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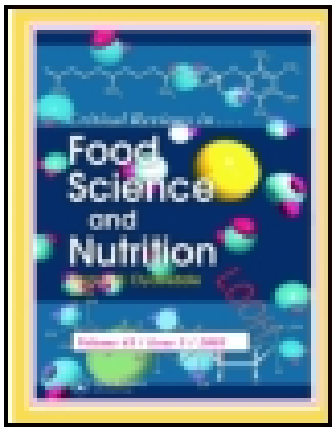

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Microbiology and food-borne pathogens in honey

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Abstract

Honey has been considered a relatively safe foodstuff due to its compositional properties, with infant botulism caused by *Clostridium botulinum* being the most prominent health risk associated with it. Our review is focused on the honey microflora along the food chain and evaluates the pathogenic potential of those microorganisms found in honey. This product may contain a great variety of bacteria and, particularly, fungi that eventually entered the food chain at an early stage (e.g. via pollen). For many of these microorganisms, opportunistic infections in humans have been recorded (e.g. infections by *Staphylococcus* spp., *Citrobacter* spp., *E. coli*, *Hafnia alvei*, *Aspergillus* spp., *Fusarium* spp., *Trichoderma* spp., *Chaetomium* spp.), although direct infections via honey were not registered.

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Keywords

food safety; microbiological criteria; opportunistic pathogens; fungi; stramenopiles.

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1. Introduction

Eusocial insects have developed feeding systems to ensure the survival of their states; many social ants and termites cultivate fungi, while bees produce a mixture of sugars and enzymes based on the nectar they feed on and on their own digestive secretions, a substance known as honey. Humans have been consuming honey from early on, as an 8000 years old drawing of a honey seeker reveals.

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On a global level, several genera of bees (family *Apidae*) are used for honey harvesting or have even been domesticated, especially those of the tribes *Apini* (ñhoneybeesò) and *Melliponini* (ñstingless beesò). Current legal definitions in many countries claim that only the honey bee *Apis mellifera* produces the foodstuff named ñhoneyò, i.e. there is no legal margin for these products. This is symptomatical for many uncommon foodstuffs which have been started to enter the markets (Grabowski et al., 2013). In order to avoid confusion, ñhoneyò from other species will not be discussed in this study. Honey may be contaminated microbiologically, physically and chemically (Al-Waili et al., 2012). The latter two contamination sources will not be addressed, either.

1.1.General considerations

As with any other foodstuff, the hygienic status of honey is the result of a) conditions of the environment along the food chain b) the beneficial or detrimental properties added to the product by the animals themselves. The environment may be divided into the surroundings of the hive which is frequented by the bees, the hive itself, the body cavities in which honey is produced and the conditions in which honey is harvested and packaged. Honey composition by itself is prone

to inhibit the growth of many microorganisms, e.g. a low protein content and a high carbon to nitrogen ratio (Snowdon & Cliver, 1996). Beneficial substances added to the honey by the bees include, among others, hydrogen peroxide (produced by the animalôs glucose oxidase) and several non-peroxide dependant mechanisms, i.e. methylglyoxal, the substance responsible for the marked *in vitro* antimicrobial activity of the mŪnuka honey from *Leptospermum scoparium* and *L. polygaliforlium*), bee defensin-1, florally derived phenolics, lysozyme, pinocembrin, lysozyme, phenolic acids, terpenes, benzyl alcohol and other substances which have not been determined fully yet. This antimicrobial activity however varies strongly and depends on the floral source (whose chemical compounds may increase or decrease this activity) and the processing and eventual dilution of the honey (Baas & Grobler, 2008; Bang et al., 2003; Chen et al., 2012; Snowdon & Cliver 1996). The floral source itself may also be subjected to changes, e.g. the season and the localisation of the plants. To state an example of these conditions, an in *vitro* effect against *Helicobacter pylori* has been described, but results varied with the honey brand (Nzeako & Al-Namaani, 2006). This system is usually very effective. Even inoculation trials with pathogenic bacteria result in the inactivation of the bacteria within 40 days at latest. Slow-growing mycobacteria and some *Enterobacteriaceae* (the latter when stored at low temperatures) however manage to grow in honey. Some *E. coli* strains also seem to do so at ambient temperatures (Snowdon & Cliver, 1996).

Chemical substances added to the honey by the worker bee are one reason why mature honey is relatively resistant towards microbiological contamination. The other are physicochemical properties, i.e. low pH (usually around 3.9), high osmotic pressure and thus low water activity (0.5 ï 0.6), low redox potential, and high viscosity. According to Snowdon and Cliver (1996),

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yeast activity is limited by the content of free water, mentioning that honey from humid regions is more prone to be contaminated with osmophilic yeasts, and citing a report that yeast growth was not reported in honey samples with a water activity > 0.65.

So, honey is one of the few foodstuffs of animal origin which has not been associated widely with a variety of significant microbiological food-borne diseases, the only exception being infant botulism. Many studies dealing with food safety of honey only state the major bacterial contamination, summarizing minor risks under ñother bacteriaò and ñyeasts and moldsò. Since opportunistic bacteria and fungi have been reported increasingly over the last years, the present paper takes up this knowledge and compares it with the findings in honey.

This review follows the introduction of microorganisms into the food chain during honey production, the stages where they are eventually eliminated and potential pathogens which occur in ready-to-consume honey.

1.2. Product range and production data

In modern bee-keeping, many products may be harvested from bees:

- *honey* (the nutrient made from flowersô nectar or honeydew [i.e. a sugar-rich liquid excreted by some insects, especially hemipterans and homopterans] which mainly contains sugars and water and which is processed by the bees, filled in cells and left there to ripen),
- *royal jelly* (the nutrient secreted from hypopharyngeal and mandibular glands of workers fed to the larvae of workers for some days and to queens all their lives which is used as a foodstuff and which is rich in proteins and sugars),

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- *beeswax* (the secretion of abdominal mirror glands which is used to build honey combs; in the food industry, beeswax [E901] is mainly used to cover foodstuffs, especially cheeses),
- *apitoxin* (the secretion of the poison gland into the venom sac, usually transmitted via the stinger apparatus; it is used in allergen immunotherapy, apitherapy, and in cosmetic anti-aging products),
- *bee brood (*bee larvae and pupae are consumed in many entomophagous areas)
- *propolis* (a resinous mixture collected from plants with antimicrobial effects used by the bees to seal the hives and to keep it apart from potential pathogen sources, e.g. dead animals within the hive), and
- *bee pollen* (pollen packages collected by the worker in the field recollected at the entrance of the hive) and bee bread (fermented pollen used as a feedstuff for larvae; consumed by humans only on a small scale, usually only the beekeepers and their families).

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According to FAOSTAT, a total of 1,540,242 tons of honey was produced in 2010. The five top producers were China (398,000 t), Turkey (81,115 t), the United States (79,789 t), Ukraine (70,900 t), and Argentina (59,000 t). However, Europe is the continent which generates most honey (31.4 % of world production), with Spain, Germany, Romania, Hungary and France at the top. The percentages for Asia, the Americas, Africa, and Oceania are 29.3, 26.0, 10.66, and 2.7 %, resp.

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For the other products, global production data is scarce. A total of 2,000 t of royal jelly is produced in China every year, assuming that this country has taken the lead (quoted in Sabatini et al., 2009). A well-managed hive is able to produce approx. 500 g of royal jelly per season.

1.3 Potential paths of contamination

Regarding bee products, contamination can take place at a pre-secretion, secretion and post-secretion level (Fig. 1).

1.3.1. Phyllosphere

The prime matter used for beeswax, honey, royal jelly, and propolis originates from the environment (Fig. 2). The primary sources for contamination originate from the phyllosphere, from the beekeepersô actions to feed the animals, and from other surfaces the bees land on.

Within the phyllosphere, the important areas are the places where bees get their food, i.e. the blossoms, honeydew-secreting insects (and the environment they interact with), sources for resins, and other nectaria. Under natural circumstances, the surface of flowers contains a wide, species-dependant variety of fungi, yeasts and, to a lesser extent, bacteria, which can be transmitted to the bees. Blossom and pollen microflora in turn correspond to that of dust, air and flowers (Kaļániová et al., 2009). Microbiological findings in nectar vary from almost sterile (Gilliam 1997) to a series of microorganisms, esp. yeasts. The flora of pollen depends highly on the plant species and contains many different bacteria, yeasts and molds (Gilliam et al., 1989). Almond (*Prunus communis*) pollen alone was found to contain several bacilli (Gilliam, 1979a, 1979b; Gilliam et al., 1989) while other pollen samples harboured mesophilic aerobic and anerobic bacteria including coliforms (Kaļániová et al., 2009). Pollen also contains Gram-variable pleomorphic bacteria. Of these, some are thought to be lactobacilli, but other strains

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reacted differently, suggesting that Gram-variable pleomorphic bacteria are in fact several genera which until now are *incerte sedis* (Gilliam, 1997). Pollen flora seems to be the most influential one for honey production (Kaļániová et al., 2009). Once pollen becomes deposited inside the cells (bee bread) it also seems to contain specific yeasts, and to lesser extent, bacteria to ensure preservation and digestibility of the pollen provided for the brood (Rosa et al., 2003). As with other animals, this flora contributes to digestion and the reduction of the growth of pathogens.

Another source for honey is honeydew. Its microbiological composition also varies greatly, depending on the intestinal flora of the secreting insect (which may contain e.g. *Achromobacter* spp., *Bacillus megaterium, Flavobacterium* spp., and *Sarcina* spp. [Gilliam 1979a; Gilliam 1979b]) and that of the environment. Besides that, there is a complex interaction between aphids which are the chief insect type to produce honeydew and their environment via this secretion, and certain ergots produce a honeydew-like secretion by themselves, possibly to attract insects. *Staphylococcus sciuri* isolated from honeydew produced a kairomone (a substance emitted by one species to provide favourable information for another species), since theses volatile compounds attract animals that feed on this honeydew. In any case, honeydew as sprayed on the host plant is predominantly associated with yeasts but also with certain bacteria and even plant viruses. It promotes the growth of bacteria, fungi and yeasts, affecting thus the phyllosphere of the host plants (Aksoy & Ozman-Sullivan, 2008; Barata et al., 2008; Douglas, 2009; Leroy et al., 2011; Serjeant et al., 2008; Srivastava & Rouatt, 1963; Stadler & Müller 1996). In this way, honeydew changes the microflora of the plant surfaces the bees feed of, and this may change the microbial flora of the bee.

As may be seen, all production areas the bees contact may pose a risk of contamination.

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Because of the variety of microorganisms encountered in honey production, the following list is divided according to the localization of the isolate. Some microbes are found in several sites at the same time and they are mentioned accordingly.

- Soil (bacteria: *Acinetobacter* spp., *Clostridium* spp., *Corynebacterium* spp., *Psychrobacter* spp., *Vagococcus* spp. [Snowdon & Cliver, 1996])
- Air (bacteria: *Bacillus* spp., *Clostridium* spp., *Enterobacter* spp., *Erwinia* spp., *Flavobacterium* spp., *Micrococcus* spp. [Snowdon & Cliver, 1996])
- Dust (bacteria: *Bacillus* spp., *Clostridium* spp., *Micrococcus* spp. [Snowdon & Cliver, 1996])
- Plants, pollen, honeydew and other feedstuffs (bacteria: *Achromobacter* spp., *Bacillus* spp., *Bacillus circulans, B. licheniformis, B. megaterium, B. subtilis, Brochotrix* spp., *Citrobacter* spp., *Clostridium botulinum, Enterobacter* spp., *Enterococcus* spp., *Erwinia* spp., *Flavobacterium* spp., *Lactobacillus* spp., *Lactococcus* spp., *Leuconostoc mesenteroides, Listeria* spp., *Micrococcus* spp., *Pediococcus* spp., *Staphylococcus sciuri, Sarcina* spp.; fungi: *Aspergillus spp., Aureobasidium spp., Candida* spp., *C. methanosorbosa, C. railenensis, C. vanderwaltii, Cladosporium* spp., *C. cladosporoides, Cryptococcus* spp., *Hanseniaspora osmophila, H. uvarum*, *Lachancea cidri, L. thermotolerans, Metschnikowia* spp., *M. pulcherrima, Meyerozyma caribbica, M. guilliermondii*, *Mucor* spp., *Penicillium* spp., *P. brevicompactum, P. chrysogenum, P. griseofulvum, P. solitum* var. *crustosum, Pichia fermentans, P. kluyveri*, *Rhizopus stolonifer, Rhodotorula* spp., *Saccharomyces* spp., *S. cerevisiae, Scytalidium* spp., *Sporobolomyces* spp., *Torula* spp., *Torulaspora pretoriensis, Wickerhamomyces*

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anomalus, Zygosaccharomyces bisporus, Z. rouxii; Barata et al., 2008; stramenopiles: *Alternaria* spp. [Aksoy & Ozmam-Sullivan, 2008; Gilliam, 1979a; Gilliam, 1979b; Gilliam et al., 198; Herrera et al., 2009; Kaļániová et al., 2009; Kaļániová et al., 2012; Leroy et al., 2010; Serjeant et al., 2008; Snowdon & Cliver, 1996; Srivastava & Rouatt, 1963; Umeda et al., 2009; Vázquez et al., 2012])

In addition, Snowdon and Cliver (1996) report *Flavobacterium lactis* in drinking water and honey; however, this species could not be ratified via LPSN.

1.3.2. Worker bee

While the phyllosphere presents a microbial flora and fauna of its own, this community is further modified by the continuous approaches by the bees that carry microorganisms on their bodies and in their mouthparts. This exchange of microorganism also takes place between honeydew-secreting insects and their environment. Thus, a great variety of microorganisms may enter the production of bee secretions, usually via the mouthparts of the animals (Fig. 3).

Regarding the animal itself (i.e. the secretory level), the relevant source for microorganisms is the gastrointestinal tract, as the body surface of adult bees is usually free of them (Gilliam 1997). Bacteria and fungi contact the animalsô mouthparts via food intake and, in case of pollen, propolis and beeswax, chewing. Cleaning also is done using the mouthparts and so, the oral cavity is expected to be an area with a significant degree of microbial growth.

The following microorganisms have been recorded for larval und adult gastrointestinal tracts:

- Bacteria: *Acetobacter* spp., *A. syzygii, Achromobacter* spp., *Actinobacillus equuli, Actinomycetes* spp., *Alysiella filiformis, Arsenophonus* spp., *A. nasoniae*, *Bacillus* spp., *B. cereus, B. pumilus*, *Bacillus subtilis, Bartonella* spp., *Bifidobacterium* spp., *B. asteroides,*

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B. coryneforme, B. indicum, "Candidatus Gilliamella apicolaò, *"Candidatus* Snodgrassella alviò, *Citrobacter* spp., coliform bacteria [not specified], *Clostridium* spp., *C. colicanis, C. diolis, Enterobacter* spp., *Enterobacteriaceae* spp., *Enterobacter aerogenes, E. cloacae*, *Enterococcus* spp., *Escherichia coli*, *Erwinia* spp., *Flavobacterium* spp., *Fructobacillus (ex Leuconostoc) ficulneus, F. (ex Leuconostoc) fructosus, Gluconacetobacter* spp., *Gluconobacter* spp., *G. cerinus, G. oxydans,* Gram-variable pleomorphic bacteria, *Haemophilus ducreyi, Hafnia alvei*, *Kingella kingae, Klebsiella* spp., *K. oxytoca, K. pneumoniae*, *Lactobacillus* spp., *L. acidipiscis, L. acidophilus, L. amylolyticus, L. composti, L. crispatus, L. farrinis, L. hamsteri, L. kunkeei, L. lindneri, L. manihotivorans, L. reuteri, L. vaginalis, Melissococcus plutonius, Micrococcus luteus, Neisseria canis, Ornithobacterium rhinotracheale*, *Paenibacillus alvei, P. larvae, Paralactobacillus selangorensis, Proteus* spp., *Propionibacterium* spp., *Pseudomonas* spp., *aeruginosa, P. indica, Riemerella anatipestifer*, *Ruminococcus obeum*, *Saccharibacter floricula, Salmonella* Typhimurium, *Serratia* spp., *S. marcescens, Simonsiella* spp., *Staphylococcus* spp., *Streptococcus* spp., *Streptomyces* spp. (Babendreier et al., 2007; Cornman et al., 2012; Crotti et al., 2010; Janda & Abbott, 2006; Kaļániová et al,. 2009; Martinson et al., 2012; Snowdon & Cliver, 1996; Vásquez et al., 2012).

- Fungi: *Aspergillus* spp., *Candida magnoliae*, *C. parapsilosis*, *C. glabrata, Chaetomium* spp., *Cronartium* spp., *Cryptococus neoformans* var. *grubii, Endocronatrium* spp., *Melanospora spp., Myceliophthora thermophila, Nosema apis, N. ceranae, Penicillium* spp., *P. brevicompactum, P. chrysogenum, P. griseofulvum, P. solitum* var. *crustosum,*

Saccharomyces spp., *S. cerevisiae, Wickerhamomyces anomalus* (Anderson et al., 2011; Cornman et al., 2012; Ergin et al., 2004; Gilliam, 1997; Herrera et al., 2009; Kaļ ániová et al., 2009; Olaitan et al., 2007; Snowdon & Cliver, 1996)

- Stramenopiles: *Peyronelia* spp. (Kaļ ániová et al., 2012; Snowdon & Cliver, 1996)

Of those, some (e.g. *Enterobacter cloacae*, *Bacillus* spp., staphylococci) have also been isolated from edible insects (Grabowski and Klein, 2006; Grabowski et al., 2014).

Most bee secretion products are generated within the gastrointestinal tract e.g. honey (proventriculus) and royal jelly (hypopharyngeal glands). Both secretions leave the animal via the mouth. As eusocial insects, bees share these secretions with other members of the hive (Fig. 4), either to produce honey or to feed hive mates, especially larvae, drones and queens.

The gastrointestinal flora of apoid bees has been studied increasingly, revealing the complexity of interactions between physiological and pathological flora. Gram-variable pleomorphic bacteria are the most numerous microorganisms encountered in the beesô guts (Gilliam, 1997). They are eventually referred to as *"Bacterium" vel "Achromobacter eurydice"* (Gilliam, 1997). Mortinson et al. (2012) charted bacteria according to the anatomical region of the bee GIT but taxonomically remained on the class level, as they used phylum and class-specific primers; thus they focused on *Beta* and *Gammaproteobacteria* and *Firmicutes*. The latter dominated in proventriculus and rectum samples, while midguts yielded high amounts of *Gammaproteobacteria*. *Betaproteobacteria* were most prominent on ileum level. These findings correspond with the findings of Vásquez et al. (2012) who detected large amounts of lactobacilli (phylum *Firmicutes*) in both stinged and stingless bees. Still, other types of bacteria may also be

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found, e.g. acetic acid bacteria. These, together with the lactobacilli, are thought to contribute to the physiology of the bee (Crotti et al., 2010).

The intestinal bee flora comprises a permanent and a temporary set of microorganisms and comprises mostly bacteria of which a high percentage is and anaerobic. Some of the yeasts present in pollen also inhabit the beesô guts, but their presence seems to be associated to the geographical region and the season; the presence of some *Saccharomycetales* was associated with distress and disease in the bees (Gilliam, 1997; Kaļániová et al., 2009). Faeces of larval honey bees contained, if any, bacilli, Gram-variable pleomorphic bacteria, Gram-negative rod-shaped bacteria, penicillia, actinomycetes, and yeasts (Gilliam & Prest, 1987). While the flora of the intestinal tract is relatively well documented, the microbiology of the hypopharyngeal gland from which royal jelly is secreted remains mostly unknown (Copley & Jabaji, 2011). It is postulated that contaminants such as antibiotics, pesti and fungicides affect the intestinal flora in its composition and diversity, impacting thus on the colonyôs performance and health (DeGrandi-Hoffmann et al., 2009). A Slovakian study (Kaļániová et al., 2009) found out that the microbiological conditions of honey are based on the microflora present in the beesô digestive tract and on the pollen.

All pre-imago bee instars and the hatching imago are usually sterile from the bacteriological point of view. In case prepupae were fed with contaminated feed, microorganisms are eliminated from the body by the single defecation just before pupation. Still, growth of yeasts on larvae and pupae has been documented for both stinged and stingless bees (Rosa et al., 2003), probably originating from the animals surroundings, e.g. royal jelly and bee bread. Adult bees start to develop an intestinal flora with feeding, their sources being the environment (see above) and

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other worker bees within the hive that share their food with them (Gilliam, 1997; Kaļániová et al., 2009).

Acetic acid bacteria such as e.g. *Gluconacetobacter* spp. and *Gluconobacter* spp. prove to be symbionts to the insects (Crotti et al. 2010). It is postulated that contaminants such as antibiotics, pesti and fungicides affect the intestinal flora in its composition and diversity, impacting thus on the colonyôs performance and health (DeGrandi-Hoffmann et al., 2009).

1.3.3. Bee hive

Bees are also in contact with the hive structure, and by cleaning themselves, their mouths also get in contact with the microbial fauna and flora of the hive (Fig. 4). The beehive provides the physical structure for the bee colony. A constant entering and leaving the hive, together with the high amount of individuals living permanently inside it poses special microbiological hazards. Along with the plant and metal components of the hive, it also contains two substances exploited in bee-keeping, i.e. beeswax and propolis which are the original construction materials. *Bacillus* spp., *Micrococcus* spp., and *Aspergillus* spp. are typical colonizers of the hive. Besides, the hive is considered as a primary source for the many yeasts (not specified) that are found within honey production; it may also yield some (sporulated) moulds (Snowdon & Cliver 1996). In addition to the germs introduced by the bees themselves, other animals can also introduce microorganisms. One group are small animals that seek shelter within the hive, but are killed by the bees and their corpses remain inside the hive (usually covered by propolis to avoid contamination). The other groups are other arthropods that live as parasites, either on/inside the bees (e.g. tarsonemid or varroid mites) or inside the hive (e.g. pyralid caterpillars, clerid larvae), feeding on hive material,

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honey or bee brood. Contamination can also occur via the beekeeperôs smoker, especially with chemicals.

Insects parasitizing honeybees include flies (apimyasis), *Meloë* spp. beetle larvae and strepsipterans (*Stylops melittae*). In addition, there are some insects that live within the hive, e.g. the fly *Braula coeca* as well as the waxmoths *Galleria mellonella* and *Achroia grisella*. The life cycle of these animals takes place (at least in part) inside the hive (Bailey and Ball 1991), and although there is no reliable data for most of theses species as to their role of pathogen reservoir for honey, waxmoths have been used extensively as a research model for many pathogens, e.g. *Enterobacteriaceae*.

1.3.4. Harvesting and processing

On the post-secretory level, honey is harvested from the hives by uncapping the frames and either crushing or centrifuging them (Fig. 5). Honey is sieved one or several times, eventually stirred over a longer period of time, graded and packaged. While honey is not submitted to any significant germ-reducing procedure, the other secretions receive thermal treatment and/or dehydration to reduce the bacterial counts. Royal jelly is cooled or lyophilized, pollen is frozen or dehydrated. Propolis may become heated but is dissolved in organic liquids and sieved to eliminate impurities before further processing takes place. While other bee products, e.g. royal jelly, receive thermal treatment to ensure food safety, honey pH (usually around 3.9) and water activity (0.5 ï 0.6) during storage are vital mechanisms to avoid excessive growth of microorganisms (Snowdon & Cliver, 1996). However, the work of Tysset and Rousseau (cited in Snowdon & Cliver, 1996) stresses the fact that harvest is more likely to lead to secondary

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contamination than other production steps making good manufacture practices a necessity. Still, the methods developed by the food industry to control these pathogens are considered sufficient. The following microorganisms have been isolated from honey:

- Bacteria: *Achromobacter* spp., *Alcaligenes* spp., *Bacillus* spp., *Bacillus cereus, B. coagulans, B. mycoides*, *B. pumilus, Bifidobacterium* spp., *Brevibacterium* spp., *Citrobacter* spp., *Clostridum* spp., *C. botulinum*, *Clostridium perfringens,* coliform bacteria (not specified), *Enterobacter* spp., *Erwinia* spp., *Escherichia coli*, *Flavobacterium* spp., *Gluconobacter* spp., *Hafnia alvei*, *Klebsiella* spp., *Lactobacillus* spp., *Leuconostoc* spp., *Micrococcus* spp., *Neisseria* spp., *Paenibacillus alvei*, *Proteus* spp., *Pseudomonas* spp., *Staphylococcus* spp., *Streptomyces* spp., *Xanthomonas* spp. (Anderson et al., 2011; Babendreier et al., 2007; Janda & Abbott, 2006; Kaľániová et al., 2009; Kokubo et al., 1984; Olaitan et al., 2007; Snowdon & Cliver, 1996; Srivastava & Rouatt, 1963; Umeda et al., 2009; Vásquez et al., 2012).
- Fungi: *Acremonium* spp., *.Ascosphaera* spp., *Aspergillus spp., Atichia* spp., *Bettsia alvei, Botrytis* spp., *Candida* spp., *Cephalosporium* spp., *Chaetomium* spp., *Cladosporium* spp., *Coniothecium* spp., *Cryptococus neoformans* var. *grubii, Debaryomyces* spp., *Eurotium* spp., *Fusarium* spp., *Hansenula* spp., *Hormiscium* spp., *Lipomyces* spp., *Mucor* spp., *Oosporidium* spp., *Penicillium* spp., *P. brevicompactum, P. chrysogenum, P. griseofulvum, P. solitum* var. *crustosum, Pichia* spp., *Rhodotorula* spp., *Saccharomyces* spp., *S. cerevisiae, Schizosaccharomyces* spp., *S. octosporus, Schwanniomyces* spp., *Seuratia* spp., *Torula mellis, Trichoderma* spp., *Triposporium* spp., *Uredinaceae* spp., *Ustilaginaceae* spp., *Zygosaccharomyces* spp., *Z. mellis, Z. priorianus, Z. rouxii;*

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(Anderson et al., 2011; Babendreier et al., 2007; Cornman et al., 2012; Ergin et al., 2004; Henrici, 1941; Herrera et al., 2009; Kaļániová et al., 2012; Olaitan et al. 2007; Searjeant et al., 2008; Snowdon & Cliver, 1996)

- Stramenopiles: *Alternaria* spp., *Epicoccum* spp., *Peronosporaceae* spp., *Peyronelia* spp., *Phoma* spp., *Pythium* spp. (Kaļániová et al., 2012; Snowdon & Cliver 1996)

Although these taxa were encounntered in honey, not all of them are able to multiply in this medium.

Comparing the lists of chapter 1 it becomes clear that some microbes only occur at a pre-harvest stage (e.g. *Acinetobacter* spp., *Aureobasidium* spp., and *Rhizopus stolonifer*) and become eliminated in honey, while others remain traceable from the pre-harvest stage on, e.g. *Clostridium* spp., *Chaetomium* spp., and *Alternaria* spp. Others have been isolated only from honey so far (e.g. *Alcaligenes* spp., *Trichoderma* spp., and *Epicoccum* spp.) which suggests a contamination at hive, harvest or post-harvest level. Naturally, this information bears a certain bias since the papers eventually did not sample the entire food chain and used different isolation methods etc.

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2. Hazards associated with microorganisms isolated from honey

This chapter focuses exclusively on those microorganisms that were isolated from honey.

2.1. Bacteria

Of those bacteria groups found in honey, all contain, with the exception of *Xanthomonas* spp., pathogenic or opportunistically pathogenic bacteria. *Paenibacillus larvae* bacteremia was diagnosed in injection drug users (Rieg et al., 2010). However, the direct zoonotic potential has

not been proven for all species. *Hafnia alvei* e.g. is known to cause nosocomial infections in immunesuppressed patients, but not associated with honey yet (Janda & Abbott, 2006).

The classical zoonotic pathogen associated with honey is *Clostridium botulinum* as it causes infant botulism in babies of less than one year of age. Discovered in 1976, the disease has been reported in many countries, predominantly in the USA; incidence however is low. Type-specific toxin types A and B (produced by *C. botulinum* type I) are the most prominent toxins that cause infant botulism, but other type-specific toxins (C, E, and F) and minor toxins (Ab, Af, Ba, and Bf) have also been involved. Clostridial spores are ingested, germinate, multiplicate and may develop and start toxicogenesis in infants because of their poorly-developed (anaerobial) intestinal flora. Toxins enter the bloodstream and attach to neuromuscular endings, with toxin A displaying the greatest affinity to them. Epidemiological differences were noted depending on whether the patient was younger or older than two months. Several risk factors have been discussed critically, especially breast-feeding. Honey is a typical source for toxicogenic *C. botulinum*, but not the only one (Midura, 1996); in the USA, only 10 % of infant botulism are truly associated with the ingestion of honey (Boulanger et al., 2006). Dust emerging from disturbed soil (by agriculture or earthquake) has also been identified as a source for infant botulism (Midura 1996). The same applies to infant milk formulae (Langström & Korkeala, 2006). In honey, *C. botulinum* spores have been identified in 0 to 62 % of samples, and it is suggested that sampling and analysis techniques play in important role in the rate of detection. Spore counts also vary strongly; in 270 honey samples, one yielded counts between 36 and 60 spores/g, while the rest contained less than 1/g. The infectious dose for infant botulism is thought to be 5 to 80 spores/g. It is suggested that *C. botulinum* may still grow in maturing honey, but

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stops its growth once the content of sugars surpasses 50 %. This is why spore counts also depend on the production stage, and while 23 % of bee hive honey samples contained them, only 5 % were encountered in processed and commercialized honey. The effect of shelf life on clostridial spores has not been established clearly (Snowdon & Cliver, 1996).

In Egypt, street-vended, ready-to-consume beverages containing honey and cream were tested positive (24 % of samples) *Listeria monocytogenes* (El-Shenawy et al., 2011). The origin of the listeria cannot be traced, but dairy products are known to harbour them.

Reagrding bacterial counts, honey should yield <9,500 cfu/g as total bacterial count, <0.3 cfu/g E. coli, <132 cfu/g anaerobial bacteria (including clostridia), and < 180 cfu/g bacilli (Snowdon & Cliver, 1996).

2.2. Stramenopiles

All heterokonts found in the honey-producing environment belong to the phylum *Oomycota*, class *Peronosporales*. These water moulds basically are plant pathogens, but are also found on nectarivorous insects and in honey (Kaļániová et al., 2012; Snowdon & Cliver, 1996). Of those, only *Peyronelia* spp. has not been associated with (opportunistic) infections in human beings; the other microorganisms are known to produce keratitis/oculomycosis, endophthalmitis, (sub)cutaneous mycosis, onychomycosis, wound infections, (non-)invasive rhinosinusitis, fungal allergic sinusitis, deep mycosis, arterial occlusion and aneurysm. *Alternaria* spp. can produce toxins with a potential mutagenic effect. One case of fatal fungemia due to *Epicoccum* spp. was also recorded. However, no data has been published proving a direct connection between honey consumption and heterokont mycosis. Besides, *Alternaria* spp. has been isolated from spoiled

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food (Calvano et al., 2011; Jay et al., 2005; Kosrirukvongs et al., 2014; Pastor & Guarro, 2008; Revankar & Sutton, 2010).

2.3. Fungi

Several fungi haven not been associated with neither spoilage nor disease, i.e. *Atichia* spp., *Coniothecium* spp., *Hormiscium* spp., *Lipomyces* spp., *Oosporidium* spp., *Schizosaccharomyces* spp., *S. octosporus*, *Schwanniomyces* spp., *Seuratia* spp., *Torula mellis*, *Uredinaceae* spp., *Zygosaccharomyces mellis*, *Z. priorianus*, *Z. rouxii*. The yeast genera *Debaryomyces* (except *D. hansenii*) and *Hansenula are* known as food spoilers, but no pathogenic potential has been described yet (Jay et al., 2005).

Unlike moulds, yeasts pose a major problem for the honey industry, especially those tolerant to sugar (osmophilic) which are therefore able to ferment honey. Once favourable conditions prevail (moderate temperatures, increased humidity, granulation of the honey and elevated yeast counts), the sugars are turned into carbon dioxide and alcohol which, by reacting with environmental oxygen, may be synthesized to acetic acid. *Saccharomyces* spp. are the most widely distributed yeasts in honey. Total yeast counts can vary greatly, typically between 0 and 100,000 cfu/g (osmophilic yeasts 0 ï 10,500 cfu/g), although high counts are not palatable because of the increased rate of fermentation and the honey is unlikely to pass industrial control. From that point a view, a few hundred cfu/g of yeast are more likely to be found in commercial samples.

Of the fungi encountered in honey, several have been associated with human disease. While infections with some of them have received their own names (i.e. mucormycosis, invasive

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aspergillosis, disseminated fusariosis and opportunistic penicillosis), others pathogens are grouped by the target tissue:

- Skin and its appendages (*Acremonium* spp., *Aspergillus* spp., *Candida* spp., *Chaetomium* spp., *Cladosporium* spp., *Fusarium* spp., *Rhodotorula* spp. [Barron et al., 2003; Bennett & Klich, 2003; Chan et al., 2011; Corry & Kheradmand, 2009; Henrici, 1941; Horner et al., 1995; Jay et al., 2005; Khan et al., 2011; Kurup et al., 2000; Nucci & Anaissie, 2007; Puel et al., 2010; Revankar & Sutton, 2010; Wirth & Goldani, 2012])
- Eyes (*Acremonium* spp., *Cephalosporium* spp., *Fusarium* spp. [Bennett & Klich, 2003; Jay et al., 2005; Khan et al., 2011; Nagre et al., 2010; Nucci & Anaissie, 2007])
- Esophagus, bones (*Acremonium* spp. [Khan et al. 2011])
- Respiratory tract (*Acremonium* spp., *Aspergillus* spp., *Chaetomium* spp., *Eurotium* spp., *Penicillium* spp., *P. brevicompactum, P. chrysogenum, Trichoderma* spp. [Barron et al., 2003; Bennett & Klich, 2003; Chan et al., 2011; Chen et al., 1982; Corry & Kheradmand, 2009; Cui et al., 2013; DôAntonio et al., 1997; De Miguel et al., 2005; Horner et al., 1995; Jay et al., 2005; Khan et al., 2011; Kurup et al., 2000; Oshikata et al., 2013; Puel et al., 2010; Revankar & Sutton, 2010; Roussel et al., 2010; Wong & Wong, 2011])
- Empyema (*Chaetomium* spp. [Barron et al., 2003])
- Central nervous system (*Chaetomium* spp., *Cryptococus neoformans* var. *grubii, Rhodotorula* spp. [Barron et al., 2003; Chan et al., 2011; Henrici, 1941; Jay et al., 2005; Litvintseva et al., 2011; Revankar & Sutton, 2010; Wirth & Goldani, 2012])
- Peritoneum (*Trichoderma* spp. [Bennett & Klich, 2003; De Miguel et al., 2005])

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- Allergy, frequently associated with asthma (*Aspergillus* spp., *Candida* spp., *Cladosporium* spp., *Fusarium* spp., *Penicillium* spp., *P. brevicompactum, P. chrysogenum, Saccharomyces* spp., *Ustilaginaceae* spp. [Bennett & Klich, 2003; Carlsen et al., 1984; Chan et al., 2011; Chen et al., 1982; Corry & Kheradmand, 2009; DôAntonio et al., 1997; Dutkiewicz et al., 1988; Henrici, 1941; Horner et al., 1995; Jay et al., 2005; Kurup et al., 2000; Nucci & Anaissie, 2007; Oshikata et al., 2013; Puel et al., 2010; Revankar & Sutton, 2010; Wong & Wong, 2011])
- Fungaemia (*Pichia* spp., *Rhodotorula* spp., *Zygosaccharomyces* spp. [Adler et al., 2007; Chan et al., 2011; Henrici, 1941; Jay et al., 2005; Meletiadis et al., 2011; Wirth & Goldani, 2012])

Besides, many fungi are capable of producing a series of mycotoxins, i.e.

- *Aspergillus* spp. (aflatoxins, citrinin, cyclopiazonic acid, ochratoxin A, patulin, stegmatocystin [Bennett & Klich, 2003; Chan et al., 2011; Corry & Kheradmand, 2009; Jay et al., 2005; Kurup et al., 2000; Puel et al., 2010])
- *Candida albicans* (gliotoxin; Henrici 1941; Horner et al. 1995; Bennett & Klich 2003, Jay et al., 2005; Chan et al. 2011])
- *Fusarium* spp. (fumonisins, sambutoxin, trichothecenes, zearalenone [Bennett & Klich, 2003; Jay et al., 2005; Nucci & Anaissie, 2007])
- *Penicillium* spp. (citrinin, ochratoxin, cyclopiazonic acid [Bennett & Klich, 2003; Corry & Kheradmand, 2009; Horner et al., 1995; Jay et al., 2005; Kurup et al., 2000; Wong & Wong, 2011])

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- *P. chrysogenum* (roquefortine C, PR toxin [Bennett & Klich, 2003; Chen et al., 1982; DôAntonio et al., 1997; Oshikata et al., 2013])
- *P. griseofulvum* (patulin [Bennett & Klich, 2003; Puel et al., 2010])
- *P. solitum* var. *crustosum* (penitrem A [Bennett & Klich, 2003])
- *Trichoderma* spp. (trichothecenes [Bennett & Klich, 2003; De Miguel et al., 2005])

As can be seen, many fungi encountered in honey have a pathogenic background. However, most of those are opportunistic, their pathogenic potential revealed moreover by sampling immunocompromised patients. Several fungi (*Aspergillus* spp., *Candida* spp., *Chaetomium* spp., *Cladosporium* spp., *Cryptococcus neoformans*, *Fusarium* spp., *Mucor* spp., *Penicillium* spp., *Ustilago* spp.) have however been considered occupational hazards, i.e. they are pathogens strongly associated with the working environment, including that of farm staff (Dutkiewicz et al., 1988).

2.4. Other microorganisms

There is a large amount of entomopathogenic viruses that may affect honeybees. The *Picornavirales* families *Dicistoviridae* and *Iflaviridae* contain some of the major pathogens, e.g. the acute bee paralysis virus (*Aparavirus*), the deformed wing virus and the sackbrood virus (both *Iflavirus*). The yet unclassified Lake Sinai virus is thought to be associated partially with the colony collapsing disorder (Cornman et al., 2012). These viruses however do not play a major role as zoonotic pathogens, as they are too adapted to the insect host. However, the human *Enterovirus* (also *Picornavirales*) is thought to survive in honey, but contamination via human faeces would occur at (post)harvest level (Snowdon & Cliver, 1996).

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Algae of the the class *Chlorophyceae* haven been isolated eventually from honey made of honeydew, if a high humidity had prevailed. Dinoflagellates have also been encountered, but not in viable forms (Snowdon & Cliver, 1996). There are several protozoan-type parasites, e.g. *Crithidia mellificae* (*Excavata: Trypanosomatida)* and *Apicystis bombi* (*Chromalveolata: Neogregarinorida*) are known to affect bees (Cornman et al., 2012; Yang et al., 2013), but these pathogens do not seem to yield any zoonotic potential.

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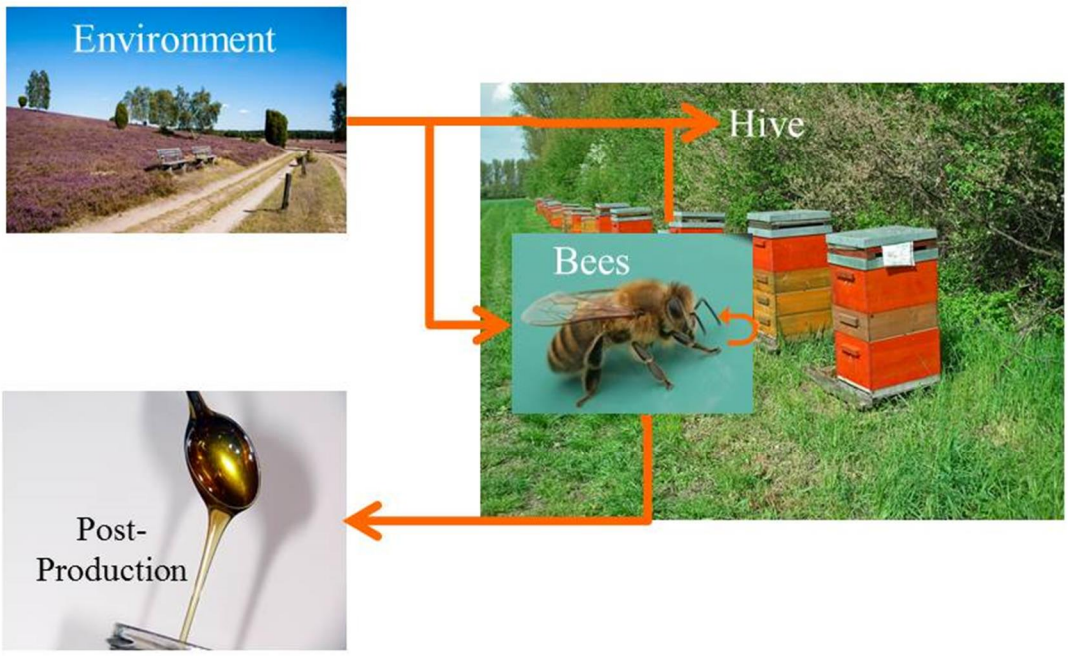

Fig. 1: General flow of microorganisms during honey production[1]

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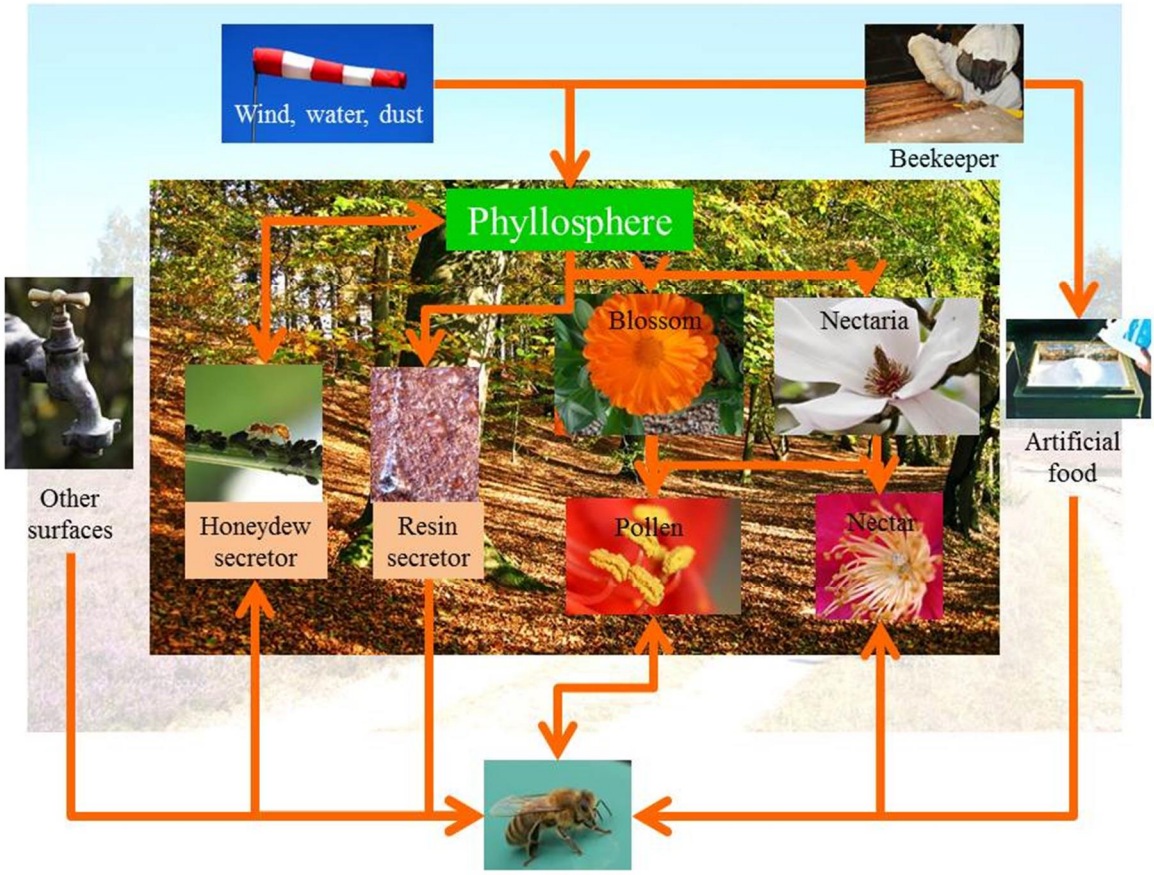

Fig 2: Flow of microorganisms within the environment[2]

[2] Images: background: Bernd Kasper / pixelio.de; from left to right and from upper to lower, upper row: Rainer Sturm / pixelio.de, Luc Viatour / www.Lucnix.be; middle row: Günter Havlena / pixelio.de, Dorothea Jacob / pixelio.de, böhringer friedrich / Creative Commons by-sa-3.0 de, kaemte / pixelio.de, Sureshbup / Creative Commons by-sa-3.0 de, Thomas Bresson / Creative Commons by-sa-3.0 de, Monika Herkens / pixelio.de, , カンツバキの蜜 / Creative Commons by-sa-3.0 de, Axel Hindemith / Creative Commons by-sa-3.0 de (modified); lower row: Lutz Stallknecht / pixelio.de

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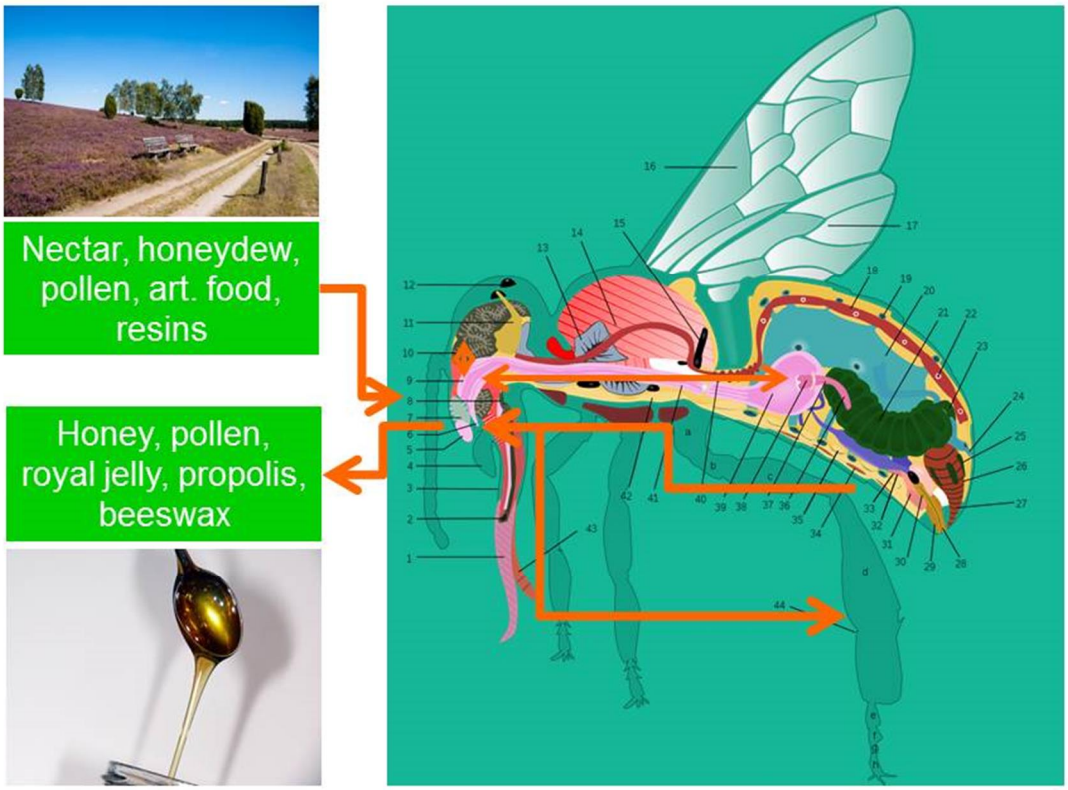

Fig. 3: Flow of microorganisms within the worker bee[3]; interactions occur, within the bee, among the mouth (9), hypopharyngeal glands (10), the upper gastrointestinal tract including the proventriculus (39), the mirror glands (34) and the legs (44)

[3] Images: upper left: Bernd Kasper / pixelio.de, lower left: Lupo / pixelio.de, right: Walké / Creative Commons by-sa-3.0 de

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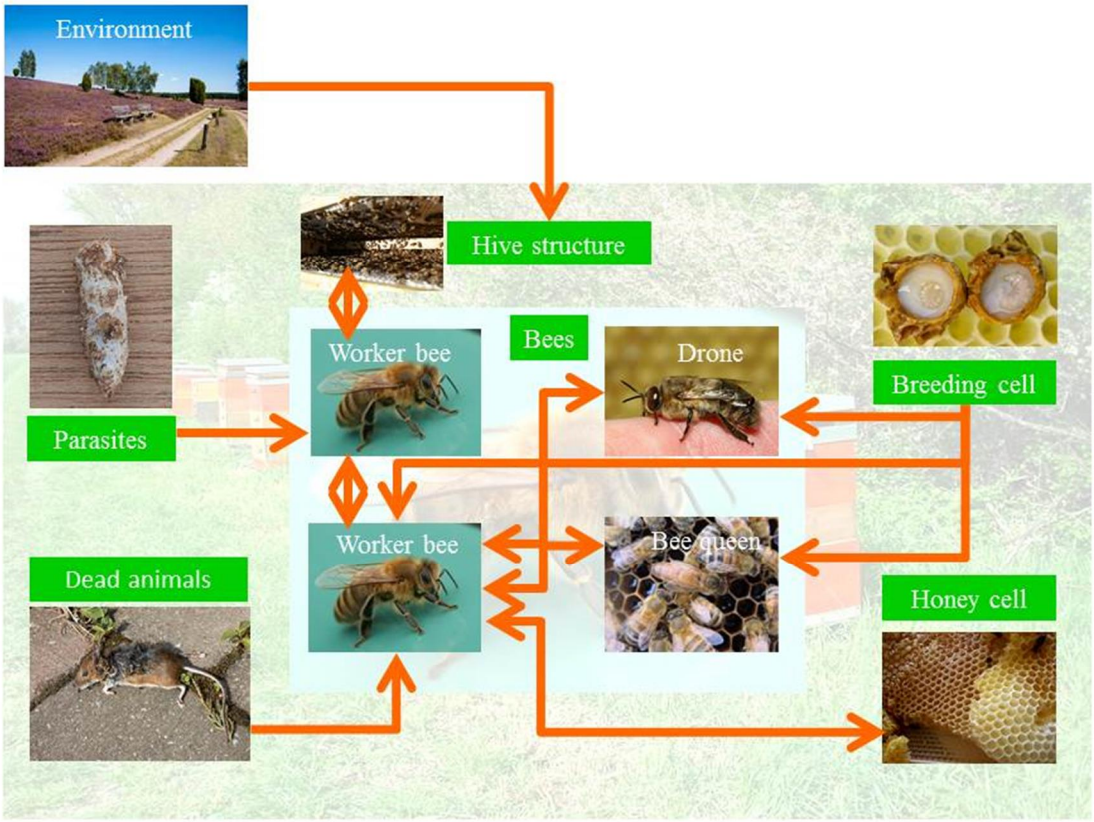

Fig. 4: Flow of microorganisms within the hive[4]

[4] Images: background: sparkie / pixelio.de, Lutz Stallknecht / pixelio.de; upper row: Bernd Kasper / pixelio.de; left to right and from upper to lower, middle row: Rasbak / Creative Commons by-sa-3.0 de, Robert Eichinger / pixelio.de, Lutz Stallknecht / pixelio.de, Waugsberg / Creative Commons by-sa-3.0 de (2x); middle row: Wouter Hagens / Creative Commons by-sa-3.0 de, Lutz Stallknecht / pixelio.de, Pollinator / Creative Commons by-sa-3.0 de, Merdal / Creative Commons by-sa-3.0 de

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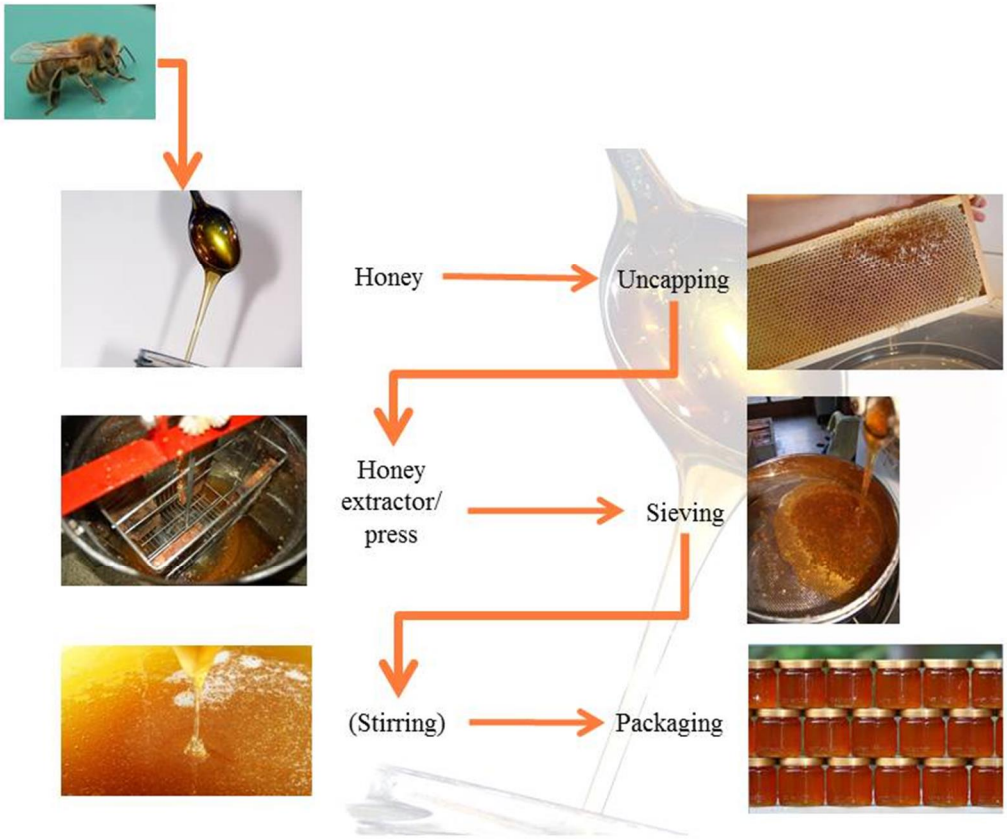

Fig. 5: Harvesting and post-harvesting flow of microorganisms[5]

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