Report of the Scientific Committee of the Spanish Agency for Food Safety and Nutrition (AESAN) on the effectiveness of maturation for more than 60 days for goat's cheese and cheese from other species produced from raw milk from herds where tuberculosis is detected, in order to ensure their safe consumption

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Working group

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Abstract

Royal Decree 1086/2020 which regulates and makes flexible certain conditions for applying the European Union's provisions on hygiene in the production and marketing of food products and regulates activities excluded from its area of application, permits the flexibility included in European Union regulations to be applied at the national level and to use milk from herds that do not fulfil the requirements with regard to brucellosis and tuberculosis, with the same requirements as in Regulation (EC) No. 853/2004.

In the case of ovine and caprine herds that do not fulfil the requirements for tuberculosis, the Regulation opens the doors to other possible processing methods that ensure food safety. Therefore,

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the Scientific Committee of the Spanish Agency for Food Safety and Nutrition (AESAN) has been requested to establish if maturation for a period greater than 60 days for cheeses produced with raw goat's milk or from other species sensitive to tuberculosis other than bovine, from females that do not have a positive reaction to tuberculosis tests or display symptoms of this disease but belong to a herd where it has been detected, may ensure the safety of these cheeses with regard to tuberculosis.

The Scientific Committee concludes that although the prevalence of tuberculosis in Europe is low, the global importance of this disease requires extreme precautions for monitoring the main sources of contagion, raw milk and dairy products being one of the main source of contamination in persons.

The special resistance of the causative agents of tuberculosis are one of the main reasons for its survival in dairy products, there being few differences between them with regard to the species that produces the milk.

Both classical references as well as more recent literature on the topic has established the survival of the causative agents of tuberculosis for periods greater than 60 days. For this reason, it cannot be guaranteed that maturation for a period greater than 60 days eliminates the members of the *Mycobacterium tuberculosis* complex.

Key words

Tuberculosis, cheese, survival, *Mycobacterium tuberculosis.*

Suggested citation

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1. Introduction

Section IX of Annex III of Regulation (EC) No. 853/2004 laying down specific hygiene rules for food of animal origin sets specific requirements for the production of raw milk, colostrum, dairy products and colostrum-based products (EU, 2004).

Chapter I of the aforementioned section lists the health requirements for the production of raw milk and colostrum which, with regard to tuberculosis, are specified as follows:

2.b) raw milk and colostrum must come from:

i. cows or buffaloes from a herd that, in accordance with Council Directive 64/432/EEC (EU, 1964) have been officially declared tuberculosis-free, or
ii. female animals of other species that belong, in this case, to species susceptible to tuberculosis, to herds regularly inspected for this disease according to an inspection plan approved by the competent authority.

(…)

3. Nevertheless, upon authorisation by the competent authority, raw milk and colostrum from animals that do not fulfil the requirements of Point 2 may be used:

a) in the case of cows and buffaloes that do not test positive for brucellosis or tuberculosis, nor display the symptoms of these diseases, after having been subjected to a thermal treatment until testing negative for alkaline phosphatase;
b) in the case of sheep or goats that do not test positive for brucellosis or have been vaccinated against brucellosis within the framework of an authorised eradication programme, and which do not display the symptoms of this disease:
   i. either for manufacturing cheeses with a maturing period of at least 2 months, or
   ii. after having been subjected to a thermal treatment until testing negative for alkaline phosphatase, and
c) in the case of female animals of other species that have not tested positive for tuberculosis or brucellosis and do not display symptoms of these diseases, but belong to a herd where these diseases have been detected by means of the tests mentioned in Point 2, letter a), clause iii), or Point 2, letter b), clause II), if they have been subjected to a treatment that guarantees their safety.

Article 12 of Royal Decree 1086/2020 which regulates and makes flexible certain conditions for applying the European Union's provisions on hygiene in the production and marketing of food products and regulates activities excluded from its area of application, permits the flexibility included in European Union regulations to be applied at the national level and to use milk from herds that do not fulfil the requirements with regard to brucellosis and tuberculosis, with the same requirements as in the regulation (BOE, 2020).

Thus, while the regulation text makes it clear that cow's milk from herds that do not fulfil the requirements relating to brucellosis and/or tuberculosis may only be used after thermal treatment, in the case of milk from other species susceptible to tuberculosis, it opens the door to other possible treatments that guarantee the safety of the food.

In the case of sheep and goat herds that do not fulfil the requirements relating to brucellosis, apart from thermal treatment, it is permitted to use the milk to manufacture cheeses with a minimum maturing period of 60 days. However, this possibility is not expressly stated in the case of tuberculosis.

This lack of clarity when drafting the regulation has led to numerous queries and different interpretations by the competent authorities.

For the interpretation of what is deemed a "sensitive species" for the application of the Regulation, we shall take the current Chapter 8.11 of the Terrestrial Animal Health Code of the OIE (2017) "Infection with *Mycobacterium tuberculosis* complex".

According to this document, for the purposes of applying Regulation (EC) No. 853/2004, sensitive species shall be those in regions or provinces that have not declared goats, camelids and cervids free of tuberculosis (EU, 2004). Sheep may be considered a sensitive species only if they are part of a herd consisting essentially of cows and/or goats that live in close quarters, provided:

a. the disease has been diagnosed in said herd, or

b. the goats do not have an inspection plan approved by the competent authority.

The Scientific Committee of the Spanish Agency for Food Safety and Nutrition (AESAN) has been requested to draft a report establishing whether maturation for a period greater than 60 days for cheeses produced with raw goat's milk or from other species sensitive to tuberculosis other than bovine, from females that do not have a positive reaction to tuberculosis tests or display symptoms of this disease but belong to a herd where it has been detected, may ensure the safety of these cheeses with regard to tuberculosis.

2. The *Mycobacterium* genre and tuberculosis

Bacteria belonging to the *Mycobacterium* genre have a series of special characteristics that are responsible, to a large degree, for the pathogenic potential of this type of agent. It is important to highlight that of the more than 170 species that are taxonomically included in this genre, those that have been the subject of the majority of research in this field are the causative agents of infection by the *Mycobacterium tuberculosis* Complex (MTC), especially *Mycobacterium tuberculosis* (Fedrizzi et al., 2017). The species included within the *Mycobacterium tuberculosis* Complex (MTC) are a group of relatively similar species that cause tuberculosis. This infection is one of the 10 major causes of mortality in persons worldwide (Gagneux, 2018) (WHO, 2020). The species that are included within the MTC are *Mycobacterium tuberculosis, Mycobacterium bovis, Mycobacterium africanum, Mycobacterium caprae, Mycobacterium microti* and *Mycobacterium pinnipedii* (Riojas et al., 2018). Of these, *M. caprae* was isolated mainly from goat lesions in Spain (Aranaz et al., 1999). Initially, it was classified as a subspecies of *M. tuberculosis* and finally, in 2003, it was reclassified as a species (Aranaz et al., 2003). As a member of the MTC, it has also been responsible for multiple cases both in humans (Cvetnic et al., 2007) (Prodinger et al., 2014) and in animals (Cvetnic et al., 2007) (Rodríguez, 2011) (Mendoza et al., 2012).

Although the disease is usually caused by *Mycobacterium tuberculosis* in humans, all the agents included in the MTC are able to infect them. Within the MTC species, *M. bovis* is usually responsible for a significant proportion of cases (de la Rua-Domenech, 2006), which are otherwise clinically indistinguishable from the ones caused by *M. tuberculosis*, and in some studies reach values higher than 30 % of tuberculosis cases in persons (Torres-González et al., 2016). *M. bovis* is the causative agent of tuberculosis in cattle, however it is considered a principal agent from a public health perspective in developing countries. In these countries, the number of tuberculosis cases in persons which may be attributed to *M. bovis* is not well known (de la Rua-Domenech, 2006). In some developed countries, a third of all tuberculosis cases in persons has also been attributed to *M. caprae* (Kubica et al., 2003). Although the transmission of tuberculosis in persons is usually by the inhalation of aerosols or direct contact due to skin abrasions, historically, the oral transmission of *M. bovis* through the ingestion of raw milk from infected cows or dairy products from raw milk has been considered one of the main entry points of these pathogens.

The specific resistance of MTC species to different exogenous elements is one of the central elements of their pathogenic potential. Undoubtedly, their characteristic lipid-rich capsule, as well as other elements, give them resistance to the host's immune cells. Additionally, their resistance to acid pH values boosts their survival in the phagosomes of the immune cells. In this way, the *Mycobacterium* bacterial capsule acts as an initial barrier to an acidic environment. A proton pump system and other elements are able to maintain the internal environment within these bacteria at a sufficiently neutral pH, even if they are in an external acidic environment (Vandal et al., 2009). The maintenance of this internal homeostasis has also been highlighted for other mycobacteria such as *M. bovis*, which forms part of the MTC, and other rapidly growing bacteria that are not part of the MTC such as *M. smegmatis* (Rao et al., 2001) or *Mycobacterium avium* (Bodmer et al., 2000). Its impermeable capsule is considered responsible for the great intrinsic resistance of *M. tuberculosis* to multiple antimicrobials, which is a key aspect in the treatment of this disease. However, it has been pointed out that the capsule characteristic of these agents cannot solely account for their high resistance to antibiotics (Morris et al., 2005). Another especially relevant aspect is the thermal resistance of mycobacteria. In this regard, while *M. bovis* has been shown to be sensitive to thermal pasteurisation treatment, even with high levels of inoculate, other species such as *M. avium* have displayed much greater resistance to thermal treatment (Grant et al., 1996).

Occasionally, only one or a few animals in a herd may shed these mycobacteria. Nevertheless, the high excretion of *Mycobacterium bovis* bacilli per ml of milk even in sub-clinically infected animals (de la Rua-Domenech, 2006) makes it easy for the contamination to spread. The ease of contamination for *M. bovis* from the milk of an animal to others in the herd from the milking equipment has also been demonstrated (de la Rua-Domenech, 2006). Thus it is shown that by virtue of the high resistance of this agent, in the event that there is one or a few infected animals, the products of the herd are prone to contamination with mycobacteria. Additionally, the use of cold tanks and mixing milk from different animals is guaranteed to contaminate the entire product. Contamination by *M. bovis* from pasture, water and other elements has also been described (Neill et al., 1994). It has also been demonstrated that animals with negative results for intradermal tests for *M. bovis* do not constitute sufficient proof to identify animals as truly negative (Zarden et al., 2013).

3. Differences between cow and goat's milk and cheese

From an organoleptic perspective, it may be pointed out that there are differences between goat's milk and cow's milk, and also between cow's cheese and goat's cheese. Nevertheless, when we look at their chemical composition, the differences are not so evident. Classically, many of the principal goat's milk proteins have been highlighted as being similar to those in cow's milk (Jenness, 1980) and even some enzymatic characteristics of the milk of these small ruminants may permit greater microbial growth, such as possessing lower lipase, ribonuclease or oxidase activity (Parkash and Jenness, 1968) than cow's milk. Regarding the lipid profile, there are differences between both types of milk, especially in the medium-chain fatty acid content (C6-C14), which is higher in goat's milk, similar to polyunsaturated fatty acids of the n-6 and n-3 series. Also, the levels of the minerals Ca, P, Mg, and Cu are higher in goat's milk ash (Sanz Ceballos et al., 2009). There are significant differences in the fatty acid profiles of cow's milk and goat's milk for some compounds. Nevertheless, the magnitude of the difference makes it almost impossible for them to affect the survival of these agents. Many fatty acids have been traditionally deemed agents with an inhibitory potential on bacteria (Fay and Farias, 1975) (Wang and Johnson, 1992) (Kelsey et al., 2006). In this regard, Sanz Ceballos et al. (2009) highlighted important differences in relation to the concentration of conjugated linoleic acid (CLA), it being significantly higher in goat's milk than in cow's milk. This difference was established at +0.23 g/100 g of total fatty acids. Differences similar to those observed between cow's cheese and goat's cheese in relation to CLA (Van Nieuwenhove et al., 2009). According to Choi (2016), the minimum inhibitory concentration of CLA against *M. tuberculosis* would be 200 µg/ml of CLA. Other authors have also established a possible inhibition of various bacteria by CLA and even bactericidal effects (Byeon et al., 2009). Nevertheless, many of these effects would be due to changes in the lipids of the bacterial wall which lead to modifications in permeability. Given the slow growth of these agents along with the significant but small difference between cow's milk and goat's milk with regard to CLA concentration, there does not appear to be differences between both types of products in relation to a greater inhibition of the growth of *M. tuberculosis* or *M. bovis.* Not only should the different biochemical characteristics between cow's milk and goat's milk be noted, but also the cultures that are added or present in fermented products may have an influence in the sense that they may be antagonists or inhibit the growth of *M. bovis* or other mycobacteria (Mariam, 2009, 2014). Although there are many inhibitory substances that are found in foods under normal circumstances, in practice their inefficiency in producing an inhibitory effect on the growth of pathogens in many foods has been demonstrated, either requiring very high doses or they simply lose their effectiveness outside laboratory conditions. Perhaps the most well-known case may be the use of bacteriocins, which are inhibitory peptides, that because of proteolytic phenomena that take place, for example, in cheese, are ineffective in these types of products. The use of concrete strategies, for example, the use of bacteriocins, essential oils, specific lactic microbiota, etc. would require additional specific tests to precisely characterise their effectiveness against mycobacteria.

4. Prevalence of tuberculosis caused by *Mycobacterium bovis* or *Mycobacterium caprae* in persons and animals

According to data from the latest European Food Safety Authority (EFSA) report on zoonosis, in 2019, the incidence of tuberculosis in the European Union caused by species of *M. bovis* or *M. caprae* was occasional in humans, with 147 confirmed cases (EFSA, 2021). The reporting rate has remained equally low between 2015 and 2019, ranging between 0.03 and 0.05 cases/100 000 inhabitants. It is worth pointing out that since 2004, there have been no reports to the EFSA of food toxiinfections linked to these species.

Most of the cases reported in 2019 (69.4 %) had their origin in the European Union. Approximately, 2/3 of the cases reported were originated in countries deemed not free from tuberculosis.

The trends demonstrated in the levels of prevalence of bovine tuberculosis in different countries of the European Union prove that there is a different evolution between those deemed officially tuberculosis-free (OTF) and those that are not. In OTF countries, there has been a decrease of 37.0 % and 14.5 % in the annual number and the prevalence of positive heads of cattle respectively, in the period between 2010 and 2019.

Conversely, in non-OTF countries, the annual general number of positive herds reported decreased proportionally by 8.6 % while the prevalence increased by 72.1 %. This increased prevalence may be explained in part by the increase in the number of positive herds detected in these countries along with a significant decrease in the total number of herds due to the changed designation of non-OTF countries to TF, over the period of 2010-2019.

In Spain, the National Programmes for the Eradication of Bovine Tuberculosis 2006-2010 entailed a qualitative change in the setting of goals, such that they set the bases for ensuring continued actions in time according to a multi-annual approach established in 5 years.

In accordance with the information compiled by the Ministry of Agriculture, Fisheries and Food (MAPA, 2021), the prevalence of herds with bovine tuberculosis in Spain has descended since 2016 to below 2 % in herds with positive animals in 2019. This decrease has been due to the increased sensitivity of diagnosis and the application of measures included in the Action Plan Against Tuberculosis in Wild Species (PATUBES, 2017).

With regard to incidence in human beings, in 2018, there were a total of 4386 reported cases of tuberculosis (9.39 cases/100 000 inhabitants) in Spain, the majority of these linked to pulmonary tuberculosis. Since 2012, the total incidence has decreased by 6 % of the annual average.

5. Transmission of tuberculosis by dairy products

Classically, milk and dairy products have been highlighted as key elements in the transmission of tuberculosis caused by *M. bovis* (Keogh, 1971) (Milian-Suazo et al., 2010). On several occasions, the need to continue to monitor for bovine tuberculosis and the pasteurisation of dairy products has been highlighted after the description of outbreaks based on the evidence of person to person transmission of *M. bovis*, after a supposed initial infection from an environmental source due to a bovine tuberculosis (Evans et al., 2007). In this manner, without any type of epidemiological confirmation, *M. bovis* has also been identified in milk (Kazwala et al., 1998) (Leite et al., 2003) and in fresh cheese made with raw milk (Harris et al., 2007), or has been identified with molecular techniques (Cezar et al., 2016), confirming the findings of the epidemiological data. For example, in this regard, the epidemiological study of tuberculosis due to *M. bovis* in the United States (Hlavsa et al., 2008) demonstrated that 82.6 % of patients with tuberculosis caused by this species revealed that they had eaten cheese produced in Mexico, linking this circumstance also to the higher incidence of tuberculosis in Mexico and the production of cheese with raw milk. There seems to be clear evidence that the consumption of raw milk with *Mycobacterium* will lead to the transmission of the disease to persons who consume this milk without thermal treatment. However, the survival of MTC agents during the maturation of cheeses must be looked into, as it may be assumed that the biochemical processes that take place during the maturing of this product will eliminate the agent. Once more, the classic literature has assessed this circumstance, reiterating the high resistance of these bacteria against different types of disinfectants, acids, alkalis and therefore, they are less sensitive to the typical acidification of fermented products. Keogh (1971) and Hammer and Babel (1957) (cited in Keogh (1971)), reviewed the survival of *M. tuberculosis* in an important variety of cheeses, and concluded that although the type of cheese has an influence on the higher or lower survival rate of this pathogen, it is able to survive for more than 2 months in most cheeses for which data currently exists. Thus, they mention 220 days of survival in Cheddar, 305 days in Tilsit, and 3 months in Camembert cheese. Other authors such as Frahm (1959), also cited in Keogh (1971), point to the viability of *M. tuberculosis* in Camembert and Edam cheeses after more than 2 months, along with other authors from the mid-20th century, while they note that there is a decline in the number of these bacteria in Emmenthal cheese made with artificially contaminated milk, they also indicate that after 3 months they were able to infect guinea pigs and cause tubercular lesions in these test animals. Their survival has been documented not only in cheese, but also in contaminated milk stored for more than 4 months. Likewise, there are references to the survival of *M. bovis* in butter for at least 100 days (Kleeberg (1984), cited in De la Rua-Domenech (2006)).

Most of these scientific documents offer data that, in spite of their age, continue to be robust owing to their use of biological confirmatory tests, in addition to the well-defined characteristic anatomopathological lesions that enable a relatively precise diagnosis. Nevertheless, it is worth verifying whether the most recent scientific literature confirms the survival of these pathogens, which otherwise seems to be firmly based on their intrinsic survival characteristics. Here we may cite the work by Forgrave et al. (2016), who researched the kinetics of survival of *M. bovis* in the preparation and maturing of lab-produced Cheddar and Caerphilly cheese with milk artificially contaminated with high and low levels of *M. bovis*. The results showed that at the level of preparation, *M. bovis* is retained in the curd, similar to other pathogens, and a lower proportion is lost in the whey, also noting, as has been mentioned in the classic literature on this subject, a decreased viability of the pathogen, albeit limited. Thus, *M. bovis* was isolated in cheeses prepared with a high level of inoculate (≈4-6 log cfu/g in the cheese of the first day of maturing) after 393 days for Cheddar cheese and 145 days for Caerphilly cheese. In the cases of low levels of inoculate (≈3 log cfu/g) Forgrave et al. (2016) detected the pathogen in Cheddar cheese for 63 days and for

56 days in Caerphilly cheese. Ramírez Starikoff et al. (2016) also studied the evolution of *M. bovis* and *Brucella abortus* during the maturation of Parmesan cheese. These researchers noted that pH acid development in cheese has a significant effect in reducing the population of *Brucella abortus*, nonetheless, it does not appear to be significant for *M. bovis*, which is in line with all that has been highlighted until now. While colonies of the pathogen could not be counted after day 32 for *Brucella abortus* in spite of using a relatively high initial inoculate (5.8 cfu/g), in the case of *M. bovis*, there was a slight reduction of 1.5 logarithmic cycles on day 63 of maturing, passing from 5.5 log cfu/g on day 1 to 4.1 log cfu/g on day 63.

Conclusions of the Scientific Committee

Although the prevalence of tuberculosis in Europe is low, the global importance of this disease requires extreme precautions for monitoring the main sources of contagion, raw milk and dairy products being one of the main sources of contamination in persons.

The special resistance of the causative agents of tuberculosis are one of the main reasons for their survival in dairy products, there being few differences between them with regard to the species that produces the milk.

Both classical references as well as more recent literature on the topic have established the survival of the causative agents of tuberculosis in cheeses for periods greater than 60 days. For this reason, it cannot be guaranteed that the maturation of cheeses for a period greater than 60 days eliminates the members of the *Mycobacterium tuberculosis* complex.

The specific characteristics of dairy products that may justify the destruction of these agents would require additional scientific testing.

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