli were enumerated on LAMVAB, an MRS-based medium supplemented with vancomycin and acidified to pH 5 (Coeuret et al., 2003; Hartemink et al., 1997; Henri-Dubernet et al., 2008). Enterococci, pediococci and lactobacilli were enumerated on acidified MRS (pH 5.5) (Carr et al., 2002; Randazzo et al., 2002). The kanamycin esculin azide agar medium (KEA, Sigma-Aldrich) was chosen for enterococci (Franciosi et al., 2009; Teuber, 1993). M17 and a PCA based-medium enriched with 10% of milk and supplemented with nalidixic acid (PCA-ATB) were used to enumerate lactococci (Corroler et al., 1998; Franciosi et al., 2009). *Leuconostoc* strains were enumerated on Mayeux Sandine and Elliker (MSE, AES Chemunex, Bruz) medium (Randazzo et al., 2002) and psychrotolerant lactic acid bacteria enumerated on MRS (pH 6.2) at 10 °C for 4 to 7 days (Teuber, 1993). Fungi were enumerated on yeast extract glucose chloramphenicol medium (yeast extract 0.5%; glucose 2%; chloramphenicol 100 mg/l) at 25 °C (Casalta et al., 2009). Depending on population tested, plates were incubated aerobically or in anaerobic jars at 10, 21, 30 or 37 °C for up to 7 days according to reference instructions. Cell counts were expressed as Log_{10} CFU/ml of milk. After enumeration, four plates of each dilution were overlaid with the four tested fungi.

2.3. Preparation of yeast cell and mold spore suspensions

Four ubiquitous fungi, commonly encountered in cheese and yogurt spoilage (Caggia et al., 2001; Hayaloglu and Kirbag, 2007; Wouters et al., 2002) were chosen as indicator microorganisms for antifungal assays. The molds *Penicillium expansum* CBS 32.548NT and *Mucor plumbeus* CBS 129.41T came from the Culture Collection of Centraalbureau voor Schimmelcultures (CBS, Utrecht, The Netherlands). The yeasts *Pichia anomala* LMSA 2.01.001 and *Kluyveromyces lactis* CLIB196 came from the "Souchothèque de Bretagne" culture collection (Université de Brest, Plouzané, France) and "Collection de Levures d'Intérêt Biotechnologique" (INRA, Thiverval-Grignon, France), respectively.

Molds were cultivated on potato dextrose agar (PDA, Difco, Le Pont de Claix, France) slants for a few days at 25 °C until spores were formed. Spores were then harvested with sterile distilled water supplemented with 0.1% Tween-80 and the suspension was spread on PDA in a Roux flask to increase spore production (Roy et al., 1996). Spores were harvested, enumerated using a Malassez cell and stock suspensions were standardized to a final concentration of 10^7 spores/ml before storage at −80 °C in glycerol (10%, v/v).

Before use, yeasts were subcultured in a yeast extract and malt based medium (YEMA) composed of yeast extract (0.3%) and malt extract (2%) for 24 to 48 h at 25 °C. One hundred microliters of this suspension were inoculated in 10 ml of YEMA broth and incubated aerobically at 25 °C for 24 h. Cells were enumerated with a Malassez cell and the resulting suspension was standardized at a concentration of 10^6 cells/ml in peptone water (0.1%). Yeast stocks were maintained at −80 °C in YEMA supplemented with glycerol (30%, v/v).

2.4. Antifungal activity assays

After enumeration of LAB, agar plates used for bacterial enumeration (except for the non-selective PCA medium) with less than 300 colonies were overlaid with 8 ml of PDA for molds or YEMA medium for yeasts (agar 0.8%) containing 10^4 fungal spores or yeast cells per ml. Plates were then incubated at 25 °C and areas of inhibition recorded after 48 h (Magnusson and Schnürer, 2001). Active colonies showing clear inhibition area, were isolated on appropriated growth medium supplemented with amphotericin (5.6 mg/ml) (Sigma-Aldrich), followed by a minimum of three successive subcultures. A second antifungal activity assay was performed on purified isolates to confirm the first test. Active isolates were maintained in growth medium supplemented with glycerol (30%, v/v) at −80 °C.

2.5. Identification of antifungal isolates

Isolates were examined by phase-contrast microscopy, Gram stained and tested for the presence of catalase. For isolate identification, total DNA was extracted from 1.5 ml of pure culture obtained after 18 h incubation in MRS broth as described previously by Randazzo et al. (2002), and used as template for the 16S rRNA gene amplification with the pA/pH bacterial universal primers pair (Edwards et al., 1989; Zamfir et al., 2006). PCR amplicons were sequenced at the Biogenouest sequencing platform in the "Station Biologique de Roscoff" (http://www.sb-roscoff.fr/SG/) and sequences were aligned on the NCBI website (http://www.ncbi.nlm.nih.gov/BLAST) (October 2010) for species assignment.

2.6. Statistical analyses

Statistical analyses were performed using STATGRAPHICS Plus 5.1 package (Statistical Graphics Corp., VA, USA). A three-way analysis of variance (ANOVA) was used to compare microbial counts on agar media and to assess the effects of animal species (*A*: cow, goat or ewe), periods of sampling (P: first, second or third), enumeration media and interactions between them. When significant effects were found, individual means were compared by one-way ANOVA using the Tukey's honestly significant difference (HSD) test. Statistical significance was set at $P<0.05$ level. Microbial counts on enumeration agar were expressed as means (Log_{10} CFU/ml) of four replicates. Dependences between numbers of antifungal isolates were obtained by Chi Square test of independence under Microsoft® Office Excel 2003 software (Microsoft Office Professional Edition 2003). Statistical significance was set at $P<0.05$ level for this test. Animals, periods of sampling, media, dilutions, fungal targets and taxa were considered as variables.

2.7. Lactobacilli biodiversity analyses

The diversity of lactobacilli was analyzed according to animals and sampling periods, by calculating the ratio (p_i) between number of antifungal *Lactobacillus* belonging to phylogenetic group i (n_i) and the total number of lactobacilli (N): $p_i = n_i/N$, for each animal-sampling period couple. Diversity was measured by the Shannon index (H) (Shannon and Weaver, 1963) using the following equation: $H = -\Sigma p_i \log_2 (p_i)$. The higher the H index is, the greater the diversity. Each animal-sampling period couple containing different numbers of *Lactobacillus* groups, the diversity within each animal-sampling period couple was also compared by calculating the equitability (E = H/log2 [S], with S being the number of *Lactobacillus* groups). The equitability can vary from 0 to 1, an E value equal to 0 means that one species clearly dominates among others, whereas an E value close to 1 indicates that individuals from each group have an equitable distribution.

3. Results

3.1. Microbial enumeration in milk

Mean total bacteria concentrations enumerated on non-selective media (PCA and Elliker) ranged from 4.7 ± 0.4 to 5.9 ± 0.5 Log_{10} CFU/ml, whereas fungal concentrations were significantly lower ($P<0.05$) and ranged from 3.5 ± 0.6 to 4.7 ± 0.7 Log_{10} CFU/ml depending on animal and sampling periods (Table 1). Milk samples, whatever their origin, were dominated by LAB cocci such as presumptive lactococci (PCA–ATB) and *Leuconostoc* (MSE) (values ≥ 4.2 Log_{10} CFU/ml). Enterococci, *Lactobacillus* spp. and psychrotolerant LAB were present in significantly lower concentrations ($P<0.05$). In most media, including fungal selective medium, cow milk samples harbored significantly lower plate counts ($P<0.05$) than goat milk, and ewe milk counts lay in between. However, LAMVAB harbored significantly higher counts ($P<0.05$) in cow and goat, compared to ewe milk samples. Consequently, the average count of lactobacilli from the three sampling periods, in cow and goat milk samples were 10 times higher than those of ewe milk samples ($P<0.0001$). Sampling periods did not have a significant influence on bacterial populations except for *Lactobacillus* spp. Indeed, their concentration significantly increased on LAMVAB medium between the second and third sampling periods ($P<0.0001$) in ewe milk' samples.

3.2. Isolated antifungal lactic acid bacteria

3.2.1. Effect of milk origin, sampling periods and targeted fungi

Bacterial colonies grown on 8 different media and originating from 27 raw milk samples collected at three different sampling periods in three animal species were screened for their antifungal activity against four targeted fungi. Among the 71,833 tested colonies, 1235 colonies (i.e. 1.7% of tested colonies) showed a detectable antifungal activity and were subsequently isolated. Most of the ALAB were

Table 1
Bacterial and fungal populations enumerated with plate counts (Log_{10} CFU/ml) during the first, second and third sampling periods in ewe, goat and cow's milk samples.

		Bacteria									Fungi
		Elliker	PCA	M17	PCA ATB	MSE	MRS pH5.5	KEA	LAMVAB	MRS 10 °C	YEGC
Cow	P1	5.2^{a}	4.4^{a}	4.7^{a}	3.8^{a}	4.1^{a}	3.6^{a}	3.3^{a}	3.8^{a}	3.6^{a}	4.0^{a}
	P2	5.0^{a}	4.8^{a}	4.5^{a}	4.6^{a}	4.5^{a}	3.6^{a}	4.4^{a}	4.0^{a}	2.7^{a}	3.0^{a}
	P3	4.7^{a}	4.9^{a}	4.7^{a}	4.3^{a}	4.3^{a}	4.1^{a}	3.9^{a}	4.0^{a}	3.5^{a}	3.4^{a}
	M	**4.9^{A}**	**4.7^{A}**	**4.6^{A}**	**4.2^{A}**	**4.3^{A}**	**3.8^{A*}**	**3.9^{A*}**	**3.9^{A*}**	**3.4^{AB*}**	**3.5^{A*}**
Goat	P1	6.2^{a}	5.4^{a}	5.5^{a}	5.0^{a}	4.9^{a}	4.4^{a}	4.5^{a}	3.3^{a}	3.7^{a}	5.0^{a}
	P2	5.9^{a}	5.9^{a}	5.3^{a}	5.3^{a}	5.1^{a}	4.7^{a}	4.8^{a}	4.1^{a}	5.5^{a}	4.3^{a}
	P3	5.8^{a}	5.7^{a}	5.3^{a}	4.8^{a}	4.2^{a}	4.4^{a}	4.2^{a}	4.3^{a}	3.6^{a}	4.8^{a}
	M	**5.9^{B}**	**5.5^{A}**	**5.4^{B}**	**5.0^{B}**	**4.7^{A}**	**4.5^{A}**	**4.5^{B*}**	**3.9^{A*}**	**4.1^{A*}**	**4.7^{B}**
Ewe	P1	–	6.3^{a}	6.2^{a}	4.8^{a}	4.2^{a}	4.1^{a}	4.2^{a}	3.1^{a}	2.8^{a}	4.7^{a}
	P2	4.8^{a}	4.3^{a}	3.7^{b}	4.1^{a}	3.9^{a}	3.2^{a}	3.7^{a}	1.7^{b}	2.3^{a}	2.8^{b}
	P3	5.6^{a}	5.4^{a}	5.2^{ab}	5.2^{a}	4.8^{a}	5.2^{a}	4.8^{a}	4.8^{c}	4.1^{a}	4.2^{a}
	M	**5.2^{AB}**	**5.4^{A}**	**5.0^{AB}**	**4.7^{AB}**	**4.3^{A}**	**4.2^{A*}**	**4.2^{AB}**	**3.2^{B*}**	**2.9^{B*}**	**4.1^{A*}**
	A	*0.04*	*0.05*	*0.04*	*0.04*	*0.1*	*0.07*	*0.003*	*0.0001*	*0.04*	*0.001*
	P	*0.4*	*0.4*	*0.01*	*0.7*	*0.9*	*0.05*	*0.07*	*0.0001*	*0.7*	*0.002*
	AP	*0.02*	*0.01*	*0.02*	*0.06*	*0.06*	*0.06*	*0.001*	*0.0001*	*0.1*	*0.5*

Data are means of four plate count replicates for each sampling period (P1: first sampling period; P2: second sampling period and P3: third sampling period) in three animals (ewe, goat and cow). Data in bold are means of the three sampling periods for each animal species (M: mean values of P1, P2 and P3). ANOVA analysis was used to assess the effects of animal species (*A*), periods of sampling (*P*) and interactions between them (*AP*).Effects of sampling periods (P1, P2 and P3) on bacterial populations for each enumeration medium: values with different letters (small letters a, b and c) in a column are significantly different by Turkey Kramer–HSD test ($P<0.05$). Effects of animal species (M) on bacterial populations for each enumeration medium: values with different letters (capital letters A, B) in a column are significantly different by Turkey Kramer–HSD test ($P<0.05$). Plate counts significantly different from PCA and Elliker counts in a row (data in bold) at the significance level: * $P<0.05$.

recovered from cow (49%) and goat (43%) milk, whereas only 8% were recovered from ewe's milk. A dramatic increase in the total number of active colonies was observed between the second and third sampling periods for cow (167 vs. 282 active colonies) and goat (102 vs. 321 active colonies), but not for ewe's milk (25 vs. 31 active colonies). Overall, around 50% of total antifungal isolates were recovered during the third sampling period.

Among the 4 targeted fungi, *P. expansum* was inhibited by the greatest number of isolates with 599 isolates representing 48.5% of total isolates. *M. plumbeus* was the second most frequently inhibited fungus with 378 isolates (30.6% of total isolates), *K. lactis* and *P. anomala* were inhibited by much less isolates with a total recovery of 150 (12.1% of total isolates) and 108 isolates (8.7% of total isolates), respectively. According to the Chi Square test of independence, isolates active against *M. plumbeus* were overrepresented ($P<0.05$) in cows and goat's milks during the third sampling period, those active against *K. lactis* were overrepresented ($P<0.05$) in goat and cow milks during the first and second sampling periods, respectively, whereas isolates active against *P. anomala* were overrepresented ($P<0.05$) in goat and cow milks in the second sampling period (Table 2). Active colonies were found in the same proportions in cow and goat milk samples with 49 and 46% of isolates active against *P. expansum*, 31 and 32% against *M. plumbeus*, and 13 and 12% against *K. lactis*, respectively. Isolates active against *P. anomala* were less numerous in cow's milk than in goat's milk (7% vs. 12%). In ewe's milk, the distribution pattern of active isolates was different from that of cow and goat's milks. A much lower number of isolates was recovered from ewe milk and the number of isolates was equally distributed in the three sampling periods. Moreover, the active isolates mainly showed antifungal activities against *P. expansum*.

3.2.2. Effect of medium on antifungal activity

Eighty six percent of the 1235 isolates came from the three MRS-based media, e.g., LAMVAB (605 isolates), MRS-5.5 (355 isolates) and MRS-10 °C (97 isolates). The remaining 178 isolates were obtained from the five other overlaid media (Elliker, M17, KEA, PCA–ATB and MSE). LAMVAB, MRS-5.5 and MRS-10 °C showed 5.5%, 3% and 1% of active colonies, respectively among ca 11,000 screened colonies for each medium. Differences were noticed in the same medium according to milk origin, sampling period and targeted fungus. The percentages of active colonies were systematically higher in cow's milk than in goat's milk for LAMVAB (9.5 vs. 5.6%) and MRS-5.5 (4.9 vs. 2.3%) media but not in MRS-10 °C (0.7 vs. 1.2%). When referring on sampling periods, an increase in the percentages of active colonies was observed for cow (plus 10 to 210%) and goat (plus 160 to 230%) milks, between the second and third sampling periods on LAMVAB and MRS-5.5, but not on MRS-10 °C. Ewe's milk showed a very different pattern to that of cow and goat milks with low percentages of active colonies for the three MRS-based media ($1.5 \pm 0.3\%$) whatever the sampling period, despite a significant increase in lactobacilli concentrations.

Table 2
Numbers of antifungal lactic acid bacteria isolates according to animals, sampling periods and targeted fungi.

Animals	Sampling periods	Targeted fungi			
		P. expansum	*M. plumbeus*	*K. lactis*	*P. anomala*
Ewe	First	18 (+6)	6 (−2)	1 (−2)	0 (−2)
	Second	**33 (+12)**	8 (−5)	3 (−2)	0 (−4)
	Third	12 (−3)	10 (+1)	5 (+1)	4 (+1)
Cow	First	**118 (+45)**	21 (−25)	12 (−6)	0 (−13)
	Second	53 (−28)	48 (−3)	**31 (+11)**	**35 (+20)**
	Third	102 (−35)	**120 (+34)**	26 (−8)	34 (+9)
Goat	First	58 (+4)	19 (−15)	**23 (+10)**	11 (+1)
	Second	45 (−4)	27 (−4)	9 (−3)	**21 (+12)**
	Third	160 (+4)	**119 (+20)**	40 (+1)	3 (−25)

Data in brackets represent the differences between obtained values and that predicted with the Chi test of independence. Data in bold are significantly overrepresented in comparison to predicted values ($P<0.05$).

3.3. Identification of isolates active against M. plumbeus, K. lactis and P. anomala

Because of the high number of isolates active against *P. expansum*, only isolates active against *M. plumbeus* (417 isolates), *K. lactis* (186 isolates) or *P. anomala* (130 isolates) were sequenced. Among them, 690 isolates (94%) belonged to *Lactobacillus*, 26 (3.6%) to *Leuconostoc*, 12 (1.7%) to *Enterococcus* and 2 (0.3%) to *Lactococcus* genera. The vast majority (92%) of antifungal lactobacilli were isolated on MRS-based media, the 8% remaining were isolated on MSE, KEA and PCA–ATB. *Leuconostoc* spp. were isolated on MRS-10 °C and MSE, and *Enterococcus* spp. on KEA and MSE. Surprisingly, the 2 isolates of *Lactococcus* spp. were not recovered on PCA–ATB nor Elliker media, but on MSE and MRS-5.5.

The 690 antifungal isolates belonging to the *Lactobacillus* genus were ranked into eight phylogenetic groups according to Felis and Dellaglio (2007) and Canchaya et al. (2006) classifications. The majority of them belonged to the *Lb. casei* group (55%), followed by *Lactobacillus reuteri* (19%), *Lb. plantarum* (17%) and *Lactobacillus buchneri* (7%) groups. The remaining 2% belonged to the *Lb. delbrueckii*, *perolens*, *salivarius* or *coryniformis* groups.

Isolates from the *Lb. casei* group were overrepresented ($P<0.05$) on LAMVAB, during the third sampling period in both cow and goat milks, and among populations targeting *M. plumbeus* (Tables 3 and 4). A very similar pattern was observed for the *Lb. reuteri* group that was overrepresented on LAMVAB, in goat milk from the third sampling period and among populations targeting *M. plumbeus*. As for the *Lb. plantarum* group, that was overrepresented on MRS-10 °C in the first sampling period for goat and second sampling period for cow, and among populations targeting *K. lactis*, whereas the *Lb. buchneri* group was overrepresented on MRS-5.5 during the second sampling period in milk's cow and populations targeting *P. anomala*.

The diversity of antifungal *Lactobacillus* groups differed according to animal species and sampling periods (Table 5). Ewe milk harbored the smallest diversity compared to goat and cow milks with H index varying from 0.0 to 1.5 compared to 1.1 to 1.8 and 1.3 to 1.8 for cow and goat milks, respectively. A transient increase in lactobacilli diversity was however observed for all tested animals between the first and second sampling periods before decreasing in the third sampling period. With the exception of the first period of sampling in ewe characterized by an overrepresentation of the *Lactobacillus* casei group, no *Lactobacillus* group clearly dominated according to E values (Table 5).

4. Discussion

Facing the need for protective cultures adapted to dairy products, we wondered whether milk could be a natural reservoir of ALAB. This study aimed at giving an overview of ALAB communities present in raw milk collected over one-year from three different dairy animals (cow, goat and ewe) in three restricted geographic areas of France.

Table 3
Numbers of antifungal *Lactobacillus* spp. isolates according to their targeted fungi.

Targeted fungi	*Lactobacillus* phylogenetic groups			
	casei	*reuteri*	*plantarum*	*buchneri*
P. anomala	62 (−9)	20 (−4)	26 (+5)	**18 (+8)**
K. lactis	88 (−12)	33 (−1)	**47 (+17)**	10 (−3)
M. plumbeus	**230 (+21)**	**75 (+5)**	42 (−21)	23 (−5)

Data in brackets represent the differences between obtained values and that predicted with the Chi test of independence. Data in bold are significantly overrepresented in comparison to predicted values ($P<0.05$).

Table 4
Numbers of antifungal *Lactobacillus* spp. isolates according to animals and sampling periods.

Animals	Sampling periods	*Lactobacillus* phylogenetic groups			
		casei	*reuteri*	*plantarum*	*buchneri*
Ewe	First	2 (+1)	0 (0)	0 (0)	0 (0)
	Second	2 (−1)	0 (−1)	1 (0)	3 (+3)
	Third	10 (0)	0 (−3)	8 (+5)	0 (−1)
Cow	First	24 (+5)	0 (−6)	6 (0)	3 (+1)
	Second	33 (−30)	10 (−11)	**54 (+35)**	**15 (+7)**
	Third	**121 (+24)**	32 (−1)	6 (−23)	13 (0)
Goat	First	21 (−6)	6 (−3)	**19 (+11)**	2 (−2)
	Second	65 (+1)	21 (−1)	19 (0)	9 (0)
	Third	**102 (+7)**	**59 (+27)**	2 (−27)	6 (−7)

Data in brackets represent the differences between obtained values and that predicted with the Chi test of independence. Data in bold are significantly overrepresented in comparison to predicted values ($P<0.05$).

In a first step, total and lactic acid bacteria (LAB) populations were enumerated, for the three animals during the three sampling periods, on common non selective and LAB semi-selective media. Total bacterial concentration was significantly higher in goat's milk compared to cow's milk, whereas concentration of total bacteria in ewe's milk lay in between. Milk samples, whatever their origin were dominated by Gram-positive catalase-negative cocci (presumptive lactococci and *Leuconostoc*), whereas enterococci and lactobacilli were present in significantly lower concentrations.

Antimicrobial activities depend on microbial community composition as well as environment and growth conditions (Pfeiler and Klaenhammer, 2007; Rouse et al., 2008; Schillinger and Villarreal, 2010). Milk samples, targeted fungi and enumeration media used for ALAB screening were then chosen to provide the highest LAB biodiversity. Significant differences in antifungal activities were observed according to sampling periods, targeted fungi, growth media and milk origins. *P. expansum* was the most frequently inhibited fungus (3% of screened colonies), followed by *M. plumbeus* (2%), *K. lactis* (0.9%) and *P. anomala* (0.6%). Low occurrence of LAB active against *P. anomala* has already been observed by Magnusson et al. (2003) who did not find any active LAB against *P. anomala* over 1200 LAB strains tested on MRS agar. In contrast, *P. expansum* is described as a sensitive target for antifungal activity screening by Hassan and Bullerman (2008). Such difference may be due to their sensitivity to lactic and acetic acids. *P. expansum* is sensitive to organic acids (Dalié et al., 2010), whereas *P. anomala* can tolerate high concentrations of them (Lind et al., 2005).

In this study, the vast majority (1057 over 1235) of active colonies were recovered on MRS based-media in the third sampling periods, with the highest frequency found on LAMVAB—pH 5 (5.5% of screened colonies), followed by MRS—pH 5.5 (3% of screened colonies) and MRS—pH 6.6 (1% of screened colonies). Indeed, it is known that sodium acetate, present in MRS agar, increases antifungal activity of LAB and that acidic pH potentiate its effect (Lind et al., 2005; Stiles et al., 2002; Schillinger and Villarreal, 2010). No clear explanation can be drawn for the increase in the percentage of active colonies observed between the second and third sampling periods in cow and goat milks for LAMVAB and MRS-5.5 media. This increase was probably not linked to animal physiology because it was not observed in ewes (in end lactation during the third sampling period, like goats) and that cow's parturition was not synchronized, but rather to feeding and/or seasonal changes since the second sampling period occurred in June, and the third in fall for both goats and cows.

Among the 733 sequenced isolates, 94% belonged to the *Lactobacillus* genus, whereas other LAB, such as *Leuconostoc* or enteroccoci, able to grow on MRS-based media and strongly represented in tested milks, accounted for less than 4% of active colonies. This suggests that strains of the *Lactobacillus* genus, despite their relatively low proportion in the raw milks tested, were more capable to inhibit fungi than *Enterococcus* and *Leuconostoc* spp. Such finding confirms literature data showing that most ALAB strains belong to the *Lactobacillus* genus (Schnurer and Magnusson, 2005; Valerio et al., 2009; Voulgari et al., 2010).

Among the antifungal *Lactobacillus*, targeted fungal species differed according to the *Lactobacillus* group tested, whose presence largely depended on the period of sampling and milk origin. Lactobacilli were ranked into eight phylogenetic groups with 98% of them belonging to four phylogenetic *Lactobacillus* groups, namely *casei*, *reuteri*, *plantarum* and *buchneri*. Their occurrence was linked to their relative abundance in milk samples with *Lb. casei* overrepresented in the 100-fold dilutions in tested selective media, and frequently described as major *Lactobacillus* group in raw milk, as well as *Lb. plantarum* group (Franciosi et al., 2009; Medina et al., 2001). When these groups were assigned to different targeted fungi, certain specificity was observed. *Lb. casei* and *Lb. reuteri* groups were over-represented in ALAB population targeting *M. plumbeus*, while the *Lb. plantarum* group in ALAB population targeted *K. lactis*, and the *Lb. buchneri* group in ALAB population were active against *P. anomala*. The antifungal mechanisms of action of these *Lactobacillus* spp. are presently not elucidated, but their apparent specialization may be linked to the organic acids and/or ethanol produced and the sensitivity of targeted fungi to these molecules. The fact that very few homofermentative antifungal lactobacilli were isolated from milk, suggests that acetic acid production certainly played a role in antifungal activity of the recovered strains. However, it was not the only produced antifungal molecule. Analyses of organic acid production of several isolates showed that certain strains produced other antifungal molecules such as phenyllactic, 2-pyrrolidone-5-carboxylic acids or caproïc acid. It is then not excluded that a mixture of molecules such as hydrogen peroxide, carbon dioxide, fatty acids or low-molecular-weight compounds (2-pyrrolidone-5-carboxylic, phenyllactic and 4-hydroxyphenyllactic acids and cyclic dipeptides) were implicated, as for example in the few ALAB targeting *P. anomala* that is highly resistant to both lactic and acetic acids. The diversity as well as the number of active isolates between animals and sampling periods is difficult to explain, but this is probably linked

Table 5
Pi values for each *Lactobacillus* group according to animals and sampling periods. Shannon index (H) and Equitability (E) for each animal relative to a sampling period.

Animals	Sampling periods	*Lactobacillus* phylogenetic groups								**H**	**E**
		casei	*reuteri*	*plantarum*	*buchneri*	*delbrueckii*	*perolens*	*salivarius*	*coryniformis*		
Ewe	First	1.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	Second	0.33	0.00	0.17	0.50	0.00	0.00	0.00	0.00	1.46	0.49
	Third	0.56	0.00	0.44	0.00	0.00	0.00	0.00	0.00	0.99	0.33
Cow	First	0.73	0.00	0.18	0.09	0.00	0.00	0.00	0.00	1.10	0.54
	Second	0.29	0.09	0.47	0.13	0.00	0.02	0.00	0.00	1.82	0.61
	Third	0.68	0.18	0.03	0.07	0.01	0.01	0.01	0.01	1.49	0.45
Goat	First	0.44	0.13	0.40	0.04	0.00	0.00	0.00	0.00	1.62	0.37
	Second	0.55	0.18	0.16	0.08	0.02	0.02	0.00	0.00	1.82	0.61
	Third	0.59	0.34	0.01	0.03	0.02	0.00	0.00	0.00	1.35	0.50

to environmental and feeding conditions. The use or not of silage, which was absent from the ewe's feed and not given in spring (second period of sampling) seems to be a possible source of ALAB (Kalač, 2011).

It appears from this study that raw milk from cow and goat, contrary to ewe's milk can be considered as reservoir of ALAB. Majority of antifungal isolates belonged to the *Lactobacillus* genus. This genus is certainly one of the most active against fungi among LAB found in raw milk. Further studies are actually performed in our laboratory to test the potential antifungal effect of different *Lactobacillus* spp. in fermented dairy products and to elucidate their mechanism of action.

Acknowledgments

This study was conducted with the financial support of the French National Agency for Research (ANR): program FUNGINIB (ANR-09-ALIA-005-01), and the French Ministry of Higher Education and Research.

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