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Current Microbiology

ISSN 0343-8651

Volume 64

Number 5

Curr Microbiol (2012) 64:486-491

DOI 10.1007/s00284-012-0098-3



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Effects of Roundup® and Glyphosate on Three Food Microorganisms: *Geotrichum candidum*, *Lactococcus lactis* subsp. *cremoris* and *Lactobacillus delbrueckii* subsp. *bulgaricus*

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Received: 25 January 2012 / Accepted: 2 February 2012 / Published online: 24 February 2012
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Abstract Use of many pesticide products poses the problem of their effects on environment and health. Amongst them, the effects of glyphosate with its adjuvants and its by-products are regularly discussed. The aim of the present study was to shed light on the real impact on biodiversity and ecosystems of Roundup®, a major herbicide used worldwide, and the glyphosate it contains, by the study of their effects on growth and viability of microbial models, namely, on three food microorganisms (*Geotrichum candidum*, *Lactococcus lactis* subsp. *cremoris* and *Lactobacillus delbrueckii* subsp. *bulgaricus*) widely used as starters in traditional and industrial dairy technologies. The presented results evidence that Roundup® has an inhibitory effect on microbial growth and a microbicide effect at lower concentrations than those recommended in

agriculture. Interestingly, glyphosate at these levels has no significant effect on the three studied microorganisms. Our work is consistent with previous studies which demonstrated that the toxic effect of glyphosate was amplified by its formulation adjuvants on different human cells and other eukaryotic models. Moreover, these results should be considered in the understanding of the loss of microbial diversity and microbial concentration observed in raw milk for many years.

Introduction

Any modification of the environment which leads to a response by living organisms may be considered as a stress [1]. Stresses affecting biological functions are classified according to two categories: (i) abiotic including physical and chemical stresses, and (ii) biotic. Furthermore, the stress response in biology, actually considered as a global phenomenon, can be extended to anthropogenic pressure such as genetic engineering or xenobiotics (including Roundup) pollution [2].

The necessity for better control of food microorganisms, which are widely used in traditional and industrial dairy technologies, requires knowledge concerning their response to numerous stresses [3]. Amongst the high number of food microorganisms found in raw or fermented milk [4], some of them, such as *Lactobacillus delbrueckii* subsp. *bulgaricus* (*L. bulgaricus*), *Lactococcus lactis* subsp. *cremoris* (*L. cremoris*) and *Geotrichum candidum* (*G. candidum*) have been deeply studied at the stress response level [5, 6], but, to our knowledge, not when the stress is induced by herbicide exposures.

The lactic acid *L. bulgaricus* is a starter lactic acid bacteria (LAB) that is commonly used in fermented food

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technology [4, 7]. Besides its primary function to produce lactic acid during the fermentation process, *L. bulgaricus* also contributes in the proper development of both flavour (production of aromatics compounds such as acetoïne and acetaldehyde) and texture (polysaccharides synthesis) [8, 9]. Amongst the LAB, *L. lactis* plays the role of model besides its role as major contributor in cheese processing [4]. Most of the food starters are based on this species which includes many subspecies and strains including the *cremoris* subspecies. The most important properties are the ability to produce acid in milk and to convert milk proteins to flavour components. *G. candidum* is a deuteromycete fungus whose teleomorph state is *Galactomyces geotrichum* [10, 11]. It is found in raw milk and is widely used for its major application as a ripening agent in cheese-making [12]. In France, *G. candidum* is the dominant yeast-like fungus from smear-ripened soft cheese and mould-ripened cheese in which it forms a substantial part of the microflora [4, 13].

It is estimated that at least 99% of the microorganisms on earth are not cultivable using current technologies. Paradoxically enough, this tremendous uncontrolled microbial diversity could be the reason for the lack of interest in the endangered bacteria hypothesis [14]. While plant and animal preservation are approached both in situ and ex situ, preservation of microorganisms is managed only ex situ [15]. Studies on the distribution of subspecies within the species *L. cremoris*, have shown the difficulty of isolating the *cremoris* subspecies from the dairy environment in some geographic areas. Some hypothetical intrinsic and extrinsic causes can be proposed to analyse this type of rarefaction. Over the past few decades, the environment of *L. cremoris* has been drastically modified by human activity, giving rise firstly to anthropogenic stress for instance pesticides and secondly to biodiversity reduction (plant varieties in pasture) [14].

Roundup is the major herbicide used worldwide partly because 70% of GM food plants are Roundup-tolerant [16]. Roundup formulations contain glyphosate, claimed active component, and also adjuvants like polyethoxylated tallowamine (POEA) for a better stability and penetration by modifying the physicochemical properties of formulations [17].

Glyphosate and AMPA (aminomethyl phosphonic acid), its main metabolite, are amongst the first major pollutants of surface waters [18]. Roundup reduces the biodiversity of species in the aquatic community [19]. Currently, the maximum tolerated glyphosate residue in milk in Europe is 0.05 mg/kg or 0.05 ppm [20]. Glyphosate is found in humans at the ppm level [21]. Some of us have previously observed that Roundup is cytotoxic to human placental, embryonic kidney and liver cell lines at very low sub-agricultural dilutions [22–25].

In vivo, effects of Roundup on male reproductive system of ducks and rabbits [26, 27] and liver of rats [28, 29] have been observed. It is known that Roundup has negative effects on fauna and flora at different qualitative and quantitative levels. In the sea urchin egg, cell cycle alteration and inhibition of transcription were observed [30, 31].

In microbiology, it was reported that glyphosate affects growth and metabolism of *Saccharomyces cerevisiae* [32]. Moreover, glyphosate is more toxic in distilled water than in seawater on prokaryotic bacteria *Vibrio fischeri* [33]. Also, *Tetrahymena pyriformis* a eukaryotic cilli protozoan is less sensitive to glyphosate and AMPA than *V. fischeri* [34].

The work described in the present article was carried out on three microorganisms of food interest, to highlight the impact of the Roundup herbicide and of glyphosate on food microbial world. The need to study and preserve the microbial diversity in dairy ecosystems (i.e. raw milk, cheese and yogurt) is of major interest for future developments in food processing.

Materials and Methods

Strains and Culture Conditions

Experiments were performed with *G. candidum* ATCC 204307, *Lactobacillus delbrueckii* subsp. *bulgaricus* CFL1 and *Lactococcus lactis* subsp. *cremoris* ATCC 19257. *G. candidum* was cultured in MSF, pH 5.6; *L. lactis* in M17 containing lactose, pH 7.1 (AES Laboratoire, Combourg, France); and *L. bulgaricus* in MRS, pH 6.4 (AES Laboratoire). Cultures recovered in stationary phase of growth were used to inoculate new media at 2% (volume of liquid culture/volume of culture broth). The initial cell concentrations before treatments are $1.95.10^4 \pm 0.36$, $6.3110^5 \pm 2.03$ and $6.70.10^8 \pm 2.52$ UFC/ml for *G. candidum*, *L. bulgaricus* and *L. cremoris*, respectively.

Roundup and Glyphosate Treatments

Roundup R400 (400 g/l of glyphosate) and R450 (450 g/l of glyphosate) (Monsanto, Anvers, Belgium) were diluted in autoclaved culture media, pH adjusted to each medium and 0.2 μ m filtered. A solution of glyphosate (Sigma-Aldrich, Saint-Quentin Fallavier, France), equivalent in glyphosate concentration and pH to R450, was diluted in different media.

Turbidimetry Measurement and Colony Counting

Experiments were performed with 96-well plates. The minimal inhibitory concentration (MIC) was evaluated,

after treatment, by turbidimetry measurement at 600 nm using a microplate reader (Metertech Σ 960, Taipei, Taiwan). The minimal microbicide concentration (MMC) which corresponds to the minimal treatment leading to 99.99% of lethality was evaluated by colony counting, after plating the previously treated micro-organisms. Concentrations between the MIC value and the MMC value correspond to cells that do not growth but are not dead. For these experiments, it is not necessary to wash the cells before plating. The final dilution is so important that the herbicide residues are then negligible.

Colonies Observations

Each microorganism was plated on Petri dishes containing agar and Roundup, and then incubated during 48 h. Colonies were macroscopically observed using a Canon EOS 350D camera and microscopically using a Leica DMLB microscope (magnification $\times 500$ for *G. Candidum* and $\times 1,000$ for *L. bulgaricus* and *L. cremoris*) after coloration with cotton blue for *G. candidum* and methylene blue for the two bacteria.

Statistical Analysis

Data were expressed as mean \pm SEM for three independent determinations ($n = 9$). Significant differences were determined by Student *t* test with $P < 0.05^*$, $P < 0.01^{**}$ and $P < 0.001^{***}$.

Results

Our first observation is the relatively comparable toxicity profiles on three essential food microorganisms, in a short 24-h period (Fig. 1a–c). Roundup is always more potent than glyphosate, and in all cases, toxic from levels 10–100 times below the lowest agricultural uses (10,000 ppm). *G. candidum* and *L. cremoris* are more Roundup sensitive than *L. bulgaricus*. Similar impacts have not been observed for glyphosate alone (except for *G. candidum* at 10,000 ppm). Roundup effect was not proportional to glyphosate concentration in the Roundup formulation, since R400 is almost 10-fold more inhibitory than R450 (Fig. 1a). A specific biphasic inhibitory effect was transiently observed at low doses (around 100 ppm) for *L. cremoris*, and possibly for *G. candidum*. Microbicide effect of Roundup was obtained at concentrations 1.2, 1.6 and 2 times higher than the measured MICs for *L. bulgaricus*, *G. candidum* and *L. cremoris*, respectively (Table 1). The macroscopic observations (Fig. 2) showed differences in sensitivity between the three micro-organisms: *L. cremoris*, *G. candidum* and *L. bulgaricus*, and no colonies were

observed at 200, 1,000 and 2,000 ppm of Roundup, respectively. Considering the size, colonies of *G. candidum* and *L. bulgaricus* decrease depending on the concentration of Roundup (Fig. 2g, i) present. Microscopically, no significant differences were observed between controls and treated colonies. However, it seems that cell contents of *L. bulgaricus* leaks out (Roundup 1,000 ppm; Fig. 2l).

Discussion

The relatively comparable toxicity profiles on these three essential food microorganisms is surprising since the fungus is phylogenetically far from the other two [35]. It has previously been shown that glyphosate and Roundup are toxic and can reduce fungal growth at concentrations above 10 ppm for mycorrhizal fungi (*Hebeloma crustuliniforme*, *Laccaria laccata*, *Thelephora americana*, *T. terrestris* and *Suillus tomentosus*) [36]. Similarly, some adverse effects were observed at concentrations below those found in the soil after typical applications. Moreover, differences in the sensitivities of the different species have also been observed towards the herbicide [36, 37]. The inhibitory and microbicide effects observed at agricultural sub-doses in this work are consistent with those previously observed in fungi [36, 37]. Glyphosate is believed to be the major active principle in the herbicide through its inhibition of EPSPS. Glyphosate metabolism varies by organism [38]. Glyphosate blocks EPSPS (5-enolpyruvylshikimate-3-phosphate synthase), a key enzyme involved in the biosynthesis of aromatic amino acids, naturally present in plants, fungi and some bacteria. EPSPS, which exists in two classes—glyphosate-sensitive and glyphosate-tolerant—is essential in plants for protein production via the shikimic acid pathway [39–41]. There could be an enzyme highly tolerant to glyphosate, which would have some genetical homologies with the first and/or the second classes of this enzyme [42, 43]. This enzyme is absent in mammals. However, other inhibition pathways are involved, like Cyp450 aromatase inhibition, since glyphosate is weakly responsible of the cytotoxicity on eukaryotic cells (human placental, embryonic or umbilical cord cells) [22, 23, 25] and cellular endocrine disruption [24]. This is in agreement with the lethality observed on the three food microorganisms studies in the present work. Moreover, like on eukaryotic cells, impact of glyphosate is not proportional to its concentration in Roundup formulations, confirming adjuvants are not inert—an observation that supports the findings of previous studies [23, 25]. Similar effects on microorganisms have been reported previously in the literature; in fact, it appeared that *Ichthyophthirius multifiliis* and *T. thermophila* tolerate glyphosate but not Roundup. The commercial formulation was then 100 times more toxic than the active

Fig. 1 Growth inhibition of the three microorganisms (**a** *Geotrichum candidum*, **b** *Lactococcus lactis* subsp. *cremoris* and **c** *Lactobacillus delbrueckii* subsp. *bulgaricus*) after 24 h of incubation in growth media supplemented with Roundup (grey circles 400 and black circles 450) or equivalent amount of glyphosate (triangles) evaluated by turbidimetry (600 nm). The herbicides' concentrations are up to the lowest agricultural uses (10,000 ppm). SEMs are shown in all instances ($n = 3$); Student test ($P < 0.05^*$; $P < 0.01^{**}$ and $P < 0.001^{***}$)

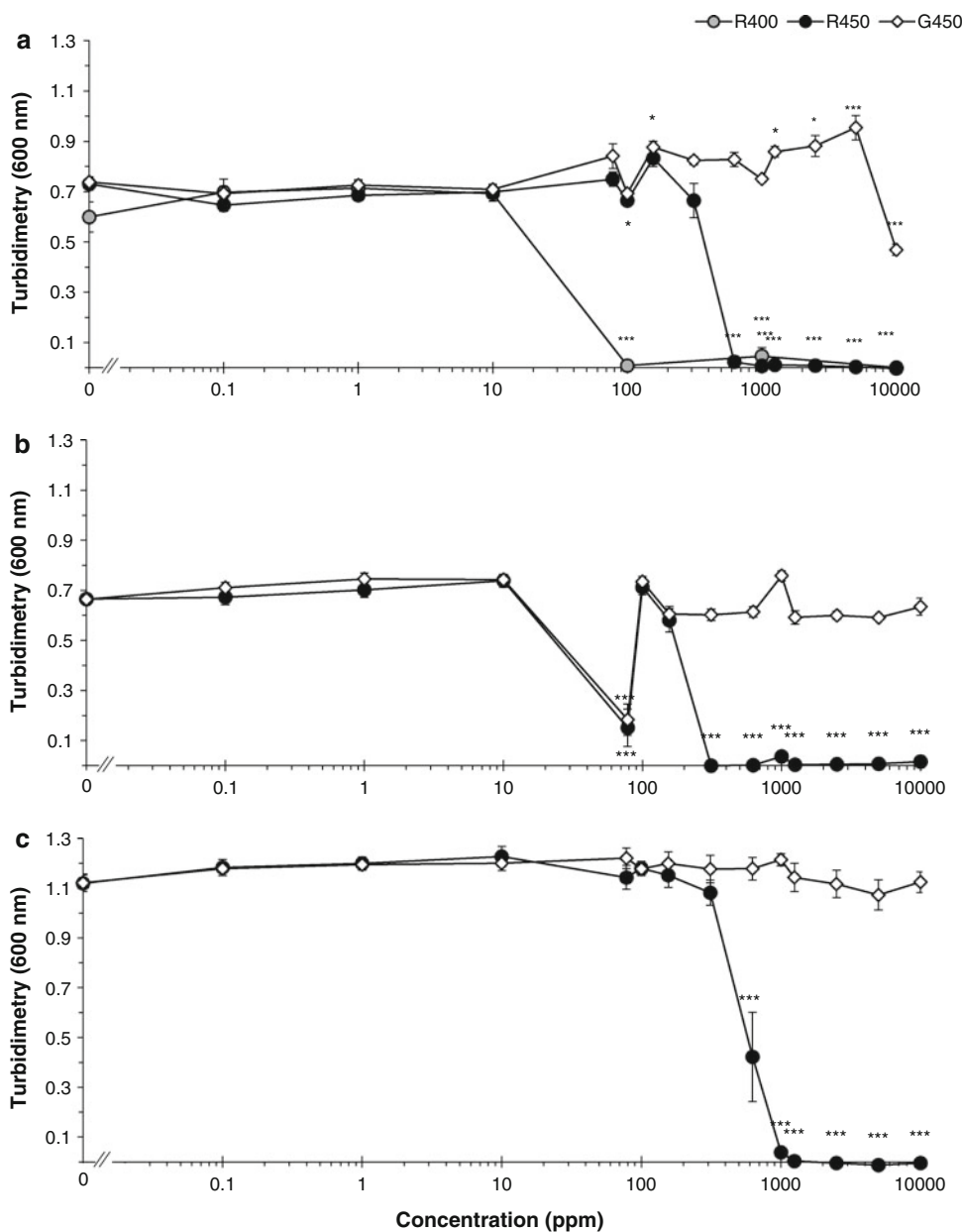


Table 1 MIC and MMC for three microorganisms (*Geotrichum candidum*, *Lactococcus lactis* subsp. *cremoris* and *Lactobacillus delbrueckii* subsp. *bulgaricus*) after 24 h of incubation in growth media supplemented with Roundup or equivalent amount of glyphosate

Strain	Glyphosate in Roundup® (g/l)	MIC (ppm)	MMC (ppm)
<i>G. candidum</i> ATCC204307	400	100	1,000
	450	625	1,000
<i>L. lactis</i> subsp. <i>cremoris</i> ATCC19257	450	312	625
<i>L. delbrueckii</i> subsp. <i>bulgaricus</i> CFL1	450	1,000	1,250

The minimal agricultural use of the herbicide is 10,000 ppm

ingredient (G) [44]. Amongst these adjuvants, POEA which promotes a xenobiotic penetration into cells, is more toxic than glyphosate [23]. Microorganisms studied here are 10 times less sensitive than human embryonic, placental and hepatic cells. At non-toxic concentrations, some endocrine biphasic disrupting effects have already been observed in human cells with Roundup [24]. This could be the case of a receptor-mediated phenomenon with a stimulation followed by internalization and desensitization. Thus, if there is a stress reaction widely distributed in evolution, we could suggest that biphasic inhibitory effects at non-toxic doses of Roundup and glyphosate on *L. cremoris* and probably *G. candidum* could be receptor-mediated.

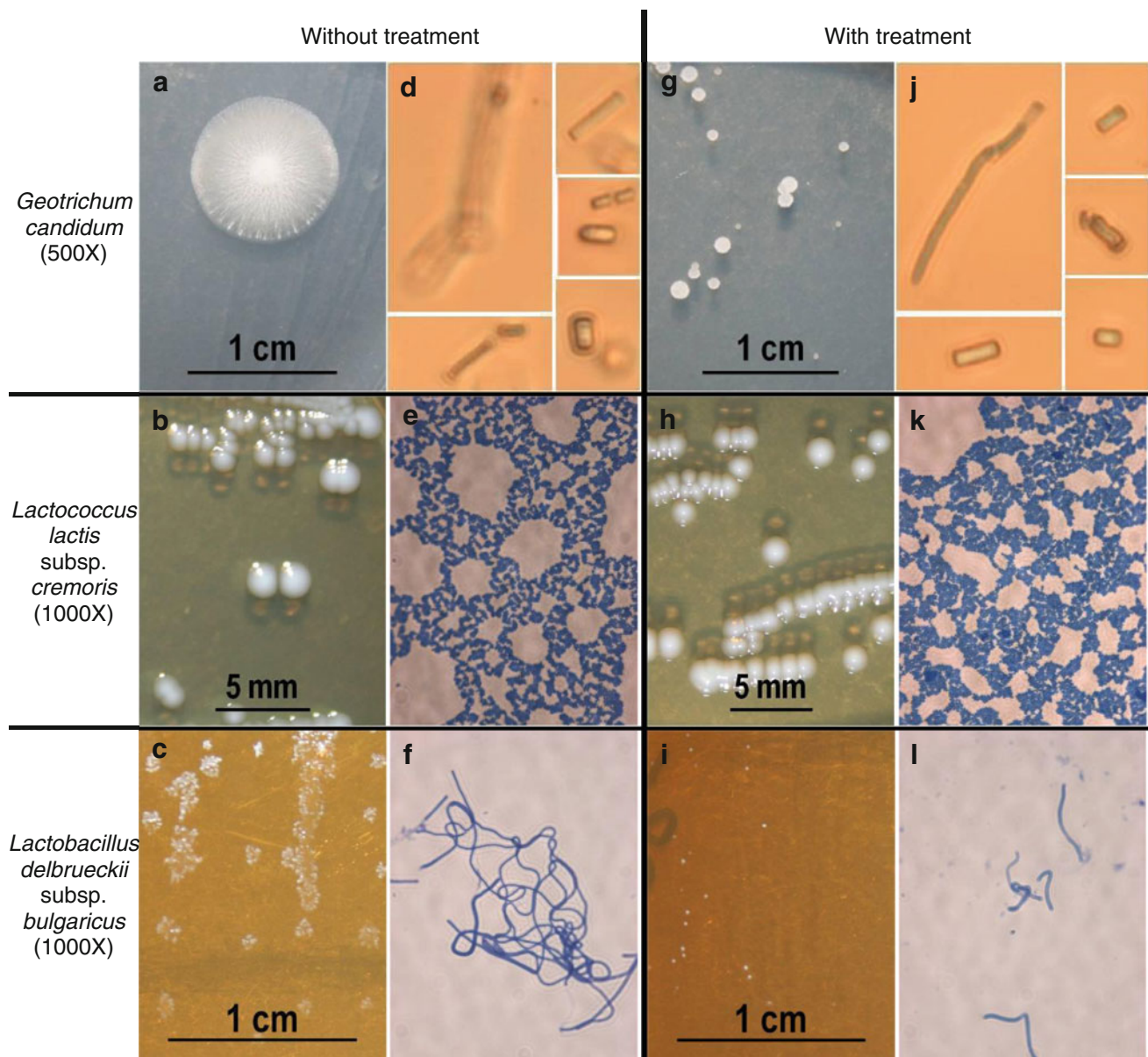


Fig. 2 Macroscopic (a–c, and g–i) and microscopic (d–f and j–l) observations of *Geotrichum candidum*, *Lactococcus lactis* subsp. *cremoris* and *Lactobacillus delbrueckii* subsp. *bulgaricus*, after 48 h of incubation on solid growth media supplemented with Roundup

(500 ppm for g and j, 100 ppm for h and k, 10,000 ppm for i and l) or without (for a, d, b, e, c and f). The herbicide concentrations are up to the lowest agricultural uses (10,000 ppm)

In conclusion, the pesticide Roundup sprayed on Roundup tolerant GMOs and on non-agricultural soils could thus impact on specific biodiversity including food interest microorganisms. This is illustrated by the fact that actual food processing requires industrial food starters for milk fermentation. Furthermore, unpredictable consequences of Roundup on soil microorganisms have to be considered.

Acknowledgments The authors thank J.-M. Bré for his technical assistance during the experimentation. This work was supported by the Ministère de l'Enseignement Supérieur et de la Recherche. E. C. holds a fellowship from the Conseil Régional de Basse-Normandie and CRIIGEN (Committee for Independent Research and Information

on Genetic Engineering). The authors would also like to thank Testbiotech for their support.

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