Alma Master Studiorum - University of Bologna

PhD Course in Food Sciences (XXV Cycle)

Development of guidelines for microbiological control in microbrewery

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DIPARTIMENTO DI SCIENZE DEGLI ALIMENTI

Assessment for admission to the final examination for the degree of PhD in Food Science (XXV Cycle)

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Over the past 15 years the Italian brewing scene showed interesting changes, especially with regard to the creation of many breweries with an annual production of less than 10,000 hectoliters (microbreweries, craft breweries). One of the major challenges that microbreweries face is the production and maintenance of beer quality.

The beers produced by microbreweries are very susceptible to attack by spoilage micro-organisms that cause the deterioration of beer quality characteristics.

In addition, most of the microbreweries do not practice heat treatments of stabilization and do not carry out quality checks on the product. The high presence of beer spoilage bacteria is an economic problem for the brewing industry because it can damage the brand and it causes high costs of product retrieval.

This thesis project was aimed to study the management of the production process in the Italian microbreweries within a production less than 10,000 hl.

In particular, the annual production, type of plant, yeast management, process management, cleaning and sanitizing of a representative sample of microbreweries were investigated.

Furthermore was made a collection of samples in order to identify, with simple methods, what are spoilage bacteria more present in the Italian craft beers.

21% of the beers analysed were positive at the presence of lactic acid bacteria. These analytical data show the importance of understanding what are the weak points of the production process that cause the development of spoilage bacteria.

Finally, the thesis examined the actual production of two microbreweries in order to understand the process management that

can promote the growth of spoilage bacteria in beer and production plant.

The analysis of the data for the two case studies was helpful to understand what are the critical points where the microorganisms are most frequently in contact with the product. The hygiene practices are crucial to ensure the quality of the finished product, especially in the case of non-pasteurized beer.

Some of the data collected during the course of the PhD project were presented as poster at an international conference.

DIPARTIMENTO DI SCIENZE DEGLI ALIMENTI

Giudizio di ammissione all'esame finale per il titolo di Dottore di Ricerca in Scienze degli Alimenti (XXV Ciclo)

Dott. Elio Moretti

Negli ultimi 15 anni il panorama birrario italiano ha mostrato interessanti cambiamenti soprattutto per quanto riguarda la nascita di numerosi birrifici con una produzione annua inferiore ai 10.000 hl che potremmo definire microbirrifici. Uno dei principali obiettivi dei micro birrifici è la produzione ed il mantenimento di un prodotto con elevate caratteristiche qualitative.

Le birre prodotte dai microbirrifici sono molto suscettibili ad attacchi da parte di microrganismi che ne causano il deterioramento delle caratteristiche qualitative. Soprattutto per il fatto che si tratta di prodotti che non subiscono trattamenti termici di stabilizzazione. Inoltre i birrai nella maggior parte dei casi non effettuano controlli di qualità sul prodotto. Spesso i micro birrifici sono costretti a ritirare dal commercio prodotti qualitativamente inaccettabili a causa dello sviluppo di microrganismi, questo provoca danni economici legati al danneggiamento del *brand* aziendale.

Il presente progetto di tesi ha avuto come scopo quello di approfondire come viene effettuata la gestione del processo produttivo nei birrifici italiani con una produzione inferiore ai 10.000 hl. In particolar modo è stata eseguita un'indagine riguardante: produzione annua, tipologia di impianto, gestione del lievito, gestione dei processi di detergenza e sanificazione.

Inoltre è stata effettuata una raccolta di campioni allo scopo di individuare, con metodiche semplici, quali batteri sono maggiormente presenti nelle birre italiane provenienti da birrifici con una produzione minore di 10.000 hl. E' stata rilevata la presenza di batteri lattici nel 21% delle birre analizzate.

Il lavoro di tesi ha poi preso in esame la realtà produttiva di due birrifici per meglio comprendere le dinamiche produttive che possono favorire lo sviluppo dei microrganismi dannosi alla qualità del prodotto in birra.

L'analisi dei dati relativi ai due casi studio è stata utile per comprendere quali sono i punti dove i microrganismi vengono più spesso a contatto con il prodotto. L'igiene degli impianti e pratiche igieniche efficaci sono fattori cruciali per assicurare la qualità del prodotto finito, soprattutto nel caso di prodotti non pastorizzati.

Alcuni dei dati raccolti durante lo svolgimento del progetto di dottorato sono stati presentati, come poster, ad un congresso internazionale.

AIM OF THE PROJECT AND ABSTRACT

Over the past 15 years the Italian brewing scene showed interesting changes, especially with regard to the realization of many breweries with an annual production lower than 10,000 hectolitres (microbreweries, craft breweries). Today, in Italy there are around 500 craft breweries and in most cases they do not perform routine microbiological controls of process and product.

Craft beer is typically more susceptible to microbial spoilage, as it is typically not pasteurised or sterile filtered. Further, microbrewers usually lack the benefits of a well resourced quality control laboratory, and cold storage is not guaranteed throughout the distribution and retail chain (Menz G. et al, 2010).

This PhD project allowed to determine how the process is managed in Italian craft breweries.
Especially investigating the following issues:
- annual production;
- layout;
- type of equipment used;
- type of yeast;
- management of the yeast;
- management of cleaning and sanitizing.

Moreover, it was carried out a collection of beers to investigate the frequency of spoilage lactic acid bacteria in beers from Italian craft breweries.
Lactic acid bacteria are of serious concern on the brewing industry, as spoilage incidents can damage brand equity and the cost of product retrieval can be high (Suzuki K., et.al. 2006).
The identification of lactic acid bacteria with simple techniques has enabled us to understand the organisms most responsible for the deterioration in the quality of the beer produced by craft brewery.

Two breweries with an annual production lower than 10.000 hectolitres were finally examined. Depth analysis of the entire production process permitted to identify weaknesses (critical points). Therefore, it was possible to improve the process (equipment, cleaning procedure, etc.) in order to obtain a low incidence in the presence of bacteria spoilage beer.
This result was obtained using simple microbiological determination to investigate the production of small breweries.
The 21% of the beers from microbreweries were positive to the presence of lactic acid bacteria. Similar levels of presence of lactic acid bacteria have been observed in other studies of unpasteurised beer. Menz in 2010 reported a contamination rate of 27.5% in Australian craft beer.

Only in one case the presence of spoilage bacteria has been detected in commercial dry yeast used for inoculation of the wort. However, in the future, this aspect should be deepened.

Analysis of production processes has highlighted weaknesses in the managment of cleaning and sanitizing of fermentation tanks. In fact, the presence of microorganisms that spoil the beer was observed from the fermentation tank.

This study, the first in Italy, allowed to describe and check the craft beer production in a scientific way.

The work done lead to control the identification of a series of measures to ensure that the beer produced by small Italian breweries able to possess high quality characteristics.

1. Spoilage microorganisms associated with beer and production plant

1.1. Regulating factors of microbial growth in beer and brewery plant

Beer has been recognized as a microbiologically stable beverage (Suzuki K. 2011). Oxygen contenent is a major factor in controlling the microflora capable of growth during beer production and storage. However, several other factors are also important for the spoilage potential of beer and include the pH (3.8-4.7), the concentration of hop bitters (approx. 17-55 mg iso α-acids/l), ethanol (0-8% w/w), CO_2 (approx. 0.5% w/v), SO_2 (approx. 5-30 mg/l), organic acids, acetaldehyde and other metabolites as well as nutrients and storage temperature (Jespersen L., and Jakobsen M., 1996).

In addition to these intrinsic factors, many stages of the brewing process reduce the potential for contamination. These processes include mashing, wort boiling, pasteurization, filtration, aseptic packaging, and cold storage (Menz G., 2009).

The microbial safety and stability, the sensory and nutritional quality, and the economic potential of many foods are maintained using a combination of preservative factors (hurdles), which is termed hurdle technology (Leistner, 2000). Beer contains an array of antimicrobial hurdles that, under most circumstances, prevent the growth of pathogenic micro organisms. Despite many of the typical hurdles that ensure the safety of beer, such as boiling in the kettle, the presence of hops, ethanol, carbon dioxide, the low pH, and the lack of available nutrients and oxygen. Beer is more susceptible to undesirable microbial growth when one or more of these hurdles are absent or at a reduced level (Menz G., 2009).

The antimicrobial properties of ethanol in beer have been recognized for some time, with the prominent brewing microbiologist Shimwell (1935) noting that beers with a higher ethanol content were more resistant to spoilage by *Saccharobacillus pastorianus* (now *Lactobacillus brevis)* than those of lower ethanol content. It can be assumed that the same applies for pathogens (Menz G., 2009).

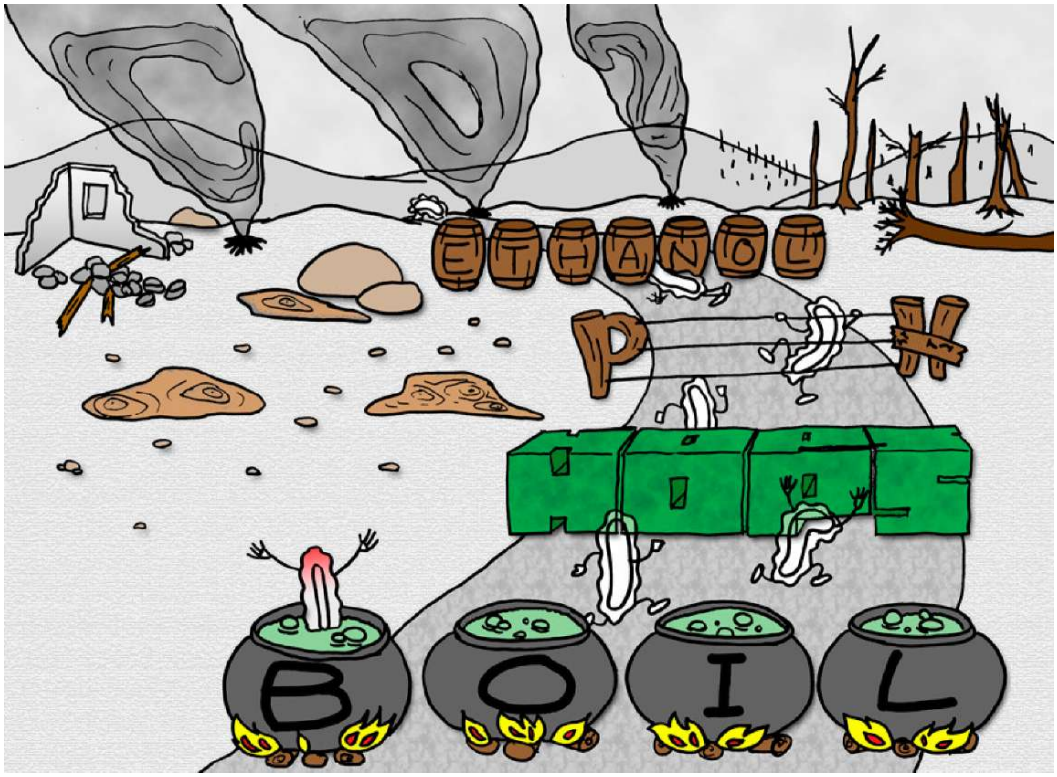

Figure 1.1. Pathogens cannot survive in beer due to the antimicrobial 'hurdles'. These include the kettle boil, hop bitter acids, low pH, ethanol, carbon dioxide (CO_2), and the lack of nutrients and oxygen (Menz G. 2009).

Ethanol inhibits cell membrane functions (Casey and Ingledew, 1986), and inactivates bacteria by inducing cell membrane leakage (Menz G. 2009).

1.2. Contamination source

The microbiological stability of the final product can be compromised from a very early point in its production, with spoilage organisms able to access the brewing process at every stage, even from to dispense (Hill A.E., 2009).

Beer may contain microbial contaminants originating from a variety of sources . Primary contaminants originate from the raw materials and the brew house vessels and secondary contaminants are introduced to the beer during bottling, canning or kegging. While approximately half of the documented microbiological problems can be attributed to secondary contaminations, the consequences of primary contaminations may be more catastrophic, with the potential loss of a complete brew (Vaughan A., 2005).

Most potential contaminants of beer originate from raw materials and/or unclean brewing equipment. Brewing raw materials, such as malt, hops and occasionally brewing water, may be infected by microorganisms and these have to be killed during the brewing process to prevent wort and beer spoilage (Hill A.E., 2009).

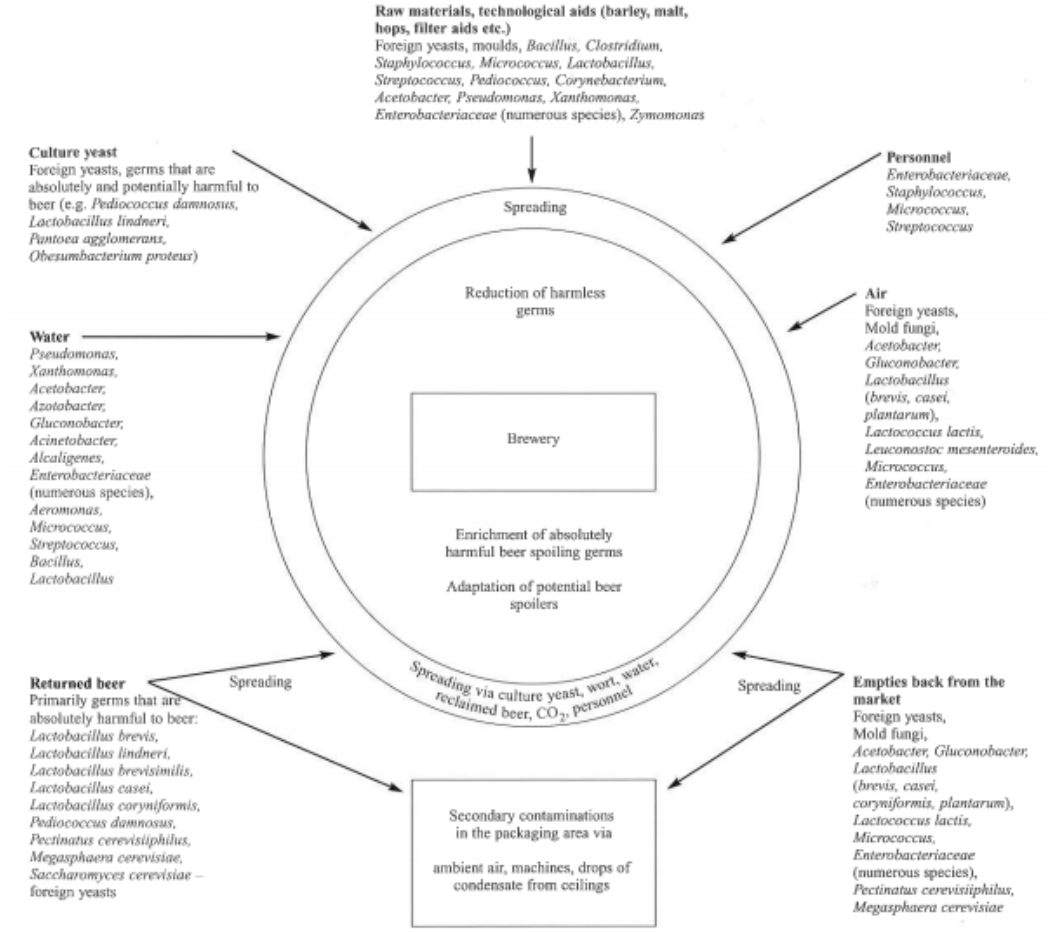

Figure 1.2. How microorganisms spread in the brewery (back W., 2005).

Malt

The effects on brewing and beer of contamination of growing, stored or malted barley are varied. The best known effect of the micro biota of both barley and malt is that of reduced gas stability or gushing (spontaneous ejection of beer from its container). A variety of different fungi have been associated with gushing, the most notable are *Fusarium graminearum* and *F. moniliforme*. Small fungal proteins, hydrophobins, present in fungal cell walls have been isolated from strains of the genera *Fusarium* , *Nigrospora* and *Trichoderma* and shown to act as gushing factors in beer (Sarlin et al., 2005).

A second consequence of fungal infection of barley and malt is the potential for release of mycotoxins, compounds toxic to man or animals. Aflatoxin B1, ochratoxin A, zearalone, deoxynivalenol (DON) and fumosins B1 and B2 are mycotoxins that may be transmitted from contaminated grains into beer. In addition to the potential harm to humans, mycotoxins may affects fermentation due to their influence on yeast activity. There is also an apparent relationship between the ability of strains to produce the mycotoxin zearalone and gushing (Hill A.E., 2009).

Water

Breweries and good water have long had a close association, and water quality is generally taken for granted (Hill A.E., 2009). Water used for brewing must be fit for human consumption (potable). As such it must be free from contaminating organisms. However, what is fit to drink is not necessarily fit for brewing use (Hill A.E., 2009).

Water for brewing is boiled during the process. From a microbiological point of view the main concern is the introduction of spoilage organisms from water introduced after fermentation, for example during dilution of beer following high gravity brewing or from vessels rinsed with contaminated water (Hill A.E., 2009).

Membrane filtration is generally used for complete removal of bacteria, viruses, proteins, salts and ions. Chlorine dioxide can be applied to water systems to reduce or eliminate brewery spoilage organisms. At levels of approximately 0.2 ppm chlorine dioxide significantly reduces microbial count while causing no off-favours or odours in the final beer (Dirksen, 2003).

Air

Air currents can carry microbial contamination to the filling area from numerous sources, including pasteurisers, floor drains, personnel, forklifts, packaging material, and the external environment (Dirksen, 2005). Bacteria of the genera *Acetobacter, Bacillus, Lactobacillus, Micrococcus, Pectinatus, Pediococcus, Pseudomonas, Shigella* and *Streptococcus,* and various yeasts and moulds have been isolated from the air around the filling line of several breweries (Henriksson and Haikara, 1991). General guidelines recommend that airborne microbial levels in breweries should be less than 5×10^2 CFU/m^3 (Dirksen, 2005). Although only a

few of the airborne bacteria are capable of causing spoilage in beer, their levels can be used as an indicator of general hygiene.

Pitching yeast

The most common source of bacterial contamination in the brewery is probably from pitching yeast, which can transfer contaminants from fermentation to fermentation. Any microbial contamination of pitching yeast compromises product quality and taste and can have a significant effect on the final beer (Hill A.E., 2009).

To prevent microbial contamination during the production process, the microbiological purity of brewing yeast starters is a necessary condition to maintain high product quality. Many strains are available on the market and their characterization is necessary for quality control in dry yeast production (Manzano et al 2005). The contamination of production strains with wild yeasts (*non-Saccharomyces* or *Saccharomyces*) and bacteria may contribute negatively or positively to beer properties and characteristics. Monitoring of microbial contaminants during the brewing process is important to obtain reproducible and high-quality beers (Tompkins, T.A. et al 1996).

Hops

Hops, is known for its antiseptic properties. As described, the majority of Gram positive bacteria are inhibited by hops, although Gram negative bacteria are unaffected. Whole hops are dried following harvesting (Hill A.E., 2009). This process reduces the chances of subsequent microbial contamination, and for brewers who do use whole hops no beer spoilage effects attributable to infected hops have been reported (Hill A.E., 2009).

Similarly, no beer spoilage organisms have been reported to have been introduced by other herbs or plant-derived products used in brewing (Hill A.E., 2009).

Sugar

Free flowing sugar, syrups or honey are commonly used adjuncts, generally added during wort boiling. The main concern in brewing involves transfer of bacterial spores, principally from Bacillus sp., which can withstand heat treatment, including boiling, and may persist into the finished beer (although beer does not support the subsequent growth of these organisms) (Hill A.E., 2009).

1.3. Lactic acid bacteria

Lactic acid bacteria (LAB) are known as predominant beer spoilers, and it has been reported that 60-90% of the microbiological incidents are cause by LAB (Back 1994, 1995, 1997). Lactic acid bacteria (LAB) are universally considered useful microorganisms in the food industry and are used in wide range in fermented products. At the same time, however, can be harmful and spoilage in many foods and beverages (Suzuki 2011).

The development of spoilage lactic acid bacteria has been associated with increased use of hops from 1400. Hop compounds added to confer bitter flavor on beer are reported to exert an antibacterial effect by acting as proton ionospheres and dissipate the trans membrane pH gradient, which prevent Gram-positive bacteria including most LAB from growing in beer. Hop resistance ability has been known as a distinguishing character of beer spoilage LAB strains. Two genes, horA and horC have been demonstrated to confer hop resistance ability on LAB. HorA acts as an ATP-dependent multidrug transporter and extrudes toxic hop compounds out of bacterial cells. HorC, on the other hand, confers multidrug resistance to various drugs, including hop compound, acting as a proton motive force (PFM)- dependent multidrug transporter (Suzuki 2005, Sakamoto and Konings 2003). Resistance to hop compounds is considered a discriminatory nature of LAB that could adversely affect the beer. These resistance mechanisms have provided selective advantages for their development in beer compared to other organisms (Fernandez and Simpson 1993). The LAB share considerable genetic and molecular homology and have long been recognized as a natural group. However, a definitive description of the LAB cannot be agreed upon and with current developments in the taxonomy of these bacteria is probably more distant now than any previous time. The typical LAB is a Gram-positive, non-

sporulating rod or coccus. It lacks the enzyme catalase and is strictly fermentative producing either a mixture of lactic acid, CO2, acetic acid and/or ethanol or almost entirely lactic acid (homofermentation) as the major metabolic end-product from sugar catabolism (Priest F.G. 1996, 2006). Since both types produce lactic acid, these bacteria have been adapted to grow in an acidic environment such as that found in fermented beverages.

Spoilage LAB produce in beer excessive acidification, haze and off-flavors (Back W. 2005).

Lactobacillus

Lactobacilli are the most common beer-spoilage bacteria, regardless of beer type (Thelen et al., 2006).

The genus Lactobacillus is the largest among the lactic acid bacteria and includes many species, but only some of them can deteriorate the beer. (Rainbow 1981, Jespersen and Jakobsen 1996). Different species vary in their ability to grow in beer and in their tolerance to hop bittering compounds.

Lactobacillus brevis

Lactobacillus brevis is the most commonly found bacteria in beer (50% of cases) (Back 2009,Vaughan 2005). This obligate heterofermentative bacterium is generally tolerant towards hops and grows optimally at 30°C and pH 4-5 (Priest 1996). *Lactobacillus brevis* is physiologically versatile and can also cause various problems in beer such as super attenuation due to its ability to ferment starch and dextrins, haze and acidification (Back 2005).

L. brevis is widespread in the food industry and in nature. In the brewery, it occurs in different types, with differing cell and colony morphology and physiological characteristics. Their gas formation (heterofermentative), pentose and usually also melibiose fermentation and the often-positive arginine cleavage are typical (Back W. 2005).

The beer spoiling activity varies considerably depending upon the strain and the origin. Some strains develop spontaneously in all kinds of beer causing haze, sediment and acidification, but no diacetyl flavor. Other strains are potentially harmful and are at best able to grow only in weakly hopped beers. Occasionally, some strains also find their way (through water and air) into the brewery, which

could be considered "foreign", but may, after a latent phase, adapt themselves to the brewery environment and thus become harmful (Back W. 2005).

In the production of "Berliner Weiss" (White, top fermented beer of Berlin), *L. brevis* also has a technological function. In order to impart the typical, pure lactic acid flavour to this beer (pH value about 3.8), the producers deliberately utilize mixed cultures of this bacterium, of top fermenting yeast and of *Brettanomyces bruxellensis* (Back W. 2005).

Occasionally, other lactic acid bacteria are also said to be involved in this fermentation process besides *L. brevis* (e.g. *L. buchneri, L. lind-neri, L. casei, L. coryniformis, L. plantarum*) (Back 2005).

Differences in cell morphology among the hetero fermentative, closely related species *L. brevis, L. brevis (frigidus)* and *L. brevisimilis*, scarcely exist. The cells of *L. brevis (figidus)* are on average a bit shorter and appear apart from one another under the microscope due to the production of slimy capsules. *L. brevisimilis* is noted for its somewhat slimmer cells, which may form short chains or, in some strains, be curved (Back W. 2005).

L. brevis (frigidus) differs from *L. brevis* mainly in the production of capsules and in the fermentation of melezitose. In beer, this species is rare, but quite dangerous (severe hazes, sediments and ropiness, increasing of viscosity). In the worst case, the latter may be so thick as to cause the beer to appear viscous at dispensing. Because of the production of capsules, this species belongs to the beer spoilers being most resistant to sanitizing agents, and may tolerate up to 25 pasteurization units. The taste of the beer turns to acidic, while no other sensory changes are detectable.

L. brevis (frigidus) contaminates preferentially the unfiltered beer in the cellars. It is capable of surviving for years at those low temperatures. Conditions may arise, under which it spreads through the filter and proliferates in beer-lines, gaskets, valves and other similar receptacles (Back W., 2005).

Pediococcus

Pediococci are important in food technology in both a negative and positive sense. *P. damnosus* is a major spoilage organism in beer manufacture, since growth may

lead to diacetyl/acetoin formation, resulting in a buttery taste (Salminen S. et al, 2004).

The most important coccus-shaped lactic acid bacterium in the brewery is *Pediococcus damnosus*. This species, mostly referred to as "Beer-sarcina" by practical brewers, is very feared because of its formation of diacetyl (unpleasant cheesy flavor). It is a typical, brewery-specific organism, which may also be found in wineries, but not elsewhere, neither in other food industries nor in nature. In contaminated beer bottles, a diacetyl flavour develops and sediment deposits on the bottom. They also can occur as punctiform or "ray-patterned" colonies. As the cells tend to settle very quickly, hazes are seldom rarely observed. Despite being one of the most frequent contaminants of the yeast and of the sections before the filter, it results in relatively few problems in the packaged beer, as it accumulates in the yeast dregs on the bottom of the storage tank and is therefore almost quantitatively eliminated before filtration. Recently, however, sarcina infections.

have been more frequently noticed in Bavarian wheat beers, to which trace contaminated yeast has been added for secondary fermentation.

The development of pediococci may also be responsible for higher than normal contents of histamine and for poor foam stability (as a result of their enzymatic activity against foam proprieties).

A feature of this species is the formation of tetrads. Cocci (singly or in pairs) as well as short chains may also occur. The diameter of the cells is between 0.7 and 1.0 μm depending upon strain, age and culture conditions. The bacteria are microaerophilic and grow preferentially in a more or less completely pure CO_2 environment. Several strains are very demanding as far as nutrients and growth components are concerned. As they often require some specific beer-components, they are unable to grow in the usual media. The optimum temperature lies around 22-25°C, the maximum for many strains is 35°C. Cellobiose, fructose, galactose, glucose, maltose, mannose, melezitose and trehalose are fermented.

1.4. Acetic acid bacteria

Acetification of beer was studied by pioneers of microbiology such as Paersoon, Pasteur, Hansen, Henneberg and Beijernck.

The acetic acid bacteria are GRAM-negative, catalase-positive, oxidase negative, non sporing, motile or non-motile short or coccoid rods, exhibiting strictly aerobic metabolism (Back W. 2005). A common characteristic of all the representatives of this family is the oxidation of ethanol to acetic acid in acid or neutral media (Back W. 2005). That characteristics is used commercially for the production of vinegar, but needless to say is very detrimental to the brewer. Because beer is should be stored with limited access of air, spoilage by these ubiquitous bacteria should not occur. However, bacteria of the genus *Acetobacter* are ubiquitarius and can cause problems in public houses dispensing cask-conditioned beer in which the ale beer is displaced by air (Priest F.G. 2006).

Their optimum temperature lies between 25 and 30°C, and the optimum pH at 5 - 6, whereas most strains are able to develop at pH-values as low as 3.6-3.8. The maximum temperature they can withstand is usually below 37°C. The family includes the two genera *Acetobacter* and *Gluconobacter*, marked by their differences in the so-called "over-oxidation" and in the cilia. *Acetobacter* species oxidise acetic and lactic acid to CO_2 and H_2O and carry peritrichous or lateral cilia. *Gluconobacter* lack the "over-oxidation" capability and are ciliated at the poles. The genus *Acetobacter* consists of 4 species: *A. aceti, A. hansenii, A. liquefaciens* and *A. pasteiuianus*, while only one species of *Gluconobacter* is described in BERGEY' S Manual (Vol. 1, 1984), namely *G. oxydans* (Back W. 2005).

Acetobacter and *Gluconobacter* spp. have been isolated from breweries, there are resistant to the bacteriostatic activity of hops, acid and ethanol and are therefore capable of growing and spoiling in beer (Van Vuuren H.J.J. 1996).

Approximately ten species of *Acetobacter* have been recognized, whereas *Gluconobacter oxydans* is the most important *Gluconobacter* in brewing microbiology (Priest F.G. 1996); *Gluconobacter oxydans* in beers with high oxygen content, they cause hazes, formation of acids, and off-tastes, their optimum temperature lies around 25-27°C, some strains grow at 34°C, none at 37°C. For most strains the pH optimum is at 5.5-6.0, some grow even at a pH of 3.6 (Back W. 2005).

Being strict aerobes, they should not grow in wort or beer once aerobic condition develop. However, beer strains probably grow under microaerophilic conditions

and have been isolated with a low oxygen continent (Priest F.G. 2003). In the brewery environment, the acetic acid bacteria are ubiquitous: in the bottling hall, on malt, in wort and brewing liquor, and also in culture yeast and beer. During fermentation and after packaging, the lack of contact with atmospheric oxygen prevents them from growing, as they are strictly aerobic. But they reproduce themselves actively wherever small residues of beer, due to poor sanitation, come in contact with air in valves, in cocks, in traps, in the beer-lines, underneath the gaskets, and so forth (Back W. 2005).

Due to the increased efficiency in exclusion of oxygen from the production lines in modern breweries, the incidence of acetic acid bacterial spoilage has decreased (Lawrence, 1988).

As the same recesses also often offer good chances for the development of beer spoilage organisms, the acetic acid bacteria are important indicator germs in biological quality control (Back W. 2005).

1.5. *Enterobacteriacee*

The family *Enterobacteriaceae* comprises numerous genera of free living and sometimes pathogenic bacteria. Fortunately, none of the pathogenic types, such as *Salmonella* or *Shigella* species, have been found in beer. The enterobacteria are facultative anaerobes able to grow in the presence or absence of air, but they are inhibited by ethanol and low pH so are only responsible for beer spoilage in low alcohol products (< 2% by vol) with a relatively high pH (>4.2) (Priest F.G. 2006).

Enterobacteriaceae are facultative aerobic, Gram negative, catalase-positive and oxidase-negative. Their morphology is slightly variable and they exhibit a marked tendency toward pleomorphic forms.

Usually, they form short rods (0.6 - 1.5 x 1.5 - 3.0 n) with weakly illuminating cell walls. The cells are often bellied, bulky or sausage shaped, with round, pointed or spindle-shaped ends. Brewery-specific strains are usually non-motile, but occasionally they carry peritrichous cilia. The optimum temperature of *O. proteus* and of *E. agglomerans* is 25 - 28°C, the maximum 32 - 37°C (Back W. 2005).

Among enteric bacteria particularly the two species, *Obesumbacterium proteus* and *Enterobacter agglomerans* show up as microorganisms indirectly harmful to beer. *Enterobacter cloacae, E. aerogenes, E. sakazakii* and *Klebsiella pneumoniae* are more seldom. In the past, these species were collectively considered "wort bacteria" or included in the coliform germs. At least for *O. proteus* and *E. agglomerans,* as these bacteria exhibit a very low temperature maximum (often below 35°C) and grow, unlike typical coliform bacteria, at temperatures as low as approx. 3°C. They scarcely occur in the wort environment, but frequently contaminate the yeast and are detected in the whole unfiltered beer area. In the culture yeast, they are able, at strong contamination levels and at temperatures of approx. 8°C, to induce heavy off-tastes through formation of acetoine, dimethylsulphide (DMS) and dimethyldisulphide ("celery-taste"). The impact of these metabolites- goes right through to the packaged beer, in which they induce more or less strong off-flavours, depending upon the degree of contamination. Moreover, these bacteria secrete peptidases and proteinases into the substrate: at high contamination rates, this may impair the foam stability of the beer (Back W. 2005).

Hafnia protea, formerly *Obesumbacterium proteus,* and *Rahnella aquatilis,* formerly *Enterobacter agglomerans,* have been detected in pitching yeasts but never in finished beer. They can retard the fermentation process. Beer produced with yeasts contaminated with *H. protea* has a parsnip-like or fruity odor and flavor (Van Vuuren, 1996; Sakamoto K, & Konings W.N. 2003).

During the fermentation process *O. proteus* grows rapidly causing the rate of fermentation to decrease and can lead to an inferior product of high specific gravity and high pH. The bacterium can cause serious problems to the fermentation. In addition, growth of *O. proteus* during early fermentation is known to produce organo-sulphur compounds, various alcohol sand diacetyl which are thought to contribute to the parsnip-like smell of *O.proteus*-contaminated beer (Maugueretand T.M., Walker J.S.L. 2002).

Significantly the growth of *O.proteus* can contribute to the formation of apparent total N-nitroso compounds (ATNC) during fermentation (Fernandez et al.1993). ATNCs are formed from the reduction of nitrates, naturally present in brewing raw materials, to nitrite during the growth of *O.proteus.* The nitrites formed can

be subsequently converted to nitrosamines by reacting with the amines present in the wort or beer (Maugueretand T.M. and Walker J.S.L. 2002).

ATNCs represent a possible risk to health and consequently their concentration is strictly monitored and limited to 20 μg/l (Maugueretand T.M., Walker J.S.L. 2002).

Abnormally high levels of diacetyl and dimethyl sulfide were detected in beer produced from wort contaminated by *R. aquatilis* (Van Vuuren, 1996; Sakamoto K, & Konings W.N. 2003). Similar problems arise in unfiltered wheat beers, if the yeast added to the beer before packaging is contaminated by these enteric bacteria (Back W. 2005). They are classified as indirect beer spoilers because the damage they induce is generated during the brewing cycle, whereas they are unable to grow in the finished beer, at pH-values below 4.8 (Back W. 2005).

1.6. Obligate anaerobic bacteria

Occasionally, beer is contaminated by mixed populations consisting of lactobacilli, pediococci, *Pectinatus* and *Megasphaera*. In such cases, the spoilage usually occurs in two phases. The lactic acid bacteria develop first, scavenging residual oxygen and producing mainly lactic acid, which is later utilised as the most important carbon source by the strictly anaerobic beer spoilers, *Pectinatus* and *Megasphaera* (Back W., 2005).

Pectinatus spp.

Pectinatus spp. are now recognized as one of the most dangerous beer spoilage bacteria. They play a major role in 20 to 30% of bacterial incidents, mainly in non-pasteurized beer rather than in pasteurized beer (Back W. 1994). *Pectinatus* species were long thought to be *Zymomonas spp*. because of their phenotypical similarities.

Pectinatus strains were initially isolated from spoiled beer in the late seventies and assigned to a new genus and specie s *Pectinatus cerevisiiphilus*. Later a new species *Pectinatus frisingensis* has been established among the initial isolates (Schleifer, K.H., et al. 1990); the type strain of *P. frisingens* is a Finnish isolate first identified as *P. cerevisiiphilus* in 1981 (Helander I.M., et al., 2004).

The first isolate was obtained from breweries in 1971 (Lee et al., 1978) and so were all subsequent isolates (Back et al., 1979; Haikara A., 1984, 1985, Haikara et al., 1987). The natural habitat of the *Pectinatus* species are still unknown (Haikara

et al, 2009). Two species are found in this genus: *P. cerevisiiphilus* and *P. frisingensis* (Sakamoto K, & Konings W.N. 2003).

Pectinatus spp. are non-spore-forming motile rods with lateral flagella attached to the concave side of the cell body (Sakamoto K, & Konings W.N. 2003).

Both *Pectinatus* species are Gram-negative, strictly anaerobic, absolutely harmful beer spoilers. The cells are slim, with parallel walls, slightly arched or snake or helicoidally shaped. On average, their diameter is 0.8 μm, and their length 4 μm and they have round or spindle shaped ends. Sometimes thread-like and bowed cells also show up but mostly the rods occur singly or in pairs. The bacteria are ciliated laterally (comb-like) and flutter rapidly when they are young, but upon growing older, they slow down and are reminiscent of snakes (Back W., 2005).

Old cultures are mostly non-motile. *Pectinatus* grows at temperatures between 15 and 40°C, its optimum being 30-32°C. It ferments several sugars, sugar alcohols and organic acids. In the presence of fermentable sugars, the medium is acidified, but when lactate or pyruvate is fermented, often a slight pH-increase can be noticed. The main metabolites are propionic, acetic and succinic acid, and acetoine (Back W., 2005).

Pectinatus spp can be identified based on production of large quantities of propionic acid and hydrogen sulphide in beer (Haikara et al 1992).

Pectinatus bacteria appear to be common inhabitants in brewery bottling hall deposits. In biofilms on bottling machines, *Pectinatus* species were regarded as occasional invaders flourishing on favorable niches rather than permanent biofilm members (Timke et al., 2004, 2005). Characterization of environmental isolates has indicated that several sources may exist in a single brewery (Sakamoto K, & Konings W.N. 2003). Moreover, *Pectinatus* bacteria have supposedly been detected in pitching yeast and in malt steeping water (Sakamoto K, & Konings W.N. 2003).

The bacteria grow in any beer, provided the pH value is higher than 4.4 and the oxygen content very low (< 0.3 mg/1). Higher values are tolerated only in cases of very severe contaminations. *Pectinatus* is also capable of reproduction in beers and utilizes glycerol or pyruvate as a C source. A combined contamination with lactic acid bacteria is not uncommon: as *Pectinatus* utilizes the lactate produced:

the spoilage occurs then in two steps. Spoilage of beer by *Pectinatus* results in the formation of high concentrations of hydrogen sulphide with its putrid odour and development of turbidity. Various fatty acids, especially propionic and acetic, together with some acetoin are also produced (Briggs D.E., et al. 2004). *Pectinatus* can be considered as a secondary contaminant only for bottled beer. The germs are probably introduced into the breweries by air currents or dirty empties and hide in various dead and difficult to clean corners in the filler and crowner area. Frequent sources of contamination are also the conveyors (and their lubricants), the sewers and loose tiles of bad floors (Back W., 2005).

Pectinatus cerevisiiphilus is difficult to detect in the brewery indicated that the most common source of contamination was bottling an d capping equipment in summer . A major concern was the slow rat e of the microbes reproduction in media a great deal of harm might be done to the product before detection (Chelack G.J., and Ingledew W.M., 1987).

New species of *Pectinatus* was first isolated only a few years ago from samples collected in breweries and has been comprehensively characterized by Juvonen (2009) and Suihko (2001, 2003). *P. haikarae* differs markedly from both of the other primary representatives of this genus, *P. cerevisiiphilus* and *P. frisingensis,* due to its positive catalase reaction (Voetz M. et al. 2010).

Megasphaera cerevisiae

Megasphaera has emerged in breweries along with *Pectinatus* and is responsible for 3 to 7% of bacterial beer incidents (Sakamoto K, & Konings W.N. 2003).

Megasphaera cerevisiae is also a dangerous, Gram-negative, strictly anaerobic, absolutely harmful beer spoiling organism. It is different from *Pectinatus,* however, as it forms oval or round cells, preferably as pairs or in chains of four. The cocci are rather large and attain diameters of 1.2 - 1.6 µm. The temperature range for this species is 15 - 37°C, with an optimum of 28 - 30°C. Fructose, lactate and pyruvate are fermented, but growth also takes place if no sugars are present. The main metabolites are butyric, caproic, acetic, propionic, and valeric acid, as well as CO_2 and H_2S is also produced (Back W., 2005).

M. cerevisiae forms only slight hazes in beer and almost unnoticeable sediments, but causes severe off-smells and off-tastes (Back W., 2005).

Megasphaera strains produce several organic and fatty acids, notably butyric acid and some acetic, isovaleric and valeric. In addition, hydrogen sulphide is generated (Engelmann and Weiss, 1985). Their potential for beer spoilage is restricted by their sensitivity to ethanol (>2.8% v/v) and acid pH (Haikara and Lounatmaa, 1987). They are common spoilage bacteria of unpasteurized packaged beers. Signs of spoilage include turbidity and off-flavours from the synthesis of organic acid sand sulphuric compounds(Haikara & Helander,2002).Nevertheless, several weeks may be required before turbidity becomes evident (Briggs D.E., 2004).

M. cerevisiae is a typical secondary contaminant in bottling-halls, its behavior being very similar to that of *Pectinatus*. Mixed contaminations of this species with *Lactobacillus brevis* or *L. casei* are also frequent. With the latter, two-stage growth occurs, *M. cerevisiae* further utilizing the lactic acid formed (Back W., 2005).

1.7. Other bacteria contaminants

Bacteria of the genus *Zymomonas* have a unique mode of catabolism among the bacteria in that they conduct an ethanolic fermentation. This is so efficient that it has been seriously considered for the production of fuel ethanol, but it is not used for potable alcohol. Nevertheless, the bacterium is tolerant of ethanol (up to about 10% by volume)and has been associated with spoilage of primed conditioning ale (Priest F.G. 2006).

The potential beer spoiling species *Zymomonas mobilis* is Gram negative, catalase-positive, oxydase-negative, not spore forming and usually immobile. The bacteria are facultatively or absolutely anaerobic and form short or long rods (1.0 - 1.5 µm x 2.0 - 6.0 µm), with rounded ends, usually occurring singly or in pairs: but bigger agglomerates are occasionally also formed (Back W., 2005).

Zymomonas mobilis tolerates oxygen but grows under anaerobic conditions. It ferments glucose and fructose but not maltose. Unlike most of the *Enterobacteriaceae* it tolerates ethanol and reportedly survives high-gravity fermentations in which 12 - 13 % v/v ethanol are formed (Briggs D.E., 2004).

It has a relatively high optimum growth temperature of 25 ± 3 °C. For this reason, it tends to be a more common spoilage bacterium in ale breweries as opposed to

those fermenting lager worts at lower temperatures. Infected worts develop a characteristic rotten apple odour due to the formation of acetaldehyde. In addition, ethanol, acetic acid, lactic acid, acetoin and glycerol are formed (Van Vuuren, 1999).

The pH value of the medium is reduced only slightly or not at all. As the sugars glucose, fructose and sucrose are essential for growth, *Z. mobilis* is irrelevant for highly fermented continental beers, but may cause problems in British ales, to which sugars are added after primary fermentation. In contaminated beers, so-called "boiling fermentations" may be triggered. An undesired, fruity flavour evolves in the beers, probably related mainly to the metabolites hydrogen sulphide and acetaldehyde. In addition, strong hazes and sediments are also formed, and the beers fail to meet consumer expectations (Back W., 2005).

1.8 Wild Yeast

Wild yeasts are generally defined as those yeasts not deliberately used and not under full control (Gilliland, 1967).

The definition of wild yeasts is diffuse and, for convenience, is traditionally divided into *Saccharomyces* and non-*Saccharomyces* (Boulton C. and Quain D., 2001).

This definition includes brewing strains that are used for a different style of beer and may have been cross-contaminated in the brewery, as well as non brewing yeasts that have gained access from the air or raw materials. It is important to emphasize that there are many genera and species of yeast with diverse physiologies; the only unifying feature is that the organisms are predominantly unicellular. However, many types of yeast have a semi filamentous lifestyle and may form mycelia under various environmental conditions (Priest F.G., Stewart G.G., 2006).

Although boiling of wort kills most microorganisms and thence is inoculated by pitching yeast, other kinds of unwanted yeasts can get into beer during fermentation; these yeasts are collectively known as wild yeasts (Deàk T. 2008).

Wild yeasts were detected in 41 % of pitching yeasts investigated (Van Der A Kühle and Jespersen, 1998).

A contamination with wild yeast results in a phenolic off - flavor in lager beers (Eβlinger H.M., 2009).

Most brewing strains are unable to utilize dextrins and these persist in beer where they contribute to fullness and mouthfeel. Some strains, originally classified as *S. diastaticus* but now placed with *S. cerevisiae*, possess glucoamylase and in consequence can utilize dextrins. Contamination of fermentations with diastatic yeasts leads to super-attenuation of the wort and beers with abnormally low present gravity. Occasionally, diastatic yeasts have been used to produce so called `light' beers (Briggs D.E. et al. 2004).

Contamination of unpasteurized bottled beer with diastatic yeast is potentially hazardous, since abnormally high concentrations of carbon dioxide can develop with the consequent risk of bottle explosions (Briggs D.E. et al. 2004).

Pichia membranefaciens is the most common contaminant of beer and wine in this category. The acetic acid-forming *Brettanomyces* and *Dekkera* species, although fermentative, do not usually cause a threat to the brewing process because they cannot flourish under anaerobic conditions (Priest F.G., Stewart G.G., 2006).

However, they form an important component of the yeast flora of fermenting Belgian lambic beers and can cause problems in ales and lagers if air should gain access. The aerobic yeasts such as *Debbaromyces, Pichia*, and *Williopsis* produce yeasty or estery flavors that are most un welcome (Priest F.G., Stewart G.G., 2006).

The fermentative yeasts such as *Kluyveromyces, Saccharomyces*, *Torulaspora*, and *Zygosaccharomyces*, on the other hand, can cause serious problems in the fermentation. They are potentially able to compete with the culture yeast, and although they cannot generally kill it, if they grow just a little faster than the culture yeast they will displace the brewing yeast over successive generations. As these wild yeasts neither flocculate well nor interact with finings, they generally pass into conditioning where they can have deleterious organoleptic effects on post fermentation beers, as well as causing haze and turbidity (Priest F.G., Stewart G.G., 2006).

Aerobic wild yeast can cause problems in beer –dispensing equipment than in the brewing process or in packaged beer. This is due to the higher oxygen levels and higher temperatures at certain points in the dispensing system. These conditions favor contamination by microorganisms such as aerobic wild yeast in addition to

the oxygen tolerant beer spoilage organisms found in the brewery environment (Storgårds E. Et al., 2006; Storgårds E. 2000).

Wild yeasts may be isolated from all stages of the brewing process, from raw materials to packaged beer, and from bar dispense equipment. However, they typically contaminate pitching yeast, and increase in number over successive re-pitching. Spoilage may occur in the finished product, during conditioning, or (to a lesser extent) during fermentation (Fleet, 1992).

The growth of wild yeasts may cause the production of off-flavors particularly phenolic compounds. The presence of these volatile phenolic compounds is considered undesirable when present in excessive concentration in bottom-fermented pilsner beers. Hence the term ‘‘phenolic off-flavor ” (POF). Despite being historically catalogued as an off-flavor, these compounds are known to be essential flavor contributors to the characteristic aroma of Belgian white beers (made with un-malted wheat), German weizen beers (made with malted wheat) and rauch beers. However, in many other top-fermented blond and dark specialty beers the phenolic flavor is essential for the overall flavor perception (Vanbeneden N. et al, 2008).

Saccharomyces wild yeasts produce phenolic off-flavors (such as 4-vinyl guaiacol) by decarboxylating various phenolic acids, due to the presence of the phenolic off-flavor gene (POF) (Ryder et al., 1978; Thurston, 1986).

1.9 Hygiene management in production facilities

The role of cleaning and disinfection for both small and large breweries has grown immensely due to production of non-pasteurized products (Kretsch, 1994) and due to new products low in alcohol and bitterness (Storgårds E., 2000).

In the 1990 edition of the “Society of Dairy Technology manual CIP”: Cleaning in Place, CIP was defined as:

The cleaning of complete items of plant or pipeline circuits without dismantling or opening of the equipment and with little or no manual involvement on the part of the operator. The process involves the jetting or spraying of surfaces or circulation of cleaning solutions through the plant under conditions of increased turbulence and flow velocity (Tiamine A.Y., 2008).

Cleaning out of place is essentially the opposite of CIP, and refers to most manual cleaning applications. Either the equipment must be broken down into pieces or major modifications must be performed before the cleaning can take place. Some equipment that is normally cleaned via CIP should be cleaned periodically in a COP mode, such as heat-exchangers. Therefore, COP cannot and should not be completely avoided. Some of the drawbacks and benefits of COP are:

- Cleaning results may vary with operator/employee;
- Exposure of employee to cleaning solutions;
- Time-consuming process;
- Easily verifiable through visual inspection;
- May expose residuals left by CIP cleaning (Loeffler D., 2006).

Soil adheres to surfaces in very complex ways. It can be trapped mechanically in pores, cracks or other inclusions, which explains the choice of hard-surface materials such as finished stainless steel. We also see electrostatic binding forces, both between the surfaces and the soil as well as between different types of soils such as protein and mineral salts. The sum of all these binding forces combined can be expressed as the adhesion energy, which is the energy that has to be achieved during the cleaning process to remove the soil. During cleaning, the adhesion energy is derived by combining the energy from chemicals, mechanics, and temperature, whereas the energy from these three components is interchangeable within certain limits (Loeffler D., 2006).

The process of soil removal can be divided into four major steps:

1. Transport of the cleaning solution to the soil with complete wetting of the soil.
2. Chemical reactions and physical processes during the cleaning process:
    - reaction of the cleaning solution with hard water constituents and/or suspended soil;
    - convective and diffusive transport of the cleaning agents from the cleaning solution to the soil;
    - wetting or transport of the cleaning agents within the soil itself;
    - cleaning reaction with the soil, both chemically and physically;

- diffusive transport of soil particles removed during the cleaning process.

3. Removal of the soil from the surface and transfer into the cleaning solution via dispersion and/or emulsification.

4. Prevention of re-depositing removed soil through stabilization in the cleaning solution and transport of removed soil away from the surface (Loeffler D., 2006).

Cleaning is the removal of contamination or undesired residues from hard surfaces with the aid of chemical and/or physical cleaning methods and agents. Factors for successful cleaning include:

- temperature (hot cleaning, cold cleaning);
- cleaning time (the longer the cleaning time, the greater the cleaning success);
- mechanics (pressure, volume flow, flow speed);
- chemical (type and concentration of the cleaning agent) (Praeckel U. 2009).

Alkaline Cleaning agents

The composition of alkaline components in a cleaner determines its alkalinity. Acidic product residues as well as carbon dioxide can partially neutralize and reduce the original free alkalinity.

Sodium hydroxide provides the most alkalinity, which is one of the key factors in removing organic soil in brewery cleaning (Loeffler D., 2006).

Sodium hydroxide exhibits an excellent emulsifying capacity for protein. Accordingly, it is used in a wide variety of applications in breweries. Caustic potash has an even greater capacity for breaking up soiling than sodium hydroxide. It is only used in limited applications, however, due to the fact that is several times more expensive (Praeckel U. 2009).

Acidic Cleaning agents

Beer-stone and mineral deposits are primarily based on mineral components. These types of deposits are virtually impossible to remove with alkaline products alone. However, acids will take water-insoluble salts and chemically transform them into a soluble, rinseable form (Praeckel U. 2009).

Mineral acids: The corrosivity as well as the incompatibility of most mineral acids with other active components commonly used in cleaning products limits their use to primarily phosphoric acid and nitric acid. Sulfuric acid may be used at temperatures not exceeding 30°C. Hydrochloric acid should be avoided at all cost (Loeffler D., 2006).

Organic acids: The most important criteria for organic acids are their odor (short-chained carbonic acids), solubility and strength. Commonly found products from this group are formic acid, oxalic acid, citric acid and lactic acid (Loeffler D., 2006).

The most suitable acidic cleaners are products with phosphoric acid. Phosphoric acid far exceeds nitric acid and sulfuric acid in its cleaning power (Praeckel U. 2009).

Disinfecting

The single most important precondition for successful sanitizing is an effective cleaning program (Loeffler D., 2006).

Disinfecting agents used in the food industry are tasked with making production equipment free of microorganisms after use and subsequent cleaning. Microorganisms can be killed physically and chemically. Physical elimination involves heat treatment, UV and X –rays and other methods. Disinfecting with chemicals is possible via a host of disinfecting agents (Praeckel U. 2009).

The most important agents disinfectants are represented in the following table.

Active ingredient	Remarks	Use
Peroxacetic acid	Acidic disinfecting agent with oxidizing effect (destroys cell membrane); conditionally stackable due to loss effectiveness; automatic dosing via inorganic conductive acids only; sealing materials may be harmed with extended contact; very broad range of effectivess.	Bottle cleaning, CIP cleaning.
Hydrogen peroxide	Neutral disinfecting agent with oxidizing effect; very environmentally and waste water friendly, since it decomposes with organic material in water and oxygen; high usage concentration; very broad range of effectiveness.	CIP cleaning; spray disinfecting
Active chlorine (sodium hypoclorite)	Alkaline disinfecting agent with oxidizing effects; danger danger of chlorophenol formation (negatively effects taste of the product); very broad range of effectiveness; ATTENTION: when mixing with acidic solutions, chlorine gas is released!	Bottle cleaning; CIP cleaning; drinking - water disinfecting
Chlorine dioxide	Disinfecting agent with oxidizing effect; two - component system that is mixed on - site when used; economical operating costs, but high investment costs; very broad range of effectiveness	Bottle cleaning; CIP cleaning; drinking - water disinfecting
Quaternary ammonium compounds	neutral disinfecting agent (surfactants); destroys the cell membrane; heavily foaming (not suitable for CIP); surface - active; relatively difficult to rinse out due to the surface activity (adheres well to the surface)	Static disinfecting; spray disinfecting

Table 1.1. Disinfecting substances (Praeckel U. 2009).

Formulation based on peracetic acid and hydrogen peroxide are frequently used for post-cleaning disinfection. Peracetic acid (PAA) penetrates the cell and oxidises enzymes and other proteins irreversibly. PAA has been shown to be effective against biofilm. The agents quickly lose their activity in a basic environment, making careful rinsing after alkaline cleaning essential (Storgårds E. 2000). Peracetic acid and hydrogen peroxide-based disinfectants also perform well in the presence of organic soil, but they are markedly less effective when the temperature is decreased from ambient (20°C) to 4°C. At low temperatures, such as in the fermentation cellar, higher concentrations are needed to obtain a good result al (Storgårds E. 2000).

CIP programmes

A typical CIP sequence will comprise the following cycles:

- pre-rinse;
- detergent circulation;
- intermediate rinse;
- additional detergent circulation (optional);
- additional intermediate rinse (optional);
- disinfectant rinse (optional);
- drain.

The pre-rinse cycle removes the loose soil: the more of this that can be removed with a simple rinse, the less the need for the chemical, mechanical and thermal energy needed later. Rinsing efficiency can be improved through 'burst rinsing' of vessels, and through ensuring that draining surfaces are on a slope. The detergent circulation is usually the key stage in removing residual soil. Circulation time is typically 10–30 min, but the choice of detergent very much depends on the particular cleaning situation. Examples of detergent/temperature solutions are shown in Table 1.2.

An intermediate rinse is then required if a further detergent circulation is going to be used. This is particularly the case if an acid treatment is necessary, usually when scale removal is required (Tamime A.Y., 2008).

Action	**Temperature**	**Duration**
Prerinsing	Cold or hot	5-10 min
Alkali cleaning; sodium hydoxide (1.4-4%)	Cold or hot (60-85°C)	10-16 min
Intermediate rinsing	Cold or hot	10-30 min
Acid cleaning; phosphoric, nitric or sulphuric acid (1-2%)	Cold	10-30 min
Intermediate rinsing	Cold or hot	10-30 min
Disinfection		
- disinfectant solution	Cold	10-30 min
- hot water	85-90°C	45-60 min
Final rinsing if necessary		
- may contain a disinfectant at low concentration	Cold	5-10 min

Table 1.2. Typical CIP programmes used in the brewery (Storgårds E. 2000).

2. MICROBREWING IN ITALY

2.1 The microbrewery industry in Italy

There is no a precise definition in Italy for a microbrewery, and this descriptor is often proclaimed, based on different factors such as size, brewing style, and ownership. Typical microbreweries are independent from large brewing companies, and they produce “craft” beer on smaller scale. Usually such beers are brewed with high malt content, no additives, hop pellet or hop flowers and they are usually not pasteurized and neither sterile filtered. Further, the majority of beers produced by microbreweries are ales (Braun J, Dishman B.H., 2006). Over recent years the number microbreweries in Italy increased dramatically. In the mid-'90s, early few pioneers made the jump from home brew to brewpub (figure 2.1); today there are almost 520 craft microbreweries and brewpubs operating in Italy, mostly in the north, but new microbreweries have also appeared in the south (figure 2.2) (microbirrifici.org). The data obtained according to the report conducted by ALTIS describe a changing landscape in which the average annual production for each craft brewery stood at 411 hectoliters. The total production of the Italian craftbreweries amounted to 137.680 hectoliters (Assobirra, Annual Report 2011), just over 1% of the national production (12.810.000 hl) (Altis, Report 2011).

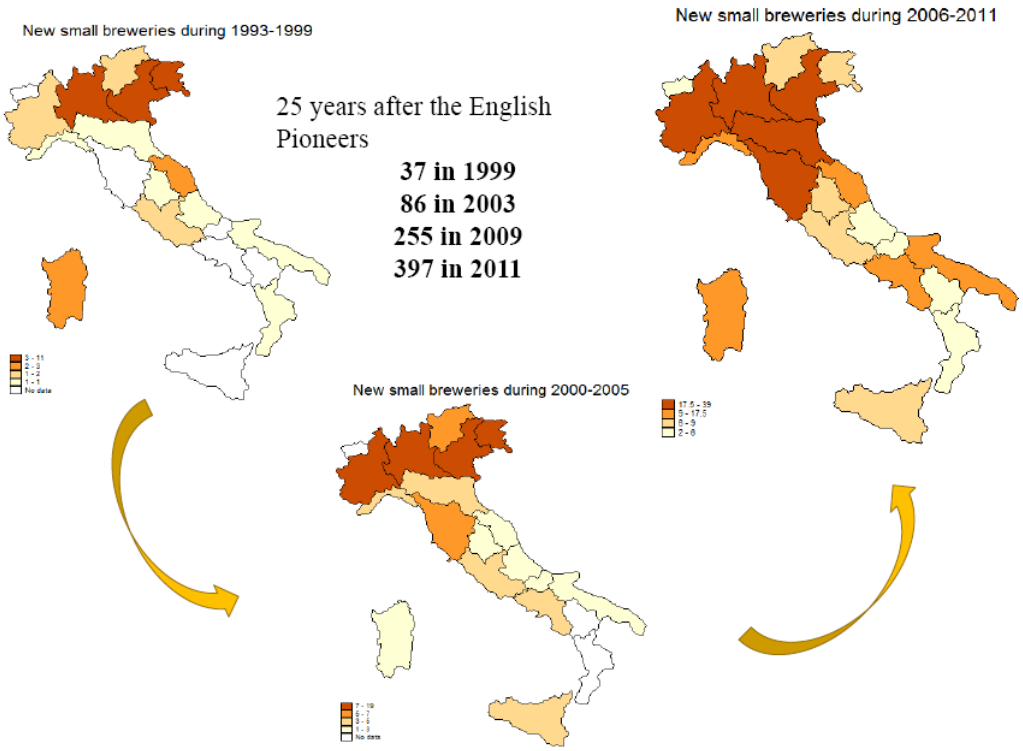

Figure 2.1. Changes in the number of breweries in the last twenty years (Savastano S., 2011).

The microbreweries in Italy are generally small businesses with an annual production ranging from 300 to 10,000 hl mainly with local distribution to restaurants and specialty shops or outlets close to the production area. The product is usually considered different and more creative compared to mass-products and it is often tied to the territory of local production according to particular ingredients that are used in the production process (Garavaglia C., 2010).

Figure 2.2. Regional distribution of small breweries in Italy (Savastano S., 2011).

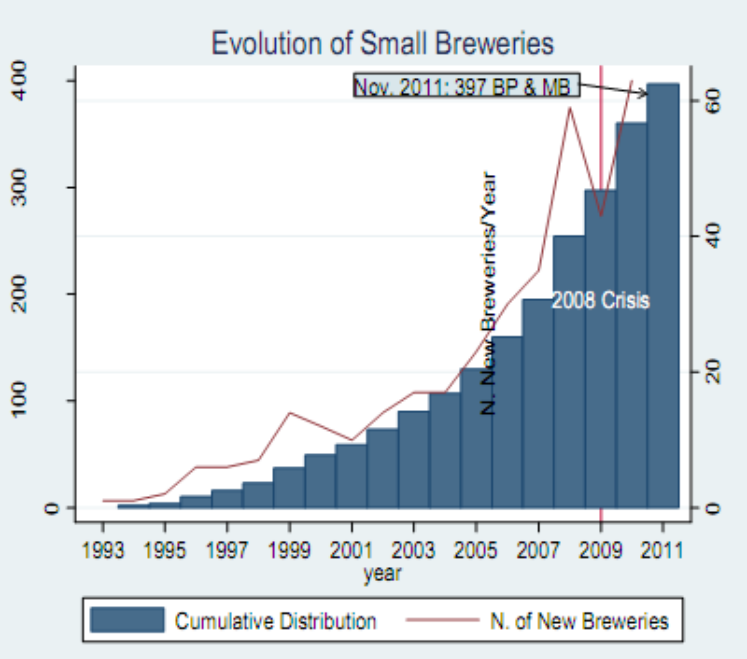

Figure 2.3. Evolution of the number of small breweries in Italy (Savastano S., 2011).

From Figure 2.3 developed by Savastano the increase in the number of small breweries and brewpubs in Italy since 1993 was highlighted.

It is difficult to imagine how the microbreweries and brewpubs situation will evolve in Italy in the coming years.

Maybe there will be a reduction in the number of microbreweries and a parallel increase in the annual production of those that remain.

One of the major challenges that microbreweries face today is the maintenance of beer quality. Due to the brewing methods used in microbreweries, their beer is inherently more prone to contamination by beer spoilage microorganism. Unlike most larger scale production, the majority of microbrewed beer is not subject to any post-fermentation microbiological stabilization process, such as pasteurization or sterile-filtration. These protective barriers are omitted in the pursit of “natural” or “real” beer with enhanced organoleptic qualities, or because of cost pressures. Further, small breweries use different brewing methods (open vessels), have less comprehensive quality control system, and produce all malt beers (Braun J, Dishman B.H., 2006). It has been suggested that the increased malt concentration in all-malt beers leads to an increase in soluble proteins and phosphate ions, which act as buffers, resulting in a beer with a higher pH (less resistant to microbial

growth) (Lewis, 2003). In addition, the increased levels of amino acids in the finished beer may later support the microbial growth.

The trend of beer consumption has been growing despite the beer industry is considered mature, enjoying an increasing success particularly in the young people. Consumers, therefore, are one of the most important elements in determining the success of craft beer in Italy. As in all the industries, they will determine the evolution of the sector in the future years. The consumer's views, is way of approaching the beer purchase and consumption, is preferences were deeply analyzed in order to understand the peculiarities and the limits of this sector (Cronache di birra, Rapporto 2010). A recent report carried out by Assobirra (Assobirra report 2011), shows that the average consumer of craft beer is male, aged between 31 and 45 years, and he is an occasional drinker which recently came in contact with microbreweries (no more than 3 years ago) and he is far from complete fidelity to craft beer. Often the consumer gives to the beer concepts like "taste", "culture" and "community". So it is a drink that enhances of the sensory perceptions of drinking and socializing whit a recognized value in the cultural background. For the consumers, the ideal beer has to be "surprising", "particular" and "complex" to capture their attention.

3 MATERIALS AND METHODS

3.1. Management of the process in Italian Microbrewery.

356 questionnaires were sent to the microbrewers in order to collect information about the process management (production volume, cleaning procedure, yeast management, etc.). Twenty breweries responded to the questionnaire and it is hen possible to analyse the Italian microbreweries situation. The questionnaire is included in Annex 1.

3.2. Identification of lactic acid bacteria mainly involved in the contamination of the finished product.

168 craft beer samples were collected and analyzed for the LAB presence. The samples were collected from 39 craft breweries.

All the beers were not pasteurised and neither sterile-filtered, and bottle-conditioned.

The analytical methods of Analytica – Microbiologica – EBC (4.2.4.2) were used for the detection of spoilage bacteria in beers. 100 mL of beer were filtered (0.45 µm, cellulose nitrate) with vacuum assistance. Some beers required multiple filtration to achieve a sample volume of 100 ml due to yeast and other particle. Each membrane was placed onto NBB-A (Döhler - Germany) and the plates were incubate in anaerobic condition at 27 ± 1 °C for 7 days. The isolated bacteria were collected on MRS agar (0.2 % phenyl ethanol) and several subculture were done to obtain pure culture. LAB were identified using the API 50 CHL kit (Bio Merieux SA, Marcy - L`Etoile, France) according to the manufacturer`s instructions. Identification of the isolates microorganisms was done with the software Apilab Plus (version 3.3.3, Bio Merieux).

3.3. Case studies

To understand where the product comes in contact with the spoilage lactic acid bacteria, it was decided to analyse the case studies. Two breweries were chosen, one with a production of less than 1.000 hl/year and one with an output between 5.000 and 10.000 hl/year.

The process water is periodically controlled and it respects the potability specifies. Both breweries produce bottle conditioning beer, with the addition of sugar solutions but without the addition of new yeast.

Description of the production process in the brewery A

The brewery has a production capacity of 10000 hl/year. The brewhouse is a traditional 4 vessel system layout: mashing vessel with a pre-mashing system, traditional lauter tun with automatic flow rate, differential pressure control and automatic spent grains removal. The boiling kettle includes an external boiler and a wort dispersion plate which allows to boil the wort at 98°C, with an evaporation rate of 3%; the whirlpool vessel has a traditional geometry, with two casting valves at different heights. A secondary wort evaporation is obtained before the cooling of the wort trough several spray nozzles where the wort is injected again inside the copper proir to be pumped into the plate cooler. The brewhouse has a cast out volume of 35 hl at 12°P.

For pitching the wort, a commercial dry yeast is used (40 g/hl).

The fermenting vessels are 10, all CCV (cylider conical vessel tank) with a nominal capacity of 70 hl (8 fermenting vessels) and 140 hl.

After the first fermentation and cold maturation of the beer, the most of the yeast are removed by a centrifuge separator, decreasing the yeast concentration from 10^6 to 10^2 CFU/ml. The centrifuged beer is then added with priming sugar into a BBT (bright beer tank) and immediately bottled.

The bottling line is composed by: a bottles transport belt, charged manually, a bottle rinser with 16 spray nozzles using ultra-filtered de-hardened water, an isobaric filling machine with 20 filling stations, a traditional single capper with magnetic caps elevator.

The filling machine is an isobaric system: a mechanical stuff holds the bottle up to the tap, where a seal guarantees that no pressure is lost, a vacuum pump line draws off the air from the bottle, immediately after the pressure is replaced with food grade nitrogen at 2 bar, this process is repeated for two times to ensure the lowest oxygen concentration inside the bottles.
After the final pressurisation at 2 bar, and the beer flows into the bottle from a tap which sprays the liquid on the surface of the bottles, in order to avoid foaming and subsequent oxidation.
When the level of the bottle is reached, the bottle is removed from the sealing by the descent of the mechanical stuff, and the bottles are transported through the capping unit.
The filling process takes place in roughly 9 seconds.

After capping, the bottles are manually picked up and put into stainless stell crates containing 600 bottles each.

The crates are transferred into the conditioning room, at 24°C, where the secondary fermentation takes place for a minimum of 15 days

The sampling points within the bottling machine were grouped and identified with the following abbreviations.

Bottle washing area:
BWM1: surface of the washer machine;
BWM2: surface of the washer machine;
PSBMW: surface of the washer machine;
CBW: conveyor belt of the washer machine.

Filling area:
FM3: surface of the filling area;
FM4: surface of the filling area;
PSBMF: surface of the washer machine;
CBF: conveyor belt of the filling machine;

FS2: filling nozzles number 2;
FS4: filling nozzles number 4;
FS6: filling nozzles number 6;
FS8: filling nozzles number 8;
FS10: filling nozzles number 10;
FS12: filling nozzles number 12;
FS14: filling nozzles number 14;
FS16: filling nozzles number 16;
FS18: filling nozzles number 18;
FS20: filling nozzles number 20;
SFH5: filling nozzle base number 5;
SFH10: filling nozzle base number 10;
SFH15: filling nozzle base number 15;
SFH20: filling nozzle base number 20;
PSBM5: surface of the filling head number 5;
PSBM10: surface of the filling head number 10;
PSBM15: surface of the filling head number 15;
PSBM20: surface of the filling head number 20.

Capping area:
CM5: surface of the capping area;
CM6: surface of the capping area;
PSBMC: surface of the capping area;
CBC: conveyor belt of the capping machine.

The process and the sampling points (red arrows) are summarized in the figure 3.1.

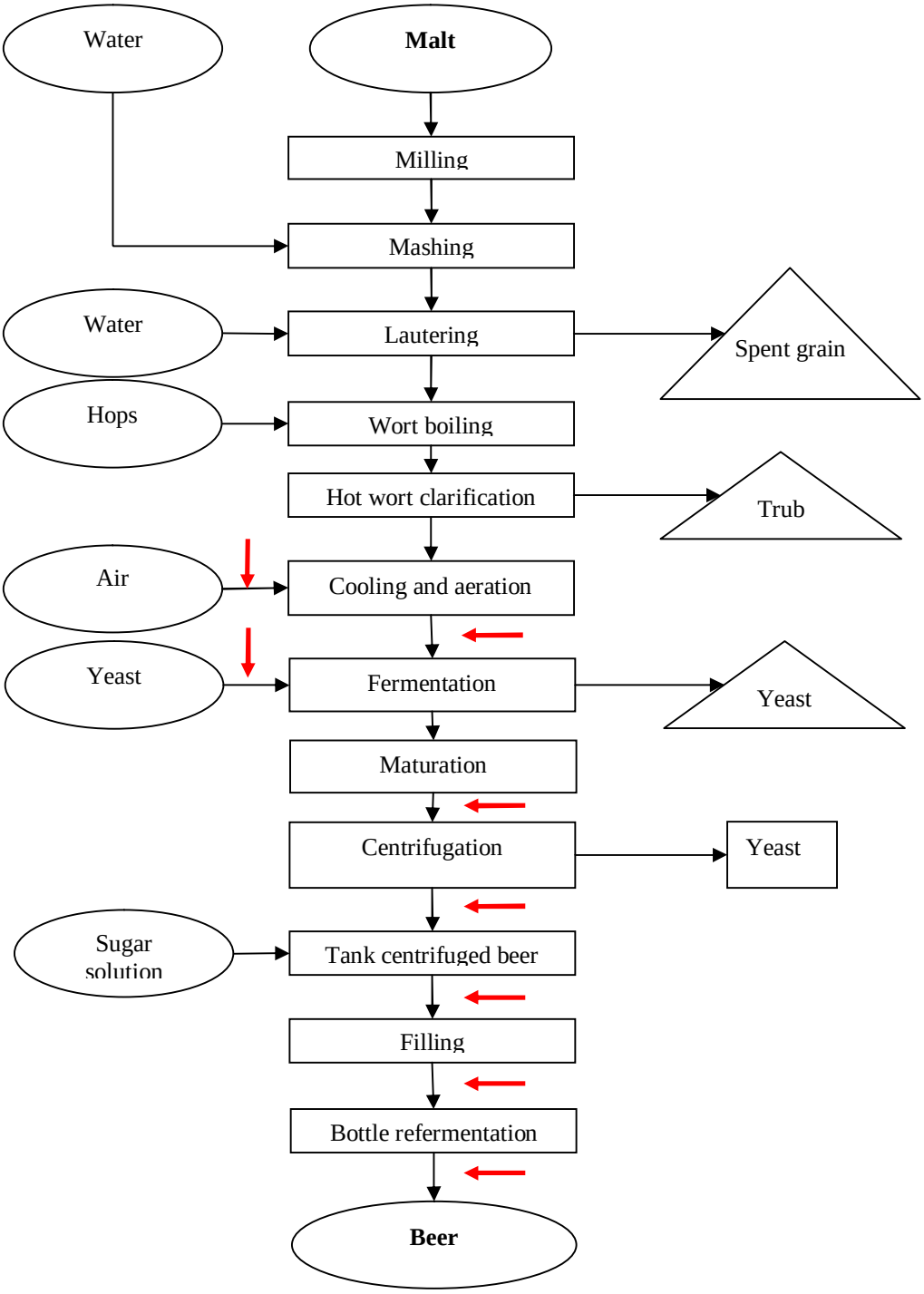

Figure 3.1 – Process in "A" brewery. The red arrows indicate the sampling points.

Description of the production process in the brewery B

The brewery has a production lower than 1000 hl/year. The brewhouse is a 2 vessel system layout: mashing/lautering vessel and boiling/whirlpool kettle.
The brewhouse has a cast out volume of 5 hl at 12°P.

The fermenting vessels are 2, all CCV (cylider conical vessel tank) with a nominal capacity of 10 hl.

For pitching the wort, a commercial dry yeast is used (40 g/hl).

After the first fermentation and cold maturation the beer is added whit the sugar and immediately bottled.

The brewery B does not perform the centrifugation of the mature beer.

The filling machine is a vacuum filler with four beaks.

After capping, the bottles are manually picked up and put into carton packing containing 6 bottles each.

The carton packing are transferred into the conditioning room, at 20°C, where the secondary fermentation takes place for a minimum of 15 days.

The sampling points within the bottling machine were grouped and identified with the following abbreviations.

The process and the sampling points (red arrows) are summarized in the figure 3.2.

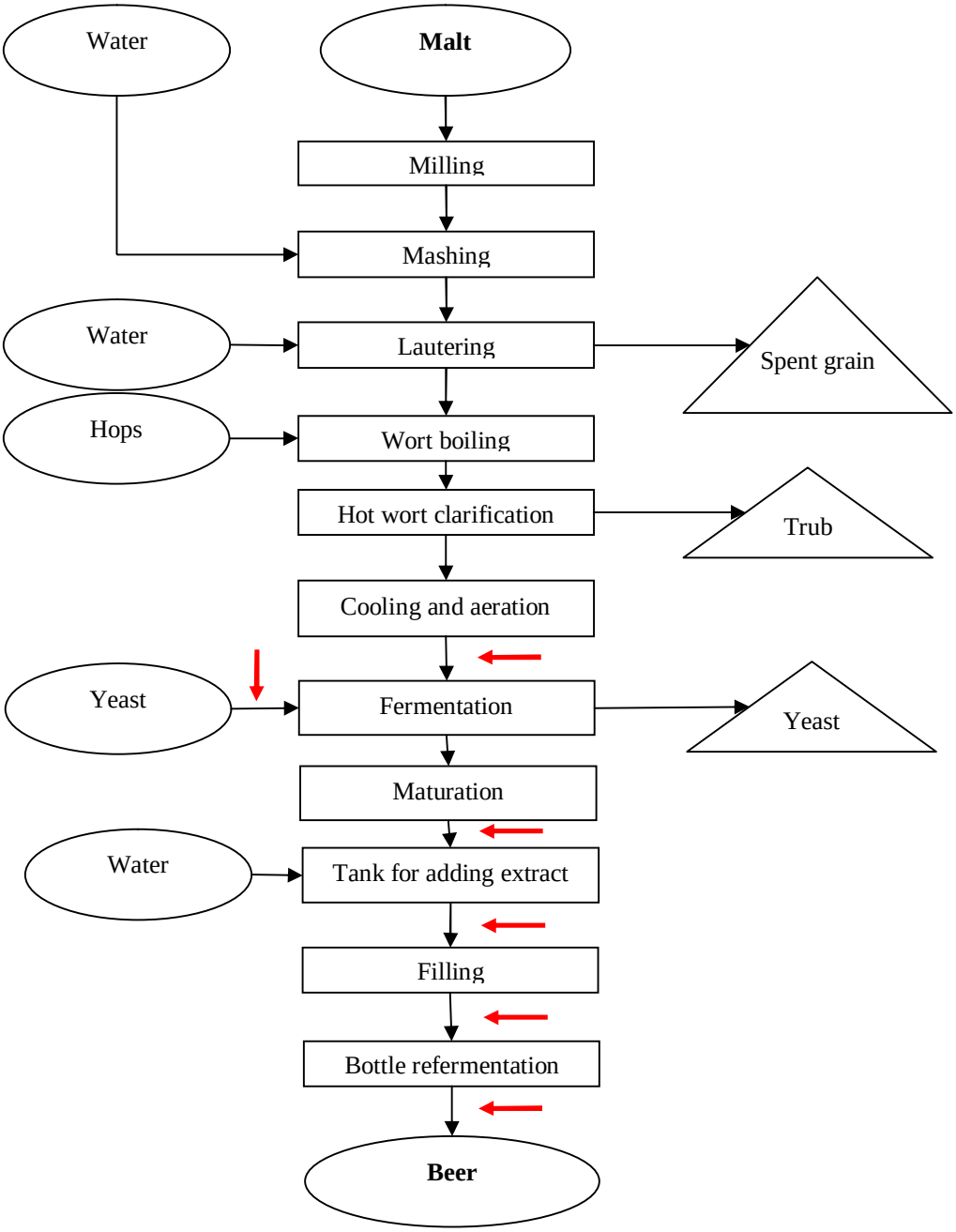

Figure 3.2. – Process in "B" brewery. The red arrows indicate the sampling points.

Analytical methods used in both breweries

The analytical methods of Analytica – Microbiologica – EBC (4.2.4.2) were used for the detection of spoilage bacteria in beers. 100 mL of beer were filtered (0.45 µm, cellulose nitrate) with vacuum assistance. Some beers required multiple filtrations to achieve a sample volume of 100 ml due to yeast and other particle. Each membrane was placed onto NBB-A (Döhler - Germany) and the plates were incubate in anaerobic condition at 27 ± 1 °C for 7 days.

For swab test analysis the analytical methods of Analytica – Microbiologica – EBC (2.2.5.6 Swab Tests) were used for the swab test. The liquid media used for incubation are NBB®-Broth-Enrichment-Media (Döhler - Germany) ready to use for detection of beverage spoiling microorganisms in brewing industry. It is used for the analysis of weak spots in filling and production sites. The swabs are incubate for a maximum of 3 days at 27°C under aerobic conditions.

The method Analytica – Microbiologica – EBC (2.2.4.2) was used to collect the microorganisms from atmospheric air. Air and dust-borne microorganisms are trapped on the surface of an agar medium.
Petri dishes with solid medium 9 cm diameter (surface area “A”), were used.
NBB-A (Döhler - Germany) was used as growth substrate and the plates were incubated in anaerobic condition at 27 ± 1 °C for 7 days. The result is expressed as "number of organisms per ‘A’ mm^2 for 15 minutes exposure”.

4. RESULTS AND DISCUSSION

4.1 Analysis of Italian microbreweries' management

The results shown below concern the information collected in some Italian microbreweries. The annual production of the microbreweries that were studied in this PhD project is shown in the following Figure 4.1.

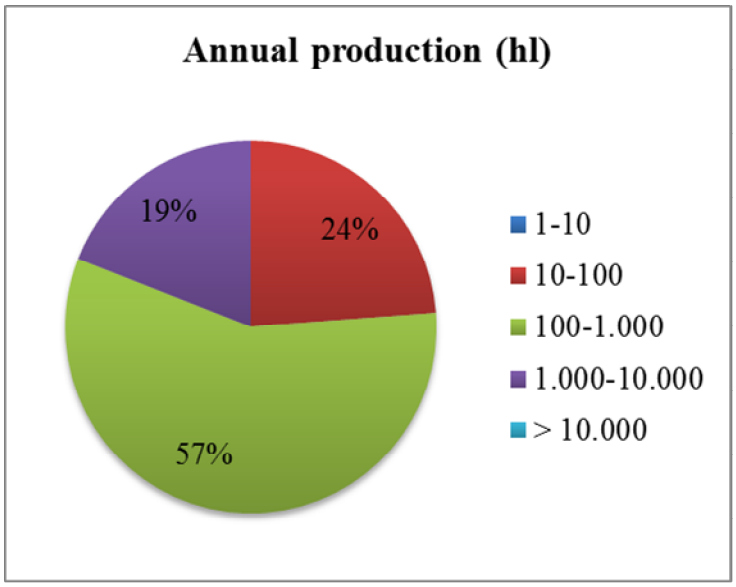

Figure 4.1 – annual production in Italian craft brewery (hl)

The figures show that the average production in Italian microbreweries is still very small compared with the U.S., U.K. and Belgian breweries.

This fact can be explained considering several factors regarding the Italian craft beer market:

- The craft beer production in Italy is still growing, because the first craft brewers opened only during the 90's. This means that this relatively new market is not yet very competitive; the technology development of the breweries still has wide limits to grow, and the technology improvement of the micro-breweries is not yet a fundamental aspect when planning and setting up a brewery.
- The beer consumption in Italy is one of the lowest in Europe with an average consumption of 29 liters per capita, where the craft beer market is only the 1% of this volume, therefore the average size of the craft brewers is quite small.

- The cost of opening a new business, the cost of workers and the taxes on alcohol products are quite high in Italy if compared with the traditional beer producing nations such as Germany, Belgium and U.K., leading to a smaller size of the craft breweries in Italy.

None of the breweries that joined the project has an annual production of more than 10,000 hl, however an increase of production volumes is expected in the next years, but also a reduction of the total number of Italian microbrewers is expected.

According to our survey, the brew house systems installed in Italian microbreweries are:

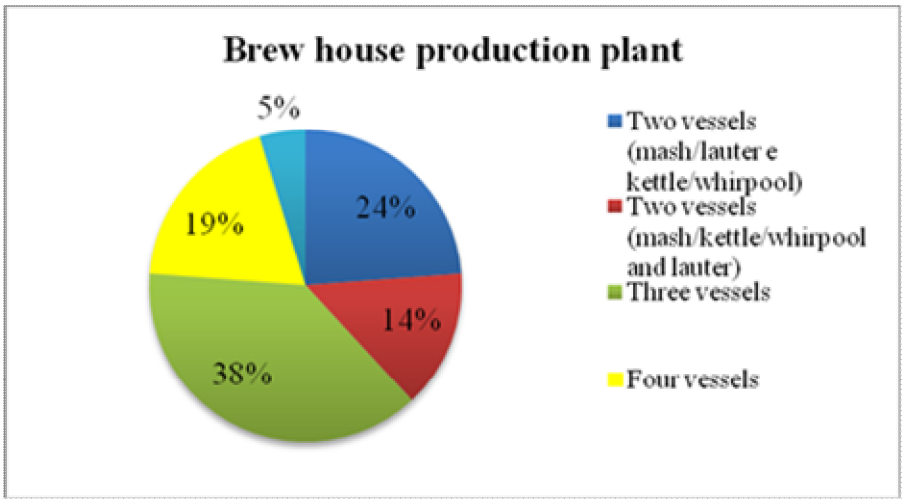

Figure 4.2 – plant types in Italian microbreweries

The brew house systems in use are very varied and depend on both the finance budget and productive capacity of each microbrewery.

Another important aspect for the beer production is the choice of the pitching yeast. The microbreweries in Italy are using the following yeast solutions.

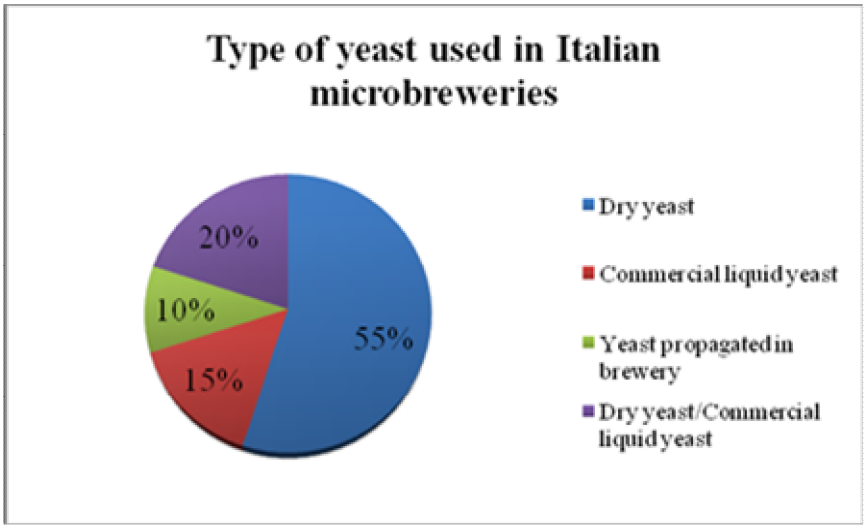

Figure 4.3 – type of yeast used in Italian microbreweries

The dry yeast is used by 55% of Italian microbreweries, for several reasons:

- convenience of use;
- easier to handle, store and transport;
- flexibility of dosing;
- possibility to produce specialty beers;
- there is no need to invest in a propagation plant (propagation is not required).

The 15% of the microbreweries uses liquid yeast; this type of yeast must be propagated in the brewery prior to pitching and it can also have some management drawbacks. In fact, a propagation carried out without the right equipment and methods can have negative effects on the quality of the pitching yeast. The 20% of microbreweries uses both dry and liquid yeast, the latter especially for specialty beers.

Only 10% of surveyed microbreweries uses propagated yeast, because propagation plant is required, but also the necessary know-how and the microbiological quality controls.

The reasons of the success of dry yeast in Italian microbreweries is due to these aspects:

- There are not yet yeast producers in Italy. All the yeast products are currently imported from Germany, France, USA and Canada. This means that it is difficult to import a fresh yeast culture, and transport costs are high.

- Due to the small amount of production of the micro breweries in many cases the recovery of the yeast for the use in subsequent batch is not possible.
- The budget needed for a proper propagation plant is not yet affordable for the most Italian microbreweries.

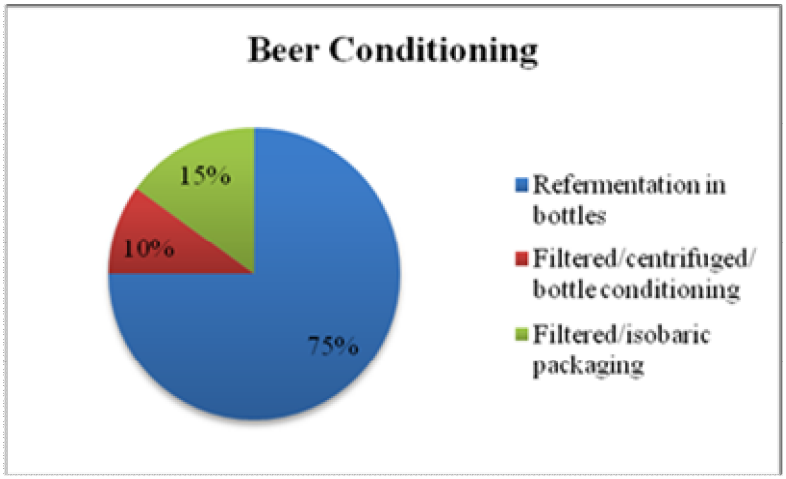

Figure 4.4 – beer conditioning

Most microbreweries in Italy (75%) perform a secondary fermentation in the bottle. During the secondary fermentation, the yeast ferments the sugars added prior to or during bottling (priming sugar). The secondary fermentation has a duration of 15 to 20 days at 20-24°C. In all cases, the brewers have claimed to use the residual suspended yeast in the beer after primary fermentation; this is the easiest way to perform the secondary fermentation.

This production method is widely use among microbrewers because of its simplicity and because there is no need for a filtration plant and no need of pasteurization. Of course this working method increases the risk of beer spoilage.

In recent years some of biggest craft breweries in Italy adopted centrifuged separators to lower the yeast concentration prior to bottling and to reduce the cold maturation steps.

Only the 10% of the breweries are equipped with a centrifuge separator for the mature beer, because this system requires investments and high operating costs. For this reason, it is not yet widely used. The use of centrifuges is advantageous because it reduces the amount of yeast concentration, but it’s still possible to carry

out the secondary fermentation in bottle. The 15% of Italian microbreweries do filter the beer and then fill the bottles using isobaric filling machines, without any secondary fermentation.
None of the surveyed breweries use heat treatments (pasteurization) to stabilize the product. That is because the Italian beer drinkers identify craft beer as a not filtered and unpasteurised product.

Another important issue involving the Italian microbreweries is how the cleaning and sanitizing of the production plants is carried out. Very often, in small breweries these operations are carried out without the use of specific C.I.P. (cleaning in place) plants and most of operations are performed out manually.
In 90% of cases the cleaning of the plants is carried out after each brew batch. The remaining 10% decide to perform cleaning on the basis of empirical parameters.

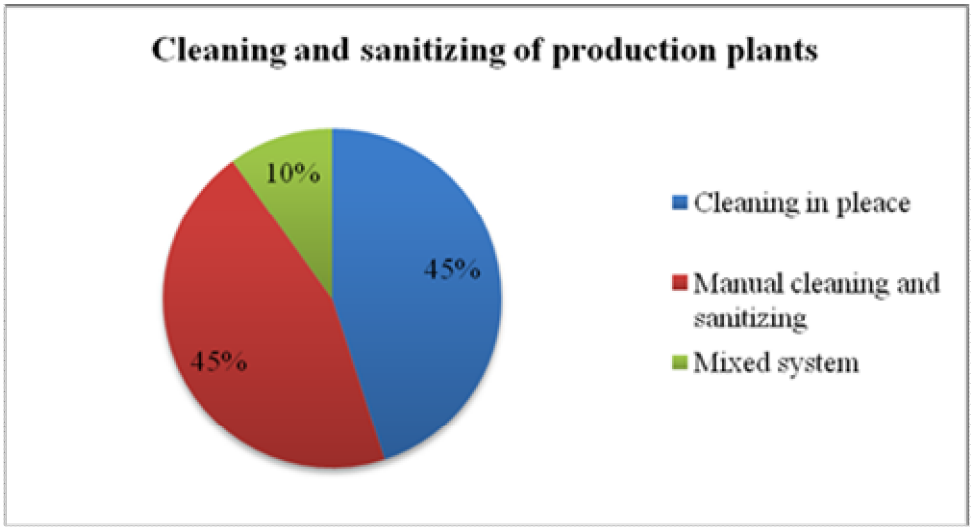

Figure 4.5 – Cleaning and sanitizing of production plant in Italian microbreweries

Almost half of the Italian microbreweries have a C.I.P. plant and this data show the concern about hygiene by both the brewers and the plant suppliers. The other half of microbreweries clean the plants facilities manually and use the brew house vessels to prepare the cleaning solutions. Most of the C.I.P. facilities are very simple machines including a hot caustic and an acid solution vessel, while the final rinse is often performed manually.

However, it is also important to understand if these systems are correctly used. For this reason, a survey was accomplished to understand what kind of detergents and sanitizers are used.

The typical CIP steps used in Italian microbrewery are: alkaline, acidic, and sanitizing agent. The sanitizing agent is used only for the points where there's a possibility for the product to come in contact with spoilage microorganisms.

For the alkaline washing, 70% of the microbreweries use a sodium hydroxide solution (1.5 -4%) at a temperature from 60 to 75 ° C, while the remaining 30% use other basic reagents.

The acid washing is carried out using the following products: 50% use phosphoric acid, 40% citric acid and 10% other acid reagents.

For the disinfecting step, in 90% of cases products containing peracetic acid and hydrogen peroxide are used (0.5 - 1%).

Regarding the correct use of the C.I.P. systems it is also to be said that very few brewers actually monitors the pH of the their cleaning solutions. Most of them just add the prescribed quantity of cleaning agents to reach the desired concentration percentage but if we considered the high variety of water characteristics, the buffer potential of this solution is almost never considered.

4.2 Microbiological Analysis of beers samples

4.2.1 Isolation of lactic acid bacteria

In this study 168 beer samples from microbreweries were tested for the presence of lactic acid bacteria. The purpose of this survey was to understand the impact of spoil bacteria in the Italian craft breweries. The samples come from 39 microbreweries located all over the country. In some cases, numerous samples from a single brewery were analysed.

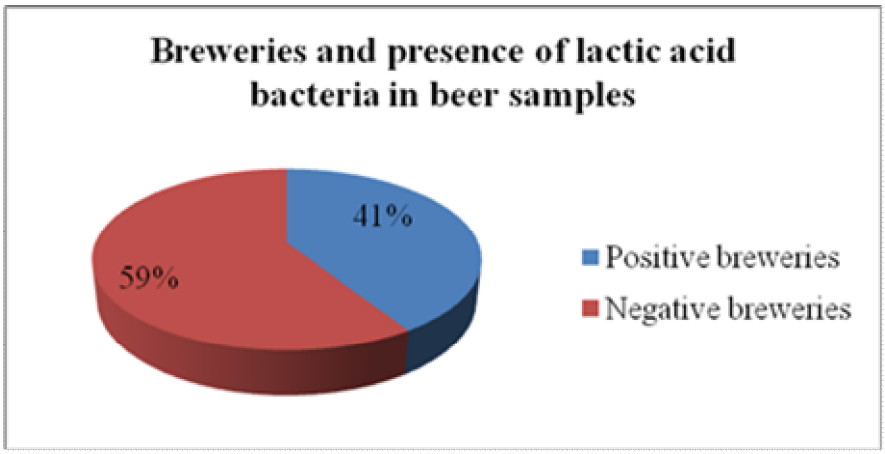

Figure 4.6 – Breweries that have at least one positive sample to the lactic acid bacteria

The 41% of the breweries have at least one sample positive for the presence of lactic acid bacteria. This result shows the importance of microbiological control and the lack of adequate procedures for cleaning and sanitizing the brewery facilities.

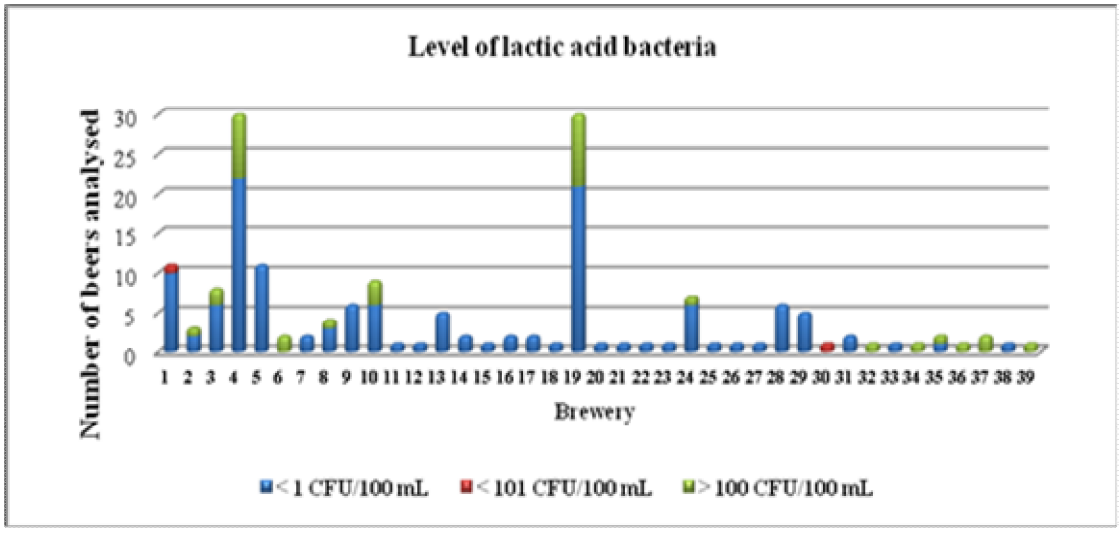

Figure 4.7 – Lactic acid bacterial contamination levels for each of the surveyed micro brewed beers.

The 21% of the beer samples gave positive result for the presence of lactic acid bacteria. Similar levels of lactic acid bacteria presence have been observed in other studies on unpasteurised beer. In 2010, Menz reported a contamination rate of 27.5% in Australian craft beer, while White reported a contamination rate of 15% in American craft beers. These data look similar and give us an idea of the importance of process management in order to avoid and prevent the development of spoilage bacteria.

Regarding pasteurized beers, the most frequently isolated beer-spoilage are lactic acid bacteria, including *Lactobacillus brevis* and *Pediococcus damnosus* (Back W. 2005,Suzuki K. Et al 2008; Hollerovà I. & Kubizniakova P. 2000).

The results of the lactic acid bacteria identification are shown in table 4.1. Spoilage bacteria detected include the *Lactobacillus* and *Pediococcus genera*. The most frequent species were *L. Brevis* (83% of cases), *L. Curvatus* and *P. damnosus* respectively in 8% and 6% of cases.

Although the number of spoilage bacteria isolated from beer samples was not very high, the most frequent occurrence of *L. Brevis* corresponds to the results published in other research studies (Menz G. Et al 2010; Hollerovà I. & Kubizniakova P. 2000).

Numero isolati	Growth on NBB	Growth on MRS	Rod/cocci	Test di Gram	Test Catalase	API ID 32 C identification	Brewery
1	+	+	Rod	+	-	*Lactobacillus brevis*	1
2	+	+	Rod	+	-	*Lactobacillus brevis*	6
3	+	+	Rod	+	-	*Lactobacillus brevis*	6
4	+	+	Rod	+	-	*Lactobacillus brevis*	4
5	+	+	Rod	+	-	*Lactobacillus paracasei ssp paracasei*	4
6	+	+	Rod	+	-	*Lactobacillus brevis*	4
7	+	+	Rod	+	-	*Lactobacillus brevis*	4
8	+	+	Rod	+	-	*Lactobacillus brevis*	12
9	+	+	Rod	+	-	*Lactobacillus brevis*	4
10	+	+	Rod	+	-	*Lactobacillus brevis*	4
11	+	+	Rod	+	-	*Lactobacillus brevis*	8
12	+	+	Rod	+	-	*Lactobacillus brevis*	3
13	+	+	Rod	+	-	*Lactobacillus brevis*	30
14	+	+	Rod	+	-	*Lactobacillus brevis*	32
15	+	+	Rod	+	-	*Lactobacillus brevis*	3
16	+	+	Rod	+	-	*Lactobacillus brevis*	34
17	+	+	Rod	+	-	*Lactobacillus brevis*	4
18	+	+	Rod	+	-	*Lactobacillus brevis*	4
19	+	+	Rod	+	-	*Lactobacillus brevis*	36
20	+	+	Rod	+	-	*Lactobacillus brevis*	37
21	+	+	Rod	+	-	*Lactobacillus brevis*	37
22	+	+	Rod	+	-	*Lactobacillus brevis*	10
23	+	+	Rod	+	-	*Lactobacillus curvatus* ssp *curvatus*	10
24	+	+	Rod	+	-	*Lactobacillus curvatus* ssp *curvatus*	10
25	+	+	Cocci	+	-	*Pediococcus damnosus*	2
26	+	+	Rod	+	-	*Lactobacillus curvatus* ssp *curvatus*	19
27	+	+	Cocci	+	-	*Pediococcus damnosus*	19
28	+	+	Rod	+	-	*Lactobacillus brevis*	19
29	+	+	Rod	+	-	*Lactobacillus brevis*	19
30	+	+	Rod	+	-	*Lactobacillus brevis*	19
31	+	+	Rod	+	-	*Lactobacillus brevis*	19
32	+	+	Rod	+	-	*Lactobacillus brevis*	19
33	+	+	Rod	+	-	*Lactobacillus brevis*	19
34	+	+	Rod	+	-	*Lactobacillus brevis*	19
35	+	+	Rod	+	-	*Lactobacillus brevis*	24
36	+	+	Rod	+	-	*Lactobacillus brevis*	39

Table 4.1 – Identification of isolates of lactic acid bacteria found in beer samples

4.3 Case study: process control of two Italian Microbreweries

4.3.1 Brewery A

Process air

No presence of spoilage microorganisms was detected in any of the 9 samples of process air (Table 4.2).

Samples	CFU/15 l of air
1	<1
2	<1
3	<1
4	<1
5	<1
6	<1
7	<1
8	<1
9	<1

Table 4.2 – Presence of microorganisms in process air.

The lack of microorganisms in the process air indicates a correct management and the efficiency of the filtration procedures adopted. That is also to be said that the occurrence of bacteria contamination in the compressed air line it's quite rare and due only to wrong practices and operations in the brewery.

Pitching yeast

Samples	CFU/g	I.F.I.	I.F.S.
1	<1	n.d.	n.d.
2	<1	n.d.	n.d.
3	2.52E+02	1.0E+02	2.52E+02
4	<1	n.d.	n.d.
5	<1	n.d.	n.d.
6	<1	n.d.	n.d.
7	<1	n.d.	n.d.
8	<1	n.d.	n.d.
9	<1	n.d.	n.d.

Table 4.3 – Presence of microorganisms in dry yeast.

Only in one case the presence of spoilage bacteria was detected in commercial dry yeast. It was a rod-bacteria, acid, Gram-positive.

Although only one sample of dry yeast resulted positive for the presence of bacteria, the dry yeast must be considered as a possible source for the contamination of bacteria into beer. This possibility has been demonstrated in other studies carried out by Manzano M. et.al. in 2005. It is important to notice that the dry yeast suppliers, when providing the quality assessments on their product, don't certified the absence of spoilage bacteria. Consequently a small amount of lactic acid bacteria (< 5 ml when dry yeast is pitched at 100 g/hl) can still be present, meaning that if the yeast has not been stored in adequate conditions, if the oxygenation of the wort is not sufficient or if there is a lacks in nutrients (FAN) the spoilage bacteria could develop in the early stage of fermentation.

Analysis of data for sampling of process

In the brewery A, nine beer batches were monitored along the production process to detect the presence of spoilage bacteria. In four cases, spoilage bacteria were detected throughout the production process of beer. This is a high incidence, which is mainly due to the management of the sanitization processes adopted by the brewers.

The cold and oxygenated wort never had spoilage bacteria, while in the fermenting wort some of spoilage microorganisms were detected. This could be due to:

- incorrect management of the cleaning operations of the fermentation vessels;
- presence of spoilage microorganisms in pitching yeast.

Only in one case the pitching yeast was found to be positive for the presence of spoilage bacteria.

So the fact that spoilage bacteria are present in beer is related mainly to poor hygiene conditions in fermentation tanks.

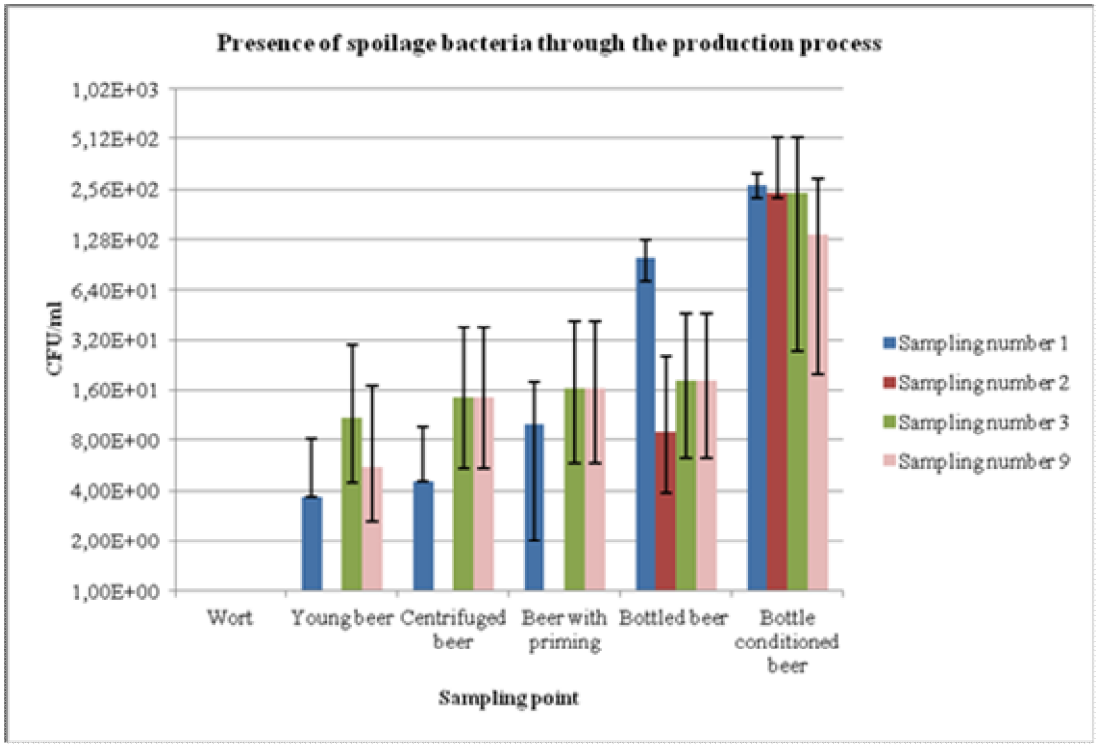

Figure 4.8 – Presence of spoilage bacteria through the production process.

As can be seen in the figure, there is an increase in the number of spoilage bacteria especially after the two weeks of secondary fermentation in bottle. This phenomenon could be due to the presence of fermentable extract. In the case of the second sampling, the presence of spoilage bacteria is evident from the bottled beer. This results means that the spoilage bacteria have come in contact with the beer during the bottling process. The filling machine was probably not correctly and that can be a source of bacteria contamination that cause spoilage of the product. The survey activity has allowed us to identify the critical points in the development of spoilage bacteria.

Regarding the samplings number 1, 3 and 9, the bacteria contamination was already detected in the fermentation vessels. The progressive increase of the contamination can be explained as follows:

- After the primary fermentation the yeast has used the most of maltose and amino acids and it has started its lag phase, in which it is not able to contrast the bacteria multiplication;
- After the addition of sugars for the priming the spoilage bacteria find new sources of nutrients for their growth.

- The secondary fermentation is carried out at temperatures in the range of 20-24°C, and in these conditions the growth of spoilage bacteria is enhanced.

Swab test results in bottling plant

A serie of samplings on the bottling machine were carried out using swab test. Samplings were carried out along 11 weeks during the bottling operations.

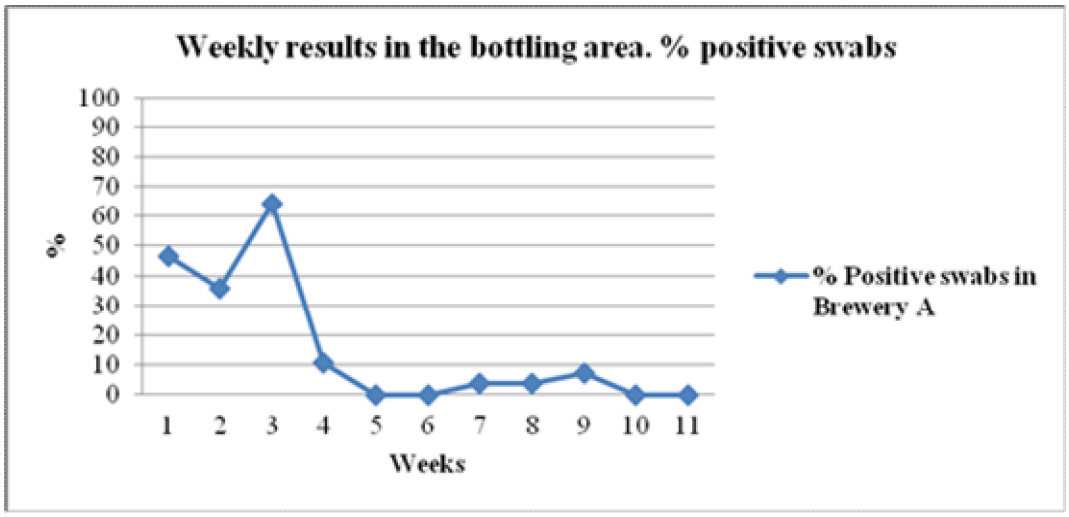

Figure 4.9 – Weekly results in the bottling area. Brewery A.

According to other authors (Back, 2005), if less than 30% of the findings are positive on average within a period of several weeks, the biological condition can be considered satisfactory. This value may be acceptable in the case that the beer is pasteurized. In the case of unpasteurized beer, the number of positive swabs must tend to zero.

As it can be seen in the figure, during the first three weeks of sampling a high incidence of positive swabs has been detected. As a conseguence of the microbiological problems encountered, a new hygiene procedures have been implemented. In particular, a new cleaning procedure has been carried out, using a solution of sodium hydroxide (2%), a water rinse and a sanitizing with a 1% solution of peracetic acid and hydrogen peroxide. The surfaces of the filling machine were cleaned using an acid foam product at 0,8% of phosphoric acid.

A decrease in the presence of positive swabs was immediately after observed.

This results us to state that the analysis carried out with swabs could quickly identify the incorrect management of the bottling plant and ensure that the new procedure for cleaning and sanitizing was effective.

Swabs referring to various areas that were found to be positive in at least 30% of cases are:

- The surface area of the bottle rinse;
- The surface area of the filling machine;
- The areas around the crowning turret;
- Conveyors in the bottle rinsing area;
- Conveyors in the filling area;
- Conveyor in the crowning area.

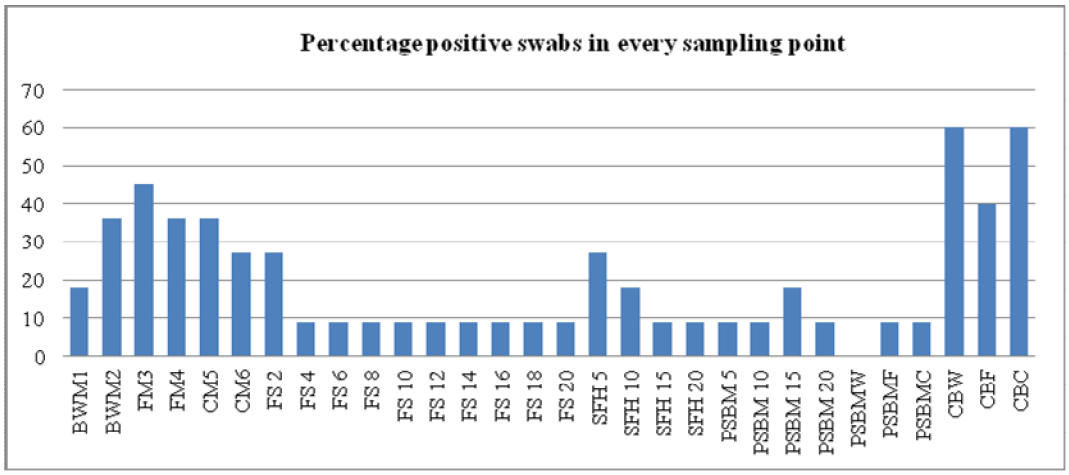

Figure 4.10 – Percentage positive swabs in every sampling point

The lack of an adequate cleaning system for the bottling machine would favor the development of spoilage microorganisms. Therefore this may represent a critical point for inoculation of spoilage microorganisms in beer, as showed in the results of sample number two.

It must be said that the length of the bottling process is also very important: during bottling the surfaces of the machinery will be inevitably sprayed with beer creating an adequate medium for spoilage microorganisms (wild yeasts and acetic bacteria), thus the time of bottling must be as shorter as possible.

The proof of this consideration can be seen in the results of sampling number 2.

Air in bottle filling plant

The results from the air samples inside the bottling machine are shown in the following chart.

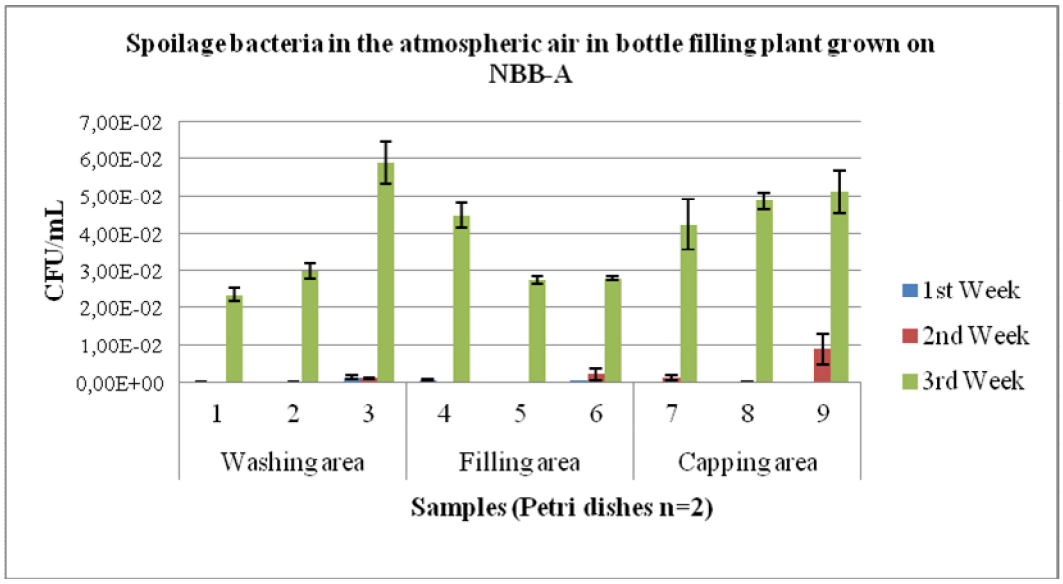

Figure 4.11 – Spoilage bacteria in the atmospheric air in bottle filling plant

The presence of spoilage bacteria was found in the first three weeks, when the new tasks of cleaning and sanitizing had not yet been implemented. In the following weeks no presence of microorganisms in the air of the bottling plant was detected.

A positive correlation between the lactic acid bacteria counts in the air of the bottling line and infected bottled beer was observed (Haikara and Henriksson, 1992). A similar observation was made by Durr (1984), noticing that increased airborne bacteria in the bottling area led to an increase in bottled beer contamination, and that temperature and relative humidity improved the prevalence of spoilage microbes (Haikara and Henriksson, 1992).

4.3.2 Brewery B

Pitching yeast

In the dry yeast sample from the brewery B the presence of spoilage bacteria was not detected.

Analysis of data for sampling of process

In the brewery B eleven complete production processes were monitored, to detect the presence of spoilage bacteria. In five cases spoilage bacteria were detected throughout the production process.

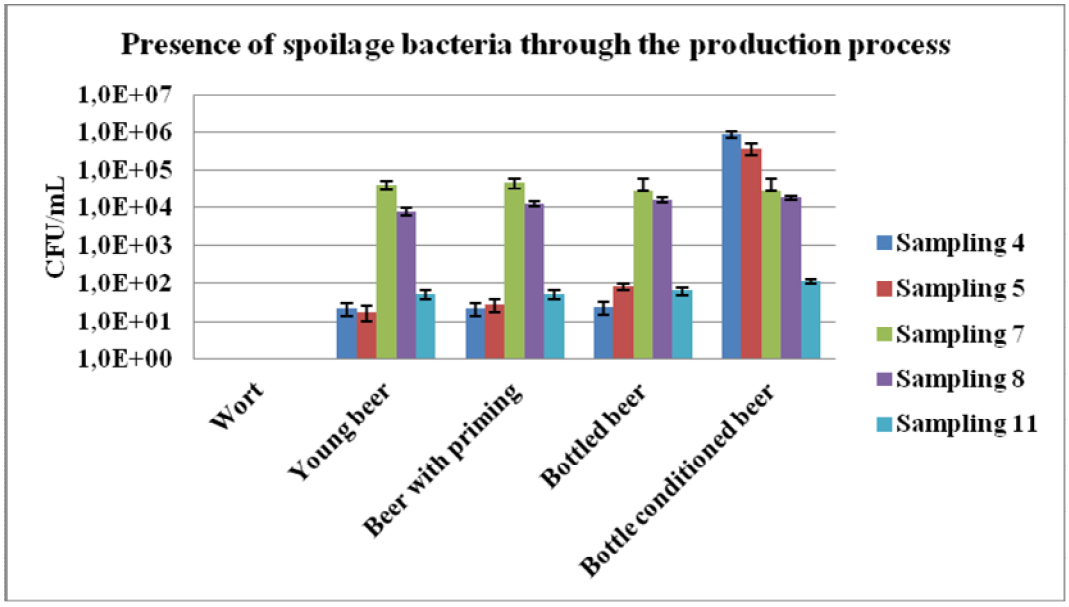

Figure 4.12 – Presence of spoilage bacteria through the production process.

The trend found in the brewery A is very similar to the one of the brewery B. The presence of bacteria was detected again starting from the fermentation tank.
Even in this case it was necessary to implement new more effective cleaning systems for the fermentation tanks.

Swab test results in bottling plant / Air in bottle filling plant

Regarding the swab testing and sampling of air in the area of the bottling line, it was not possible to detect any positive sample. This may depend on the simplicity of the machine (filling machine with four filling caps) and the relative ease in cleaning.

5. CONCLUSIONS

This PhD work was useful to investigate the process issues (brewery layout, technology, process management), related to the development of spoilage microorganisms in the Italian craft breweries.

The most interesting information emerged from the result of the questionnaires are certainly those related to the use of dry yeast.
The dry yeast is used by the 55 % of Italian microbreweries, for several reasons:
- practicality of use;
- ease to handle, store and transport;
- flexibility;
- possibility to produce specialty beers;
- no need to invest in a plant propagation (propagation not required).

In addition, as already suggested by several authors as Mc Caig R. and Dirk S. Bendiak, the product consistency for quality and flavour depends essentially on the stability of the biochemical properties of the brewery yeast. During storage the yeast is kept in a nutrient deficient medium. Under storage conditions, the yeast utilizes endogenous reserves to produce the needed energy for maintaining the essential cellular metabolic functions.
For brewers who do not have the appropriate instrumentation for the management and analysis of yeast it is impossible to perform its recovery.

Most microbreweries in Italy (75%) perform a secondary fermentation in the bottle. This production method is widely use among microbrewers because of its simplicity and because there is no need for a filtration plant and pasteurization. Of course this working method increases the risk of beer spoilage.
In recent years some of biggest craft breweries in Italy adopted centrifuged separators to lower the yeast concentration prior to bottling and to reduce the cold maturation steps.

The 41% of the breweries have at least one sample positive for the presence of lactic acid bacteria. This high percentage of craft breweries positive for the presence of lactic acid bacteria is indicative of the fact that this problem is widespread. In the case of the breweries number 4 and 19 (see figure 4.7) after a series of negative samples, in the last samples analyzed there was an increase of positivity in the presence of lactic acid bacteria.

The 21% of the beer samples gave positive results for the presence of lactic acid bacteria. Similar levels of lactic acid bacteria have been observed in other studies on unpasteurised beer. In 2010, Menz reported a contamination rate of 27.5% in Australian craft beer, while White reported a contamination rate of 15% in American craft beers. These data look similar to ours and give us an idea of the importance of process management in order to avoid and prevent the development of spoilage bacteria.

Menz in 2010 found that whilst the process hurdles were significant in preventing the growth of pathogenic bacteria in standard beer, they had little effect on the prevalence and growth of spoilage lactic acid bacteria.
Consequently, it is not possible to reduce the development spoilage bacteria only with obstacles process; the objective must be the implementation of effective hygiene and sanitation systems.

The analysis of the data shows that the presence of spoilage bacteria is mainly due to:

- mismanagement of sanitation procedures;
- lack of adequate microbiological control;
- failure to identify the causes that lead to the development of spoilage bacteria.

This leads to the sudden onset of microbiological problems which subsequently have an impact in additional costs for microbreweries (withdrawal of the product, damage to the image of the brand, disposal costs, lost earnings, etc.).

Procedures for prevention of microorganisms infection should be activated by the brewers, focusing the attention on the following points:

- Good quality equipment and hygienic design;
- Effective cleaning and disinfection (removal of microbes and their nutrient source);
- Good yeast management;
- Microbiological quality check (control and monitoring);
- Good Manufacturing Practice for the workers of the brewery;
- Prevention with process management (hurdle technology) and beer characteristics (low pH, higher levels of alcohol, higher bitterness, low nutrient availability, cold storage).

Today the most of the microbreweries could implement microbiological control with a low cost investment. In this thesis, easily reproducible methods have been used in order to provide useful tools for microbiological control in microbreweries.

Currently, the Italian craft beer consumer is willing to accept the presence of defects and off flavours in the product because they are associated with artisanal production, but in the coming years the expansion of the craft market will lead to a greater awareness.

In the future, the craft beer market will occupy a larger market share and will entrust the distribution to the operators of the Organized Retail, HORECA, etc..

Therefore, the challenge of the artisan producers will be to provide a product free from defects and stable over time.

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