

Microbiological Origin of “Framboisé” in French Ciders

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ABSTRACT

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In this work, populations of acetic acid bacteria and zymomonads were studied and the kinetics of several parameters followed in three ciders: one “healthy”, one “framboisé” and one stabilized. In the “framboisé” cider, correlations were observed between cider-sickness symptoms (reduction of sugars, production of CO₂ and increase of ethanol and acetaldehyde content) and the presence of a bacteria growing on a zymomonads medium. When inoculated in a “healthy” cider at pH 4.1, the bacteria induced cider-sickness symptoms while no symptoms were observed at pH 3.7. The bacteria were characterized and identified by sequencing of the 16S rDNA region and corresponded to a strain of *Z. mobilis pomaceae*. A detection study on 17 spoiled ciders and 5 “healthy” ciders appeared to indicate that there was a high prevalence of this microorganism involved in “framboisé” cases in France. To our knowledge this is the first description of the involvement of *Z. mobilis* strains in French “framboisé” ciders.

Key words: Cider, cider-sickness, framboisé, spoilage, *Zymomonas mobilis*.

INTRODUCTION

Cider is a complex product elaborated from apple must by a fermentation process. Although the presence of microorganisms (yeast and lactic acid bacteria) is necessary for the production of the final product, cider is susceptible to the growth of spoilage microorganisms because of its sugar richness and low acidity. One of the problems encountered in French cider production is the spoilage called “framboisé” or cider-sickness in English. Early descriptions of this spoilage have been reported in England² and in France¹⁸.

This cider-sickness is observed in sweet ciders with a pH greater than 3.7 and can occur in industrial as well as in traditional cider companies, in tanks, in wood casks or in bottles. It has been known for a long time that pH is an important parameter for development of the spoilage⁸. This can be explained by the sensitivity of the microorganisms to low pH (<3.5). Recently, a study from ARAC⁶

(Association de Recherche Appliquée à la transformation Cidricole) showed that no “framboisé” cases were observed for ciders with pH lower than 3.75 while 92% of bottled ciders with pH > 3.90 developed the sickness. This spoilage is virtually unknown in English cidermaking today since the ciders are generally at a pH < 3.5 and are never stored sweet.

The spoilage is characterized mainly by the production of an off-flavor described as a “banana-skin”, “rotten lemon” or “raspberry” (framboise in French) aroma. This flavor is correlated with the accumulation of high concentrations of acetaldehyde in the medium (150 to 400 mg/L and up to 1000 mg/L while the legal limit in France is 120 mg/L for cider and 100 mg/L in “cidres bouchés”⁷). Acetaldehyde is probably not the only compound responsible for this spoilage, as under production conditions, a decrease of acetaldehyde is observed, but the spoilage is still detectable to a lesser extent and is designated as “framboisé passé” or “faded” cider-sickness. Other features of “framboisé” are an “almost explosive” production of gas generating high pressure (3 to 9 bars) in bottles, accompanied by a thin and persistent foam and a dense white turbidity of the beverage, due to the combination of phenolic compounds with acetaldehyde¹².

These different features make the product undrinkable and not saleable from an organoleptic point of view as well as from a legal one. The frequency of this spoilage is not constant and can range from 5 to 17% of the annual farm production (ARAC, CTPC – Centre Technique des Produits cidricoles – personal communication). For example, in 2002, the ARAC¹⁰ showed that 7% of 193 tested ciders exhibited acetaldehyde levels higher than the legal limit.

The origin of this spoilage is now considered to be microbial, but the microorganism responsible and the spoilage mechanisms are not certain. So far two hypotheses have been proposed. According to Maugenet¹³, the spoilage could be caused by the production of D- and L-lactic acids in the cider that would be metabolized to acetaldehyde by *Acetobacter rancens*. In England, Barker *et al.*³ discovered a micro-organism, *Zymomonas mobilis pomaceae*, responsible for cider-sickness showing the same symptoms as “framboisé”. This bacterium is able to ferment glucose into ethanol and CO₂ with an accumulation of acetaldehyde via the Entner-Doudoroff pathway. So far, this bacterium has never been isolated from French ciders.

The aim of this work was to determine the microbiological origin of “framboisé” in French ciders by isolating and identifying the bacteria able to create this kind of spoilage.

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Table I. Evolution of different parameters in ciders A (“healthy”), B (“framboisé”) and C (stabilized).

	Days						
	0	3	6	9	13	16	20
Acetaldehyde (mg/L)							
Cider A	14	16	16	17	14	26	12
Cider B	53	29	52	313	365	193	205
Cider C	41	28	35	51	53	65	50
Reducing sugars (g/L)							
Cider A	34.3	34.8	34.5	36.3	35.6	34.1	34.2
Cider B	50.6	56.0	54.7	49.5	38.9	35.4	35.2
Cider C	54.1	55.4	56.0	57.1	55.8	53.6	56.1
Ethanol (% v/v)							
Cider A	4.20	4.23	4.30	4.30	4.30	4.30	4.30
Cider B	3.70	3.62	3.70	4.08	4.50	4.70	4.70
Cider C	3.60	3.56	3.50	3.60	3.60	3.60	3.60
pH							
Cider A	3.42	3.43	3.53	3.58	3.65	3.61	3.77
Cider B	4.14	4.17	4.11	4.16	4.16	4.16	4.18
Cider C	3.89	3.91	3.89	3.91	3.90	3.84	3.92
Total acidity (g H ₂ SO ₄ /L)							
Cider A	3.14	3.43	3.60	2.94	2.80	2.70	2.65
Cider B	2.10	2.23	2.28	2.35	2.28	2.33	2.47
Cider C	2.74	2.99	3.19	2.72	2.94	2.94	3.09
Volatile acidity (g H ₂ SO ₄ /L)							
Cider A	0.08	0.11	0.14	0.06	0.13	0.10	0.19
Cider B	0.29	0.37	0.40	0.34	0.34	0.38	0.47
Cider C	0.29	0.24	0.27	0.30	0.33	0.28	0.41
Polyphenols (g/L)							
Cider A	2.51	2.47	2.25	2.70	2.57	3.00	3.00
Cider B	1.62	1.63	1.56	1.70	1.90	1.90	1.90
Cider C	1.99	1.82	1.95	1.80	2.10	2.10	2.40

MATERIALS AND METHODS

Materials

The selection of “framboisé” and “healthy” ciders was done by the ARAC using the predictive test for the early detection of “framboisé” developed by the association¹¹. This test consisted of the incubation of a cider bottle at 25°C for a period of one month. At 15 and 30 days there was a check for the symptoms of spoilage. Several liters from these original tanks were used to perform the experiments reported in this paper. This test, based on the temperature difference between the tested bottle and the cider tank, allowed for early detection of spoilage, and the cider could be studied before any sign of the spoilage appeared and then during the entire spoilage process.

Media and cultural conditions

A series of 10-fold dilutions in TS (Tryptone Salt, AES Laboratoire, France) was carried out for each sample. Aliquots (0.1 mL) of the appropriate dilution were plated onto specific media. Acetic acid bacteria were grown for 6 days under aerobic conditions at 30°C, on MRS agar (AES Laboratoire, France) with the pH adjusted to 4.8 using 10% citric acid. Growth of yeast and lactic acid bacteria were inhibited by adding 100 mg/L pimaricine (Delvolid, DSM Food Specialties, France) and 30 mg/L penicillin (Sigma-Aldrich, France). Zymomonads were grown for 4 days at 30°C on ZPP (*Zymomonas Pimaricine Penicillin*) medium (glucose 20 g/L, peptone 5 g/L, yeast extract 3 g/L; malt extract 3 g/L and agar 15 g/L), a medium developed by our laboratory for the detection of these organ-

isms. Growth of yeast and lactic acid bacteria were inhibited as described previously and the growth of acetic acid bacteria was inhibited by incubation under anaerobic conditions.

Characterization and identification of microorganisms

Bacteria isolated were characterized using the Gram stain, oxidase and catalase tests, as well as API50 CH galleries (API-System-Biomérieux, France). Furthermore, the bacteria of interest were identified by the sequencing of their 16S rDNA. The bacterial DNA was extracted using the DNeasy Tissue Kit (Qiagen, France) according to manufacturer’s instructions. The PCR mix, 100 µL final volume, contained: 1.5 mM MgCl₂, 0.2 mM dNTP (Sigma-Aldrich, France), 20 pmol of each primers, 10 mL 10× PCR buffer (Invitrogen, France), 25 ng of extracted DNA and 2.5 units *Taq* polymerase (Invitrogen, France). The PCR amplifications were performed¹⁹ using primers BSF8 and BSR1541 in a Gradient Master Cyclor (Eppendorf, France) under the following conditions: denaturation at 95°C for 5 min and 39 cycles of denaturation 95°C for 45 s, primer annealing 59.5°C for 45 s and DNA extension at 72°C for 2 min. A final extension was completed at 72°C for 5 min. Ten µL of each reaction mixture was run on a 0.8% agarose gel (w/v) (Invitrogen, France) in the presence of 100 bp ladder (Invitrogen, France) to evaluate the quality and the size of the amplified fragment. The amplified fragment was then purified using the GenElute PCR Clean-up kit (Sigma-Aldrich, France) according to the manufacturer’s instructions and sequenced by the MWG Biotech company (Germany).

Analytical methods

Total reducing sugars, total acidity, volatile acidity and alcoholic degree were analyzed using the methods described in "Recueil des méthodes internationales d'analyse des vins et des moûts"¹⁶ (Compendium of International Methods of Analysis of Wine and Must). Glucose, fructose and sucrose were all enzymatically analyzed using the same kit (ref. E-0716260, Roche Diagnostics Technologies, France) according to the manufacturer's instruction; polyphenols were measured by the Folin-Ciocalteu reagent method¹⁵ and acetaldehyde by the method of Jaulmes and Espezel¹.

RESULTS AND DISCUSSION

Two ciders, one "healthy" and one "framboisé", were selected by the predictive test. The ARAC provided the bottles of the "healthy" cider (cider A), bottles of the cider susceptible to cider-sickness (cider B) and bottles of the same cider stabilized by the addition of 100 mg/L of D/L malic acid (Standa Industrie, France) and 120 mg/L of SO₂ (Littoral Œnologie, France) (cider C). These conditions have been shown to stop spoilage of the cider (ARAC, personal communication).

The evolution of acetic acid bacteria and of bacteria capable of growing on ZPP medium was followed and several chemical and physical parameters were measured.

Kinetics

For the three ciders, acetaldehyde, reducing sugar, ethanol and polyphenol concentrations, as well as pH and total and volatile acidity, were followed during this experiment. The results are presented in Table I.

In cider A and C, the acetaldehyde concentrations were stable over the 20 day period of the experiment. However with cider B, an evolution of this concentration was observed. Between day 0 and day 6, the acetaldehyde content was stable, then between day 6 and 13, it rapidly increased to 365 mg/L. Between day 13 and 20, the concentration decreased and stabilized at around 200 mg/L.

Concerning the reducing sugars and ethanol, differences were observed. While in ciders A and C, reducing sugars and ethanol concentrations were stable during the experiment, in cider B, a decrease of about 15 g of sugar was observed during this time period, and this decrease was accompanied by a 1% (v/v) increase in ethanol.

No major differences between the three ciders were observed for pH, total and volatile acidity and polyphenol content.

The results for the acetaldehyde concentrations showed that cider A was a "healthy" cider and cider B was "framboisé", therefore confirming the results of the predictive test. The fact that cider C didn't show any sign of "framboisé" confirmed that the addition of 100 mg/L D/L malic acid and 120 mg/L SO₂ to a cider presenting a high risk of "framboisé" was able to prevent spoilage. In parallel to the acetaldehyde production in cider B, a decrease of sugar and an increase of ethanol were observed accompanied by high pressure and persistent foam. The observed pH levels confirmed the importance of this parameter re-

garding the risk of "framboisé". Cider A exhibited a pH of 3.42 at day 0 and of 3.77 at day 20. The difference in pH was probably due to the completion of the malolactic transformation in this cider. Cider B exhibited a pH of 4.14. The pH observed for cider C, the stabilized cider, remained at 3.90 (the observed difference of pH between ciders B and C is certainly due to malic acid addition). No

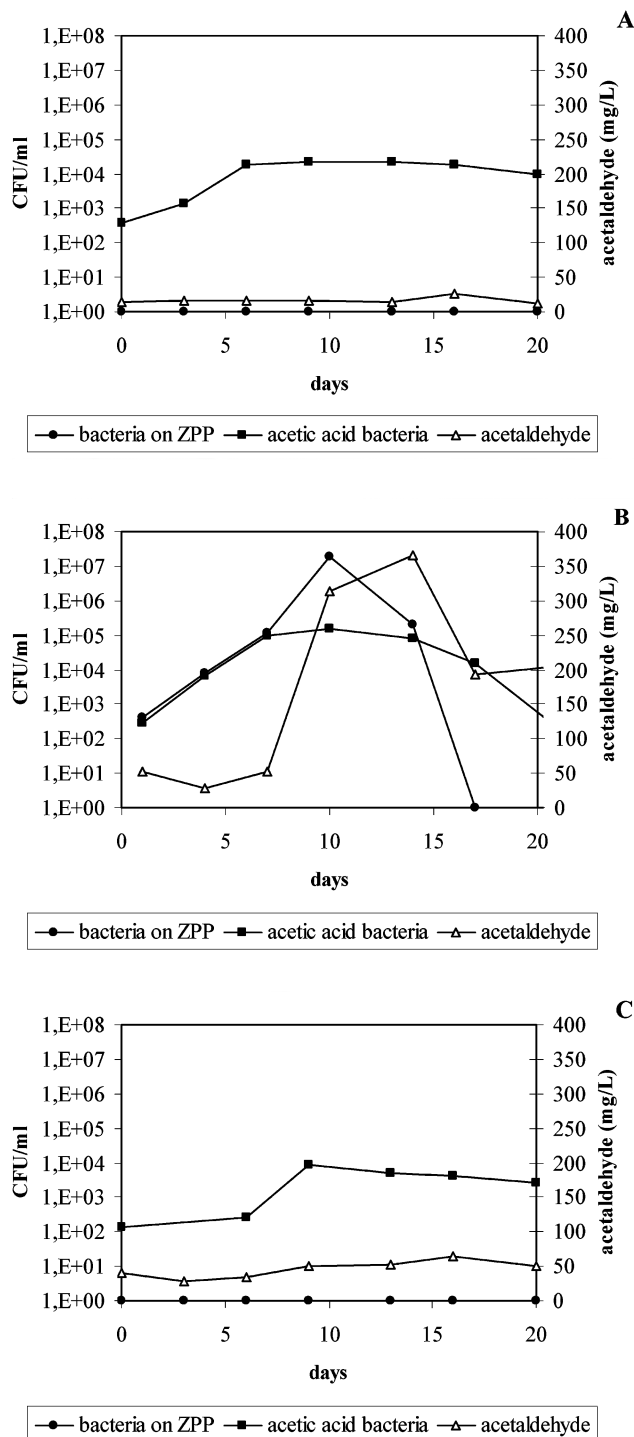


Fig. 1. Evolution of bacteria growing on ZPP (●, CFU/ml), acetic acid bacteria (■, CFU/ml) and acetaldehyde concentration (△, mg/L) in three ciders: (A) "healthy", (B) "framboisé" and (C) stabilized.

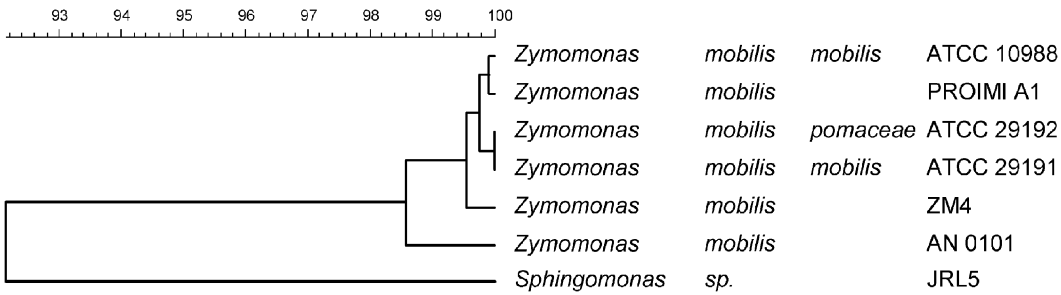


Fig. 2. Dendrogram of 16S rDNA sequences of *Zymomonas mobilis pomaceae* AN 0101, *Zymomonas mobilis mobilis* ATCC 10988, *Zymomonas mobilis* PROIMI A1, *Zymomonas mobilis pomaceae* ATCC 29192, *Zymomonas mobilis mobilis* ATCC 29191, *Zymomonas mobilis* ZM4 and *Sphingomonas* sp. JRL-5 obtained by using the Pearson correlation on Bionumerics (Applied Maths, Belgium).

clear correlation was observed between the “framboisé” and total and volatile acidity or polyphenol content.

The evolution of the acetaldehyde concentration, as a marker of “framboisé”, and the populations of acetic acid bacteria and other bacteria which grew on ZPP, were plotted to look for correlations (Fig. 1). In cider A, acetaldehyde was stable, and acetic acid bacteria were present at 10^3 CFU/mL on day 0 and stabilized at 10^4 CFU/mL at day 6. No bacteria grew on ZPP medium. In cider B, the acetaldehyde concentration was 350 mg/L on day 13. Acetic acid bacteria increased from 10^3 CFU/mL on day 0 to 10^5 CFU/mL on day 6, and remained stable. Bacteria which grew on ZPP medium were detected at around 10^3 CFU/mL on day 1 and reached 2.10^7 CFU/mL on day 9. Then the population decreased rapidly and was no longer detectable after day 16. In cider C, the evolution was similar to cider A.

These results showed a strong correlation between classical symptoms of “framboisé” (acetaldehyde formation, decrease in sugar content, increase of ethanol content, high pressure and persistent foam) and the presence of bacteria growing on ZPP media. Sensory evaluation of the ciders during the experiment confirmed the “framboisé” character of cider B, while no alteration in flavor was detected in ciders A and C. The one log difference of acetic acid bacteria populations observed between ciders B and C was probably due to the addition of SO_2 in the latter. The results of this experiment suggest that the bacteria which grew under anaerobic conditions on ZPP medium were able to form ethanol, CO_2 and acetaldehyde from sugar, and thus could be responsible for the “framboisé” spoilage in French ciders.

Identification of bacteria

The bacteria isolated on ZPP, coded AN 0101, appeared as typical colonies on this media; round, white, glossy, 0.5 to 2 mm diameter, with a small peak in the middle. The colonies resembled an anaerobic Gram negative large bacillus. The catalase test was positive and the oxidase test was negative. An API gallery 50CHE was employed to investigate the metabolism of AN 0101, and after 48 h, only a strong assimilation of glucose and fructose and a

weak assimilation of 5-ceto-gluconate were observed. To better identify the bacteria, the gene corresponding to the 16S rDNA was obtained after a PCR experiment and then sequenced. The 1436 bp long sequence (Genbank accession number: AY350737) was compared to international sequences in databases and showed 98% identities with the sequences of 5 strains of *Zymomonas mobilis*, namely ZM4 (AF117351), PROIMI A1 (AF088897), ATCC 10988 (AF281033), ATCC 29191 (AF281034) and ATCC 29192 (AF28281032), and only 92% with *Sphingomonas* sp. JRL-5 (AF1851572) (Fig. 2). Taxonomically, *Zymomonas mobilis* has been divided into two subspecies *Zymomonas mobilis mobilis* and *Zymomonas mobilis pomaceae*¹⁷. *Z. mobilis mobilis* has been isolated from bees, from ripening honey in Spain, from fermenting sap of *Agave americana* in Mexico, from fermenting palm juice (*Arenga pinnata*) in Java and Indonesia, and from *Elaeis guineensis* and *Raphia vinifera* in Zaire and Nigeria¹⁷. It has also been isolated from spoiled beers in England⁵. *Z. mobilis pomaceae* was isolated in England from sick cider^{3,14} and apple pulp⁴. No differences in the 16S rDNA sequences were observed between *Zymomonas mobilis mobilis* phenotypic centrotypic strain ATCC 29191 and *Zymomonas mobilis pomaceae* type strain ATCC 29192, while 20 differences were observed between the *Z. mobilis* AN 0101 sequence and the sequences of the previously mentioned strains. To identify the strain at the subspecies level, two tests (growth in presence of 0.5% NaCl and growth at 36°C), as described by Swing and De Ley¹⁷, were used. According to the authors, strains of *Z. mobilis mobilis* can grow in the presence of salt and at 36°C while *Z. mobilis pomaceae* cannot. Strain AN 0101 showed no growth after 48 h in liquid medium in the presence of 0.5% NaCl and at 36°C, while the type strain of *Z. mobilis pomaceae* ATCC 29192 and the reference strain of *Z. mobilis mobilis* ATCC 29191 grew in the presence of 0.5% NaCl and at 36°C. These results combined with the DNA sequence of the reference strain *Z. mobilis pomaceae* ATCC 29192 brought into question how to differentiate the two subspecies and the validity of the identification of this strain as a *Z. mobilis pomaceae*. The strain isolated during this study was designated as *Zymomonas mobilis pomaceae* AN 0101.

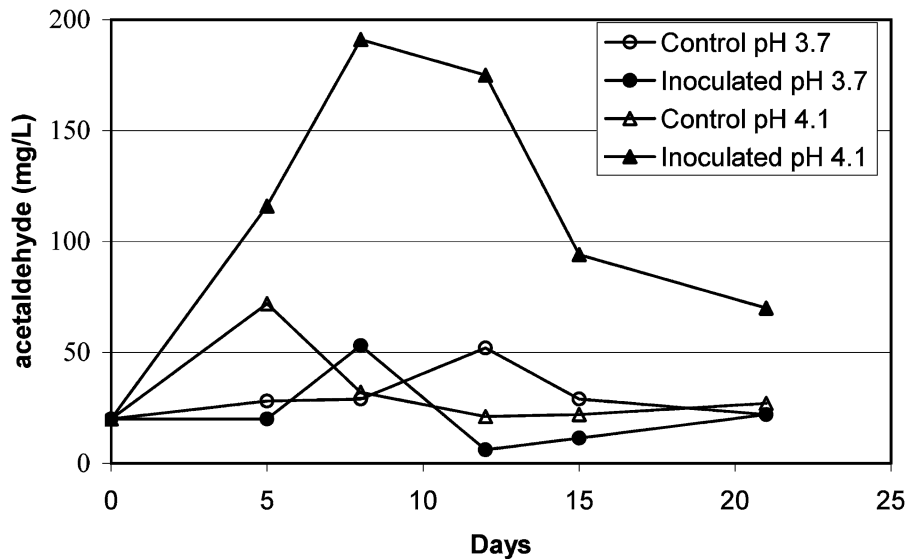


Fig. 3. Evolution of acetaldehyde concentrations under four different conditions: control cider pH 3.7 (○), cider pH 3.7 inoculated with 2% *Z. mobilis pomaceae* AN 0101 (●), control cider pH 4.1 (△) and cider pH 4.1 inoculated with 2% *Z. mobilis pomaceae* AN 0101 (▲).

Reproduction of the “framboisé” spoilage

To confirm the role of the isolated strain in “framboisé”, attempts were made to reproduce this spoilage in a “healthy” cider. The ARAC provided 20 liters of a “healthy” cider (no sign of framboisé after predictive test). This cider was characterized as follows: density was 1022, alcohol content 3.8% (v/v), total sugars 47.7 g/L and acetaldehyde 20 mg/L. Ten liters was adjusted to pH 3.7 and 10 liters to pH 4.1, and 12 bottles of each were filled. For each pH condition, 6 bottles were inoculated with 2% (v/v) of an overnight grown culture of *Z. mobilis pomaceae* AN 0101. The inoculum was approximately 10^5 CFU/mL. The bottles were incubated at 25°C and acetaldehyde concentrations were followed over a 21 day period. The results are presented in Fig. 3.

At pH 3.7, no significant differences between the control cider (non-inoculated) and the inoculated cider were observed, and the acetaldehyde concentration stayed at low levels during the experiment. At pH 4.1, a large difference was observed. The acetaldehyde concentration in the control rose to 72 mg/L at day 5, but stayed at a low level during the rest of the experiment. The cider inoculated with strain AN 0101 showed a peak of acetaldehyde of approximately 200 mg/L at day 7 and then decreased slowly to reach 70 mg/L at day 21. At this point the cider was qualified as “framboisé passé”. This behavior has previously been observed (ARAC, personal communication) and may be explained by the use of acetaldehyde by different cider microorganisms and by acetaldehyde combination with cider polyphenols⁸. Other symptoms of the spoilage were also noticeable during the experiment. Only the inoculated bottles at pH 4.1 were cloudy, and when opened, high pressure and foaming resulted in loss of cider from the bottles. The same results were observed on different ciders (data not shown). These results confirmed that strains of *Zymomonas mobilis pomaceae* AN 0101 can be responsible for “framboisé” and that pH is of great importance in the development of this spoilage.

Prevalence of *Z. mobilis pomaceae* in “framboisé” in French ciders

To estimate the prevalence of *Z. mobilis pomaceae* in the French cases of “framboisé”, 17 spoiled ciders and 5 “healthy” ciders from different cider producing regions of France (Calvados, Côte d’Armor, Morbihan, Maine et Loire and Oise), were obtained and examined for the presence of *Zymomonas mobilis* using ZPP medium. In 13 out of 17 cases of “framboisé”, colonies isolated on the ZPP medium exhibited the same characteristics as *Z. mobilis pomaceae* AN 0101 (Gram negative bacilli, catalase positive, oxidase negative), and sequencing of their 16S rDNA region confirmed the identification as strains of *Z. mobilis*. None of the 5 “healthy” ciders showed the presence of this type of bacteria.

These results suggest that *Z. mobilis pomaceae* was responsible for a majority of cases of “framboisé”. It must be noted that the 4 “framboisé” ciders negative for *Z. mobilis pomaceae* were obtained later and this delay in analysis could explain the fact that no additional *Zymomonas* strain was detected. Moreover, if the time of analysis is of such importance it could also explain why, to date, no *Zymomonas* has been observed in French ciders. However the possibility that the “framboisé” can result from the metabolism of lactic acid bacteria and acetic bacteria cannot be fully ruled out, as was demonstrated by Maugenet *et al.*^{9,13}. To evaluate definitively the prevalence of *Z. mobilis pomaceae* in “framboisé” sickness, a detection study of this microorganism should be carried out on a large number of ciders representative of French cider production.

In this study, we have described for the first time, to our knowledge, the identification of strains of *Zymomonas mobilis* involved in cases of “framboisé” or cider-sickness in French ciders. Several questions arose from the results of this study. The origin of contamination of the cider is still unknown. The pH appears to be an important factor for the expression of the spoilage, but other environmental

factors could also be of importance (nitrogen content, polyphenols, etc.). Occurrence of *Z. mobilis* in ciders and its prevalence in cases of “framboisé” requires further study. Field observations showing that SO₂ can be used to reduce the risk of cider sickness do not correlate with previous findings showing that *Z. mobilis* is not inhibited by 0.02% SO₂ (legal limit in the United Kingdom)¹⁷. As SO₂ is supplemented along with D/L malic acid in production conditions, further studies are necessary to understand the role of SO₂, malic acid and pH reduction in *Zymomonas* inhibition. Taxonomy of *Z. mobilis* species also needs to be further studied to confirm (or not) the results obtained for *Z. mobilis pomaceae* type strain ATCC 29192.

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