Research and Toxinogenic Characterization of Penicillium Contaminating Goat's Traditional Dairy Products in Northern Morocco

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Abstract

The goat rearing in northern Morocco had known a very important evolution with the introduction of European breeds of goats more productive of milk compared to local breeds. Moulds are common contaminants in many traditional dairy products. Some species are responsible for significant economic losses and major public health problems by producing toxic metabolites like mycotoxins. This work aims to isolate, identify and

characterize the mycotoxin-producing species of *Penicillium* from milk and traditional cheese (Jben) in the different northern region of Morocco, based on microbiological analysis and *in vitro* identification of *Penicillium* toxinogenic by fluorescence detection and HPLC analysis. Our microbiological analysis clearly show that the fungal flora is highly developed in goat's milk and Jben: 85 mould and yeast isolates from 57 samples collected. The rate of fungal flora (mould and yeast species) is high, it is around 2,6 10^4 CFU/ml in milk and 5,90 10^4 CFU/g in Jben. *Penicillium* isolates were purified and identified according to macroscopic and microscopic criteria. The proportion of *Penicillium* (61,53 %) in milk is more than Jben (16,94 %). The most important *Penicillium* species in two dairy products are *P.italicum* (23,1% in milk against 5,1 % in Jben), *P. cyclopium* (7,7 % in milk against 5,1% in cheese), and *P. expansum* (7,7% in milk against 3,4 % in cheese). According to in vitro identification of *Penicillium* toxinogenic by fluorescence detection, the majority of *Penicillium* isolates were able to produce mycotoxin type Citrinin and Ochratoxin A. The capacity of mycotoxin production by the identified strains was confirmed by High-Performance Liquid Chromatography Analysis. The patulin was produced by all *Penicillium* isolates, while the citrinin was produced by *P. nalgiovense*, *P. simplicissimum*, and *P. dipodomyis*. Unlike the ochratoxin A was produced by all isolates, except the *P. nalgiovense* and *P. dipodomyis*. The presence of toxicogenic *Penicillium* species in Moroccan traditional goat's dairy products suggests that contaminated products according to processing conditions and after storage traditionally could be a risk to the health and safety of consumers. The awareness-raising measures, the professional guidance on the concept of hygienic quality and the tendency to semi-industrial production remain work-sites to work more efficiently by the state.

Keywords: Milk, Cheese (Jben), Toxinogenic *Penicillium*, Citrinin, Ochratoxin A, Patulin, HPLC

Introduction

In recent years, goat breeding in northern Morocco has undergone a very important evolution with the introduction of European breeds of goats (Alpine, Murciano-Granadina) more productive of milk compared to local breeds. The ecosystems of the Rif chain offer geographical and nutritional favourable conditions for wild development of goats, whose main objective is milk production and consequently the repercussions on the local socio-economic development.

The major destination of milk production in the northern region of Morocco is self-consumption and the traditional manufacture of a single type of fresh milk cheese, known as "Jben". It is a flat, unpackaged cheese,

generally resulting from natural fermentation and draining, sold directly in the traditional way on dwarf palm leaves at roadsides and in the weekly markets.

Extensive farming and artisanal practices ranging from animal husbandry, to milk production, to the manufacture Jben and its marketing, mean that hygienic quality requirements are often far from being met (Zantar *et al.,* 2007). The lack of hygiene has been well demonstrated by microbiological analyses carried out on milk and Jben from Morocco (El Marrakchi 1996; Zantar *et al.,* 2007).

Fresh cheese like any other fermented product is characterized by the presence of bacterial and fungal microorganisms, however Jben and milk from northern Morocco have faecal parasites and a high level of lactobacilli and fungal flora ranging up to 7.9. 10^4 CFU/g (Zantar *et al.,* 2007).

Having regard to the traditional chain of collection and marketing of milk and Jben in northern Morocco, the risk of contamination of these dairy products by mycotoxins produced by some toxinogenic fungi of this fungal flora will be higher.

Penicillium is one of the types of moulds found in milk and cheese, some are useful in the refining of cheese (Hidalgo *et al*., 2017) , but others are toxinogenic. The *Penicillium* genus contains approximately one hundred toxic species and the range of mycotoxin classes produced is much wider (Sweeney and Dobson, 1998; DeVries *et al*., 2002; Pitt and Hocking, 2009). The production of secondary metabolites such as mycotoxins represents a major risk for human and animal health (Sweeney and Dobson, 1998; Boudra *et al*., 2007; Galtier *et al*., 2008).

Northern Morocco is characterized by a temperate, humid and subhumid bioclimate that is favorable for moulds growth and mycotoxin production. In fact, a review study carried out by Zinedine and Mañes, (2008) revealed the occurrence of mycotoxins in more than twenty food and feed in Morocco.
Despite the problems of toxinogenic *Penicillium* related to foodstuffs, Moroccan milk and Jben have not yet been deeply studied. For all we know, the only work available focuses on pasteurized milk (Zinedine *et al.*, 2006). However, no studies have thrown much light on *Penicillium* in the northern region of Morocco.

This study aimed to isolate and identify *Penicillium* species from traditional raw goal's milk and fresh goat's cheese and to evaluate their mycotoxin-producing potential.

This mycological analysis specific to *Penicillium* then makes it possible to monitor toxin-producing species, to protect the consumer by preventing intoxication and to consider an improvement in working conditions and marketing local dairy products.

Material and Methods

Sampling

In total, 57 samples of raw goat's milk and traditional goat's cheese (Jben) were collected from different commercial locality in the northeast region of Morocco "Tangier-Tetouan- Al Hoceima" during the period of 2016-2017. The Geographic coordinates and localization of collected points are respectively presented in The **Table 1 and Fig. 1**. The Number of sample is also noted in the same **Table 1**. Each sample collected were quickly analysed or stored at 4 °C until analysis.

Isolation and identification of *Penicillium* strains

One ml of diluted sample of milk or Jben in solution (dissolution of 1 g of Jben in 1 ml of sterile distilled water) are deposited on a MEA medium (Malt Extract Agar) in triplicate and incubated at 25 °C for 7 days (Maouni, 2002, Pitt and Hocking, 2009). After incubation, *Penicillium* strains were selected and purified. The species identification are based principally on colonial characters (Diameter on mm, Texture, Obverse and Reverse colours) on three mediums CYA (Czapek Yeast Agar), MEA and G25 N (Glycerol 25% Nitrate Agar) and microscopic characters (length of the Conidiophore, Stipe aspect, divergent Penicilli, number of phialides carrying conidia, Phialide size and shape, Conidia size, shape and aspect). Several keys have been used in the identification of *Penicillium* species (Pitt and Hocking, 1985; Peberdy, 1987; Pitt, 1988; Botton *et al.*, 1990; Frisvad and Samson, 2004; Compaoré *et al.*, 2016).

Table 1: Geographic coordinates of collected points and number of sample of traditional dairy products

				Number of simple	
Number of collected point	**Locality**	**Latitude**	**Longitude**	**Raw goat's milk**	**Fresh goat's cheese**
1	Farm El Menzla, **Tangier**	N 35°55'2	W 5°93'4	**1**	**1**
2	Weekly Sunday market, **Tangier**	N 35°70'7	W 5°91'1	/	**26**
3	Souk Diki, **Ksar Seghir**	N 35°84'4	W 5°56'2	**1**	**1**
4	Souk Jbel zem-zem, **Ksar Seghir**	N 35°70'1	W 5°677	/	**1**
5	Souk Khmis Anjra, **Ksar Seghir**	N 35°66'3	W 5°527	**1**	**1**
6	Souk Wadras, **Tetouan**	N 35°54'9	W 5°52'4	**2**	**2**
7	Souk Central **Ouad laou**, **Tetouan**	N 35°44'8	W 5°11'1	**2**	**3**
8	Souk Central, **M'Diq**	N 35°68'3	W 5°32'5	**1**	**1**
9	Souk Khmiss-Bni Arous, **Larache**	N 35°30'5	W 5°62'9	**3**	/
10	Souk Lbali Jnan Francis, **Larache**	N 35°16'9	W 6°17'5	**4**	**4**
11	Souk Bou Jedyane, **Ouezzane**	N 34°95'8	W 5°52'3	**1**	**1**
	Total samples			**16**	**41**

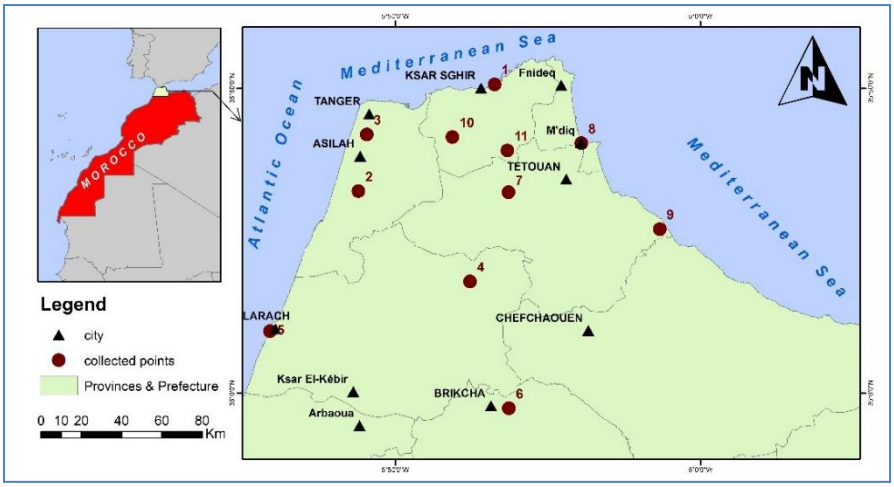

Fig. 1: Geographic localization of collected points in the northeast region of Morocco "Tangier-Tetouan- Al Hoceima" (**SASPlanet 15.11 and ArcGIS 10.3)**

In vitro identification of *Penicillium* toxinogenic by fluorescence detection
***In vitro* mycotoxins extraction and quantification**

Strains of isolated *Penicillium* were grown on MEA and CYA to detect Citrinin (CIT) and on the Yeast Extract Sucrose Agar (YES) to detect Ochratoxin A (OTA) for 7 days at 25 °C. The fluorescence of mycotoxins has been detected under UV light using a transilluminator (UVP, Benchtop 3UV, Transilluminator) as described by Frisvad, 1987; Nguyen, 2007; Rasch *et al.*, 2010; Hruska *et al.*, 2013 and Smeesters *et al*., 2014 and 2015.

***In vitro* mycotoxins extraction and quantification**

25 mL of MEA and CYA were poured into 100 mL Erlenmeyer flasks and inoculated with the isolated *Penicillium* strains; the Erlenmeyer flasks were then incubated at 25 °C for 7 days.

Extraction and purification of mycotoxins were performed as described by Nguyen *et al*. ,2007, with some modifications. The mycotoxins were extracted with 30 mL of acetonitrile (Chromasolv, Sigma-Aldrich) and 4% aqueous solution of potassium chloride (9:1 at pH 1,5). The Erlenmeyer were shaken for 20 min. The extract was then passed through Whatman n° 4 papers under vacuum. 30 mL of hexane (Sigma-Aldrich) was added to the filtrate and stirred for 10 min. After separation, the upper phase (hexane) was discarded. This step was repeated with 30 mL of hexane. In the lower phase, 30 mL of deionized water and 30 mL of chloroform (Merck, Germany) were added. The solution was stirred for 10 min.

After separation, the lower phase was collected. The upper phase was re-extracted with 20 mL of chloroform using the same conditions. The chloroform extracts have been grouped together. Then, the chloroform was

evaporated in vacuum using a rotary evaporator (Bücher, Rotavapor R-200, Switzerland) at 40 °C. 2 mL of methanol (Merck, Germany) were added and the solution was sonicated (P-Selecta), filtered and evaporated under nitrogen gas. The quantitative determination was performed by High Performance Liquid Chromatography (HPLC) system.

The dry residue obtained was taken up in 500 µL of methanol and was stirred gently before putting it in the HPLC system (SIL-20A / 20AC, Shimadzu, Kyoto, Japan) which consisted of a high-pressure pump (LC-20AD, Shimadzu, Kyoto, Japan) and an auto-sampler (SIL-20AC HT, Shimadzu, Kyoto, Japan). The column (EC 250 mm/4 mm Nucleodur 100-5 C18 EC (Marcherey-Nagel) was equilibrated with H_3PO_4 (0.33 M)/acetonitrile/propanol-2 (650/400/50 v/v/v) as mobile phase, 10 µL of the samples were injected and eluted at a flow rate of 0.5 mL/min and the system was conducted isocratically. The different excitation and emission fluorescence parameters were used according to each toxin with the detector (RF-10 AXL, Shimadzu, Kyoto, Japan), the OTA 335 and 465 nm and CIT 331 and 500 nm (Nguyen, 2007), and a UV-VIS spectrophotometer (SPD-M20A, Shimadzu, Kyoto, Japan) at 276 nm, used to detect patulin (Fuchs *et al*., 2008). This analysis was repeated twice.

Results And Discussion

Penicillium species isolated from raw milk and fresh cheese.

All samples of raw milk and traditional cheese collected in the Tangier-Tetouan region (57 samples) were analyzed and were demonstrated contamination with molds**.** **Table 2** shows the results of number and proportion of different *Penicillium* species isolated: 16 isolates from raw goat's milk and 10 isolates from fresh goat's cheese (Jben).

Table 2: Number and proportion of different mold species isolated from raw goat's milk and fresh goat's cheese

Identified strain of *Penicillium* (P)		Raw goat's milk		Fresh goat's cheese (Jben)	
		Number	**Proportion (%)**	**Number**	**Proportion %**
Sp1	*P. italicum*	6	23,1	3	5,1
Sp2	*P. cyclopium*	2	7,7	3	5,1
Sp3	*P. expansum*	2	7,7	2	3,4
Sp4	*P. nalgiovense*	1	3,8	0	0
Sp5	*P. dipodomycola*	1	3,8	0	0
Sp6	*P. simplicissum*	1	3,8	0	0
Sp7	*P. brevicompactum*	1	3,8	0	0
Sp8	*P. dipodomyis*	0	0	1	1,7
Sp9	*P. chrysogenum*	1	3,8	0	0
Sp10	*P. griseofulvum*	1	3.70	1	1,7
Total *Penicillium*		**16**	**59,26**	**10**	**16,94**

Other mold and yeast species	**11**	**40,74**	**49**	**83,05**
UFC/g total moulds	**2,6 10^4 /ml**		**5,90 10^4 /g**	

Our microbiological analyses have clearly shown that the fungal flora is highly developed in goat's milk and forming (85 mould and yeast isolates from 57 samples).

The rate of fungal flora (mould and yeast species) is high, it is around 2,6 10^4 Colony-Forming Units (CFU) per ml in milk and 5,90 10^4 CFU/g in Jben.

The fungal flora in raw milk from northern Morocco is slightly higher (2,6 10^4 CFU/ml) than that obtained for the raw milk from the Gharb and central region of Morocco (1,2 10^4 CFU/ml) reported by Labioui *et al.*, and 1,5 10^4 CFU/ml reported by Ouazzani *et al.*, 2014.

Other analytical results showed that the fungal flora is particularly numerous in the northern jben (from 7,90 10^4 CFU/g to 9,4 x 10^6 CFU/g) compared to 3.0 x 10^4 CFU/g (El Marrakchi, *et al.*,1991; Zantar *et al.*, 2007).

The massive presence of moulds and yeasts in raw milk and Jben of Northern Morocco may be due to a strong external contamination and insufficient hygiene of women working in a traditional way in this sector.

The percentage of *Penicillium* isolated in raw milk (61,53 %) is, higher compared to other moulds (42,3 %) and also higher than Jben (16,94 %).

The results of *Penicillium* species identification showed that the proportion in raw goat milk was 23,1% for *P. italicum,* 7,7% for each *P. cyclopium and P. expansum,* 3,8% for each of *P. nalgiovense, P. dipodomycola, P. chrysogenum P. simplicissum, P. brevicompactum and* 3,7 % for *P. griseofulvum*. While the Jben presented the following proportions: 5,1% for each of *P. italicum* and *P. cyclopium,* 3,4% for *P. expansum,* and 1,7% for each of *P. griseolflum* and *P. dipodomyis,* and 0% for five strain of *Penicillium*

The main criteria used to identify *Penicillium* are consistent with other studies by Frisvad and Filtenborg, 1989; Frisvad and Samson, 2004). *P. brevicompactum* and *P. chrysogenum* are ubiquitous on spices, dry cereals, cheese and meat, tropical seeds, and other foodstuffs (Pitt, 1979).

Detection of mycotoxins by fluorescence under ultraviolet light

The ten *Penicillium* species isolated were tested for their ability to produce Citrinin and Ochratoxin A mycotoxins using fluorescence detection of the medium under ultraviolet light.

Mycotoxin production was detected in all *Penicillium* colonies by the visibility of fluorescence under ultraviolet light in three medium studies (CYA, MEA and YES), except *P. italicum* in YES medium (Table 3). The more concentrated of Citrinin was noted for *P. simplicissimum* in CYA

medium and *P. cyclopium, P. dipodomyis, P. expansum and P. chrysogenum* in MEA medium.

Nguyen, (2007) found that CYA is the best medium used to detect toxinogenesis of *P. citrinum.*

Table 3: Detection of mycotoxins by fluorescence under ultraviolet light

	Citrinin		**Ochratoxin A**
Strains	CYA	MEA	YES
P. griseofulvum	(+)	(+)	(+)
P. cyclopium	(+)	(++)	(+)
P. italicum	(+)	(+)	(--)
P. nalgiovense	(+)	(+)	(+)
P. dipodomyis	(+)	(++)	(+)
P. simplicissimum	(++)	(+)	(+)
P. expansum	(+)	(++)	(+)
P. chrysogenum	(+)	(++)	(+)
P. dipodomycola	(+)	(+)	(+)
P. brevicompactum	(+)	(+)	(+)
These assays were performed in duplicate	+: Mycotoxins fluorescence intensity --: Absence of mycotoxins		

Identification and quantification of mycotoxins by HPLC

According to HPLC results **(Table 4**), each *Penicillium* strains was able to produce more than one toxin at the same time. These results are in agreement with those obtained by (Erdogan *et al.*, 2003; Nguyen, 2007; Lecellier, 2013).

Table 4: Production of mycotoxins by *Penicillium* strains cultivated at 25 °C for 7 days

	Mycotoxin mean (µg/g medium)					
	PAT		**CIT**		**OTA**	
Strains	**MEA**	**CYA**	**MEA**	**CYA**	**MEA**	**CYA**
P. griseofulvum	143,70	57,80	4,48	26,7	0,00	0,00
P. cyclopium	230,80	0,00	0,00	0,00	11,30	0,53
P. italicum	66,70	0,00	0,00	0,00	10,50	0,40
P. nalgiovense	115,00	27,50	0,00	0,00	11,00	12,80
P. dipodomyis	81,40	12,50	3,80	3,80	0,00	0,00
P. simplicissimum	67,10	0,00	0,19	22,1	0,98	0,00
P. expansum	93,70	21,60	0,00	0,00	1,72	8,10
P. chrysogenum	126,10	0,00	0,00	0,00	5,85	251,50
P. dipodomycola	73,10	47,80	0,00	0,00	0,00	66,80
P. brevicompactum	57,30	30,30	0,00	0,00	87,60	175,10

PAT: Patulin; CIT: Citrinin; OTA: Ochratoxin A
CYA: Czapek Yeast Agar; MEA: Malt Extract Agar;
This analysis was performed in duplicate

In the MEA culture medium, PAT is secreted by all *Penicillium* species with a concentration higher than 57,30 µg/g medium. The highest

concentration is founded by P. *cyclopium (*230,80 µg). *P. griseofulvum (*143,70 µg), *P. chrysogenum (*126,10 µg), *and P. nalgiovense* (115,00 µg).

In CYA culture medium, PAT is secreted by most *Penicillium* strains; *P. griseofulvum, P. cyclopium, P. nalgiovense, P. dipodomyis, P. expansum, P. dipodomycola* and *P. brevicompactum,* with a concentration of 12,5 µg to 57,8 µg. On the other hand, *P. cyclopium, P. italicum, P. simplicissimum and P. chrysogenum* do not produced PAT in CYA medium. *P. griseofulvum* and *P. expansum* are very efficient producers of high levels of PAT in pure culture and they have the potential to produce PAT in cereals, fruits, pasta and similar products (Frisvad *et al*., 2006 and Tannous, 2015). In addition, there are 14 *Penicillium* species patulin-producing (Samson *et al.*, 1984 and Tannous, 2015).

Only three strains of *Penicillium* (*P. griseofulvum, P. dipodomyis* and *P. Simplicissimum)* produced CIT in the MEA and CYA medium, with low concentrations ranging from 0,19 to 4,48 µg, noted by *P. griseofulvum.*

P. citrinum mainly produces CIT (Sweeney and Dobson, 1998; Frisvad *et al*., 2006), while, other author showed that CIT was produced by *P. expansum* and *P. verrucosum* (El-Banna, *et al*.,1987).

Six strains of Penicillium (P. cyclopium, P. italicum, P. nalgiovense, P. expansum, P. chrysogenum and P. brevicampactum) produce OTA in MEA and CYA, with a dose range from 0,40 µg to 251,50 µg. The highest concentration was founded with P. chrysogenum.

P. simplicissimum produce OTA in MEA but not in CYA and *P. dipodomycola* produce *OTA in CYA* but not in *MEA.* These results confirm the hypothesis that many species among the *Penicillium* genus may be ochratoxinogens (Bejaoui, 2005), with the exception of *P. griseofulvum* and *P. dipodomyis* do not produce OTA in MEA and CYA medium.

Conclusion

In conclusion, the fungal flora of milk and Jben from goats in northern Morocco remains higher than normal and that toxigenic *Penicillium* is present and potentially able to produce mycotoxins that have harmful effects on human's health. It seems that the respect of good hygiene during milk collection from goats, the manufacture of Jben and their marketing in northern Morocco remains relatively unsatisfactory, despite the efforts of the state in this direction. The sensibilization, the professional guidance on the concept of hygienic quality and the tendency to semi-industrial production remain work-sites to work more efficiently by the state.

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